Extracellular Vesicles in Human Reproduction in Health and Disease

Carlos Simon,[1,2,3,4]* David W. Greening,[5]* David Bolumar,[1,2]* Nuria Balaguer,[1,2] Lois A. Salamonsen,[6,7,8] and Felipe Vilella[1,2,4]

[1]Igenomix Foundation, 46980 Valencia, Spain; [2]Instituto de Investigación Sanitaria Hospital Clínico (INCLIVA), 46010 Valencia, Spain; [3]Department of Pediatrics, Obstetrics and Gynecology, School of Medicine, Valencia University, 46010 Valencia, Spain; [4]Department of Obstetrics and Gynecology, Stanford University, Palo Alto, California 94304; [5]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria 3086, Australia; [6]Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria 3168, Australia; [7]Department of Molecular and Translational Science, Monash University, Clayton, Victoria 3168, Australia; and [8]Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria 3168, Australia

ABSTRACT Extensive evidence suggests that the release of membrane-enclosed compartments, more commonly known as extracellular vesicles (EVs), is a potent newly identified mechanism of cell-to-cell communication both in normal physiology and in pathological conditions. This review presents evidence about the formation and release of different EVs, their definitive markers and cargo content in reproductive physiological processes, and their capacity to convey information between cells through the transfer of functional protein and genetic information to alter phenotype and function of recipient cells associated with reproductive biology. In the male reproductive tract, epididymosomes and prostasomes participate in regulating sperm motility activation, capacitation, and acrosome reaction. In the female reproductive tract, follicular fluid, oviduct/tube, and uterine cavity EVs are considered as vehicles to carry information during oocyte maturation, fertilization, and embryo–maternal crosstalk. EVs via their cargo might be also involved in the triggering, maintenance, and progression of reproductive- and obstetric-related pathologies such as endometriosis, polycystic ovarian syndrome, preeclampsia, gestational diabetes, and erectile dysfunction. In this review, we provide current knowledge on the present and future use of EVs not only as biomarkers, but also as therapeutic targeting agents, mainly as vectors for drug or compound delivery into target cells and tissues. ***(Endocrine Reviews 39: 292 – 332, 2018)***

Intercellular communication is an essential process both for multicellular organisms and for the relationship of unicellular organisms with the environment and hosts (1). Classically, communication has been identified as indirect as endocrine, paracrine, and autocrine or direct via cell-to-cell contact, secretion, release, and uptake of chemical moieties such as hormones, growth factors, or neurotransmitters (2, 3). According to the Human Protein Atlas, nearly 39% of the human protein-coding genes are annotated to give rise to membrane (28%) and secreted (15%) forms of signaling protein variants, some producing both isoforms and posttranslational modifications that can alter function. These molecules, which constitute potential therapeutic targets, include cytokines, growth factors, and coagulation factors, among others, playing physiological and pathological roles in processes such as immune defense, blood coagulation, or matrix remodeling. Of note, >500 of these proteins are currently known as pharmacological targets, with already approved druggable targets available commercially.

A new mechanism has recently been in the spotlight for cellular communication: the release of membrane-enclosed compartments, most commonly regarded as extracellular vesicles (EVs). EVs can act to convey molecules from one cell or tissue to another. Importantly, their contents (cargo) are protected from extracellular degradation or modification. They exert their biological roles by either direct interaction with cell surface receptors or by transmission of their contents by endocytosis, phagocytosis, or fusion with the membrane of the target cells. Recipient cell specificity appears to be driven

ISSN Print: 0163-769X
ISSN Online: 1945-7189
Printed: in USA
Copyright © 2018 Endocrine Society
Received: 13 October 2017
Accepted: 25 January 2018
First Published Online: 30 January 2018

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

 doi: 10.1210/er.2017-00229

ESSENTIAL POINTS

- Extracellular vesicles are a newly identified mechanism of cell-to-cell communication, recently discovered as a communication between the mother and the embryo
- Extracellular vesicles play an important role in normal physiology and in pathological conditions in human reproduction
- Prostasomes participate in regulating sperm motility activation
- Different extracellular vesicles and their cargo are implicated in promoting oocyte development and maturation
- Exosomes and their cargo in microRNAs play an important role in embryo implantation
- Extracellular vesicles are involved in the triggering, maintenance, and progression of reproductive and obstetric pathologies
- The participation of extracellular vesicles in human reproductive health has made them appealing players as biomarkers and to carry therapeutic agents

by specific receptors between the target cells and EVs (4–6). EVs have been described in different body fluids, including semen (7), saliva (8), plasma (9), breast milk (10), urine (11), and amniotic fluid (12), among others (4).

EVs can be classified in different populations based on their biogenetic pathway, composition and physical characteristics, such as size or density, giving rise to three major categories: apoptotic bodies (ABs), microvesicles (MVs), and exosomes (EXOs) (5, 13, 14).

EV content is complex as a continually progressing field with new cargos being identified continually. Regrettably, owing to technical limitations in methods of isolation and differentiation of the different populations of EVs, mixed, heterogeneous populations are often used, making interpretation of their content and functionality difficult (15–17). This constitutes a salient notion in the field at present, that populations of EV subtypes must be considered when reviewing published literature. With homogeneous sample preparation and key developments in characterization of EVs, we now hold important insights into defining these selected communicators in far greater depth. With the implementation of high-resolution and sensitive instrumentation for characterization such as mass spectrometry and next generation deep sequencing, it has been possible to develop databases gathering information about protein, lipid, and RNA content of EVs from different sources, including ExoCarta (online source: www.exocarta.org) (18), EVpedia (online source: www.evpedia.info) (19), and Vesiclepedia (online source: www.microvesicles.org) (20).

In recent years, EVs have been shown to participate in different processes committed to the maintenance of the normal physiology of the organism such as tissue repair, maintenance of the stem cell status of progenitor cells, platelet and immune function, and nervous system homeostasis. The potential role of EVs in the pathogenesis of different diseases has also been studied, with cancer, autoimmunity, neurodegeneration, HIV-1 infection, and prion diseases being the widest studied areas (1, 6, 21). In all of these cases, EVs are unique, as they became small indicators of an organism's homeostasis that can stably travel over the body fluids. That their content reflects cell of origin and pathophysiological states highlights their usefulness as biomarkers. Importantly, EVs are attributed with the potential to cross tissue barriers, such as the blood–brain barrier, possibly by transcytosis. This fact makes them appealing targets for therapeutics development (22). EVs can be released in response to cell activation, pH changes, hypoxia, irradiation, injury, exposure to complement proteins, and cellular stress (23–25). Interestingly, EVs are also secreted by plant cells (26, 27), as well as pathogens (28, 29), including bacteria, mycobacteria, archaea, and fungi (30, 31), suggesting an important evolutionary conserved mechanism of intercellular signaling.

In the field of reproductive biology there is growing interest in understanding the role of EVs within the male and female reproductive tracts, as they may constitute a new mechanism of communication between the reproductive tract and the immature germ cells, or between the mother and the developing embryo. Such developments offer great potential implications in the establishment of a successful pregnancy or implications with understanding associated pathological conditions (32). In the present review, we address current knowledge on the existence and functionality of EVs as cell-to-cell messengers in normal human reproductive physiology, as well as their contribution in the triggering, maintenance, and/or progression of pathological conditions in the functionality of the reproductive tract. Furthermore, we discuss their usefulness as biomarkers of altered reproductive conditions such as preeclampsia, spontaneous preterm birth (SPB), or polycystic ovaries syndrome. Finally, we summarize current knowledge on the present and future of the use of EVs as therapeutic agents, mainly as vectors for drug or compounds delivery into target cells and tissues.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Types, Isolation, and Characterization of EVs and Cargo

EV heterogeneity

EVs can be classified into selected subtypes according to different criteria, that is, cellular origin, biophysical (density and size) and biochemical (biological markers) characteristics, biological function, and biogenetic pathway. According to their biogenetic mechanism of formation and release, three main classes of EVs are defined: ABs, MVs, and EXOs (Fig. 1).

Apoptotic bodies

ABs are EVs produced by plasma membrane blebbing in cells undergoing programmed cell death. This term was coined by Kerr *et al.* (33) who defined them as "small, roughly spherical or ovoid cytoplasmatic fragments, some of which contain pyknotic remnants of nuclei." Indeed, one of the events that characterizes ABs is the fragmentation and packaging of cellular organelles such as the nucleus, endoplasmic reticulum (ER), or Golgi apparatus into these vesicles (34, 35).

ABs have widely been described as 1 to 5 μm in diameter, thus overlapping with the size range of platelets (36, 37), although some groups extend this range to 50 nm (16, 38, 39). Their buoyant density in a sucrose gradient is in the range of 1.16 to 1.28 g/mL (40, 41).

This vesicle population is characterized by cytoskeletal and membrane alterations, including the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the lipid bilayer (42). In this way, PS serves as an "eat me" signal for phagocytes to target and clear apoptotic debris (43, 44). Moreover, PS can naturally be recognized by annexin V, which is a useful marker of ABs (45). Nevertheless, care should be taken when using annexin V for this purpose, as PS flipping can also be triggered by other stimuli such as mechanical disaggregation of tissues, enzymatic treatments for detachment of cells, electroporation, chemical transfections, or retroviral infections, and PS exposure has also been described in healthy cells (46). PS flipping also induces MV formation, so these can also be recognized by annexin V detection (47, 48). Another specific feature of ABs is the oxidation of surface molecules, creating sites for recognition of specific molecules such as thrombospondin (49) or C3b complement protein (50), which are also useful as markers of ABs.

Included in newly identified potential molecular markers of ABs, VDAC1 is a protein that forms ionic channels in the mitochondrial membrane and has a role in the triggering of apoptosis. It proves to be a useful AB marker, as its biological function and subcellular localization are characteristic of this vesicular fraction (39). Calreticulin is an ER protein that could also work as an AB marker due to its subcellular localization (15), although it has also been observed in the smaller sized MV fraction (39). It is possible that, during the apoptosis process, the ER membrane is fragmented and forms vesicles smaller in size than ABs, which would contain calreticulin and would sediment at higher centrifugal forces (51, 52). Indeed, proteomic studies have related calreticulin with vesicular fractions across the full size range of MVs (53) and ABs (54).

Different functions have been attributed to ABs, although most are also features of other EVs. DNA can be horizontally transmitted between somatic cells, with possible integration of this DNA within the receptor cell where it can be functional (55). These vesicles are also a vehicle for the horizontal transfer of oncogenes, which are internalized by target cells and consequently increase their tumorigenic potential *in vivo* (56, 57). ABs have also been related to the immune response where they are associated with an underactivation of the immune system (58) and with antigen presentation with special regard to self-tolerance (59–61).

Microvesicles

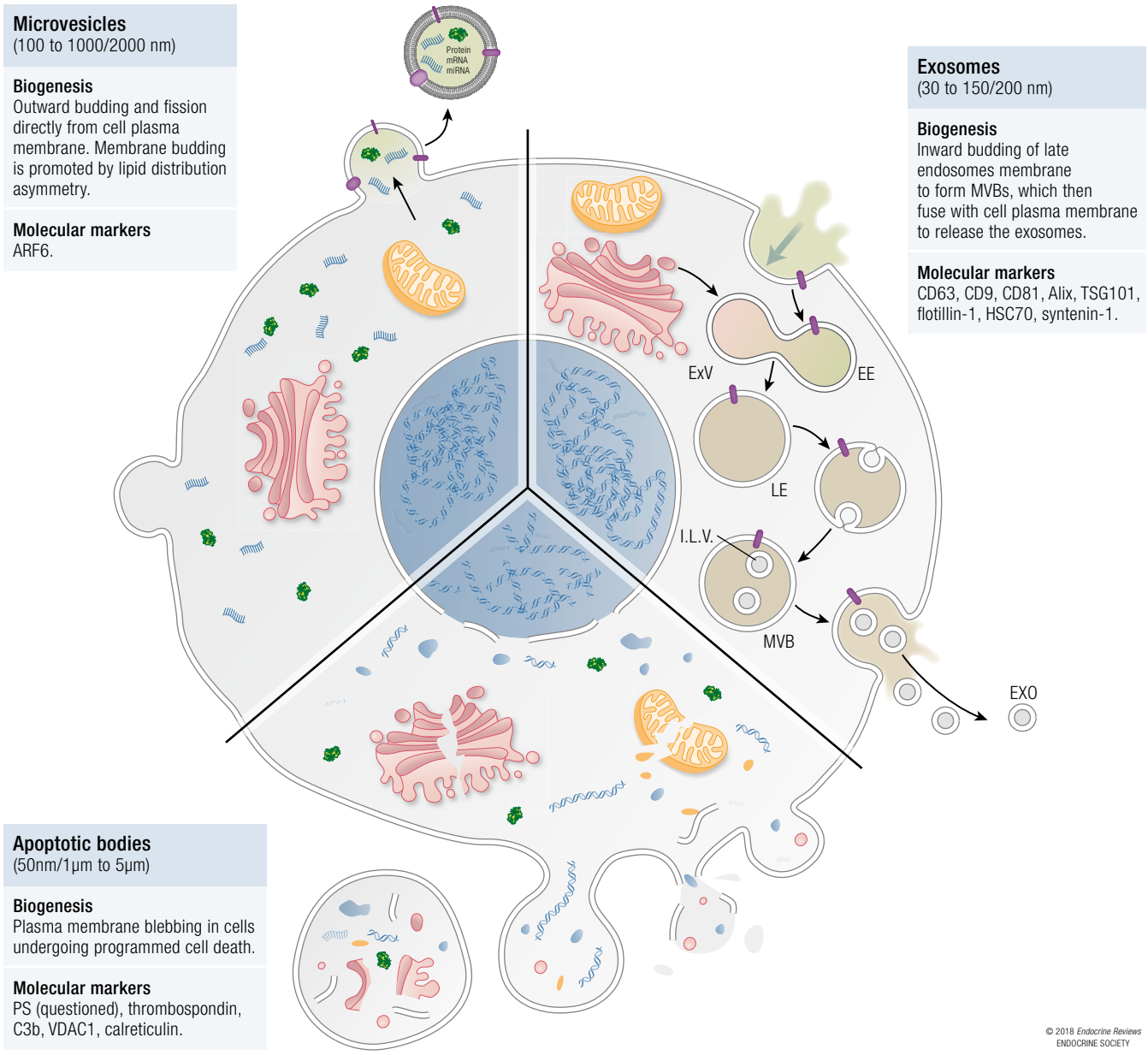
MVs were reported for the first time by Chargaff and West (62) as being sedimented at high-speed centrifugation (31,000 × *g*) (not specifically at lower speeds such as 5000 × *g*). MVs are a population of EVs that are formed and released directly from the cell plasma membrane by outward budding and fission from viable cells (63, 64). Plasma membrane blebbing is triggered by different mechanisms that are accompanied by the remodeling of the membrane proteins and lipid redistribution, which modulate membrane rigidity and curvature (65). Such changes within the periphery of the plasma membrane have been associated with cargo sorting in MVs (66).

The size range of MVs has been classically established between 100 and 1000 nm (67), thus overlapping with that of bacteria (13). Some groups extend this range up to 1500 nm (68) or even 2000 nm (69–71). The buoyant density of MVs is not as clear as that of other vesicle populations: ~1.16 g/mL in sucrose gradient (71) or 1.04 to 1.07 g/mL (72). The flotation density in iodixanol gradient is between 1.18 and 1.19 g/mL (73).

As a proposed marker for MV populations, ARF6 is a guanosine triphosphate–binding protein that is implicated in the regulation of cargo sorting and promotion of the budding and release of MVs through activation of the phospholipase D metabolic pathway (65, 74). Additionally, data coming from our current knowledge on proteomic studies suggest numerous proteins (*e.g.*, KIF23, RACGAP1, exportin-2, chromosome segregation 1–like protein) as unique and/or enriched for MVs and potentially discriminatory markers (75). Nevertheless, care should be taken with these results, as different EV cell sources and

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Figure 1. Main types of EVs present in body fluids and culture media. EVs are classified in three groups according to their biogenetic pathways. EXOs are produced in the endosomal pathway by invagination of the membrane of late endosomes to form intraluminal vesicles (ILVs) enclosed in multivesicular bodies (MVBs). MVBs can then fuse with lysosomes and degrade their content, or fuse with cell plasma membrane to release ILVs, now regarded as EXOs. MVs are produced directly from the cell plasma membrane by outward budding. ABs are generated as blebs in cells undergoing programmed cell death. EE, early endosome; ExV, exocytic vesicle; LE, late endosome.



techniques to selectively enrich may lead to differences within EV populations.

Among the functions described for MVs are pivotal roles in cancer cell invasiveness (76, 77), transformation potential (78), progression (63, 79, 80), and drug resistance (81). MVs have also been implicated in autoimmune diseases (82–84), immune system modulation and coagulation (67, 85, 86), embryo–maternal crosstalk (87), and embryo self-regulation (88).

Exosomes

The first description of EXOs in 1981 described them as a second population of vesicles that appeared in the preparations of MVs, and the term "exosome" was coined (89). Two years later, their biogenetic pathway was formally described by transmission electron microscopy (TEM), trying to follow the pathway of uptake and trafficking of transferrin molecules within reticulocytes in an anemic mice model (90). EXOs constitute a population of nano-sized EVs that arise

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

and are trafficked through the endosomal pathway. Endosomal sorting complexes required for transport (ESCRTs) are important for the biogenesis of multivesicular bodies (MVBs, which include EXOs). MVB inward budding of the limiting membrane of late endosomes facilitates formation of intraluminal vesicles (ILVs) that remain enclosed inside the greater membrane compartment of MVBs. ESCRT-independent mechanisms, including neutral sphingomyelinase/ceramide formation and ARF6/PLD2, have been reported to also occur (73, 91). The formed MVBs can then be targeted to plasma membrane to release ILVs, now known as EXOs, or otherwise fuse with lysosomes to degrade their content (92). Members of the Rab guanosine triphosphatase (GTPase) family have been shown to modulate EXO secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. It is likely that ESCRT-dependent and ESCRT-independent MVB/EXO biogenesis machineries vary from tissue to tissue (or even cell type) depending on specific metabolic needs. There are several molecular mechanisms, both canonical and alternative, implicated in the formation, release, and extracellular fate of EXOs [see reviews in (5, 75)].

Most studies place EXOs in a size range of 30 to 150 nm (5, 93) or even 200 nm (94), thus establishing an overlap with viruses in terms of size (14). The buoyant density of EXOs in sucrose gradients has been set in a wide range of 1.10 to 1.21 g/mL (38, 95), and 1.10 to 1.12 g/mL in iodixanol gradients (96).

The classically associated markers of EXOs are molecules mainly implicated in the biogenesis of this population, which are incorporated during this process: tetraspanins (CD63, CD9, CD81), Alix, TSG101, and flotillin-1, among others (5, 95). Nonetheless, with the emerging interest in studying different EV populations as isolated entities, many of these classical markers have been identified as widespread between populations, although with different relative abundances. This is the case for at least CD9, CD63, HSC70, and flotillin-1. Other molecules such as TSG101 and syntenin-1 have been ratified as markers of only this vesicle population (39). PS, while being described as a broad marker of EVs, has also been reported as exposed on the surface of EXOs produced by different cell types (92, 97). Accumulating evidence from *in vitro* studies using cells grown in culture and ex vivo body fluids indicates the existence of more than one EXO subtype (98–105). For example, EXOs contain subpopulations, including the study of EXOs derived from apical (EpCAM-Exos) or basolateral (A33-Exos) surfaces of highly polarized cancer cells, which indicated the presence of two distinct subtypes with distinct protein (98) and RNA cargo (99, 106). The biological significance of these findings awaits further investigation.

Because of the high expectations and efforts dedicated to the study of the role of EXOs in different biological processes, both in physiological or pathological conditions, the field of EXO biology has experienced an exponential growth in recent years, with a wide range of functions identified (1, 107). EXOs are implicated in cancer physiology, participating in tumor progression and maintenance, resistance, immune modulation, and angiogenesis (108). Their function in immune regulation has also been well studied in antigen presentation modulation, immune activation, and suppression (109, 110). Importantly, knowledge of the seminal role of EXOs in reproductive biology is expanding rapidly. Such studies and the molecular markers and mechanisms identified have the potential for use as markers to discriminate between EV subtypes, as well as various applications of EXOs in clinical diagnosis.

Methods of isolation and purification of EVs

The main experimental problem when studying EVs is to achieve a homogeneous separation with appropriate yield of the EV population of interest. Different methods of isolation and purification have been developed; however, to a varying extent, all carry the bias of providing completely homogeneous EV populations of any one vesicle type (summarized in Table 1). In the field, there is a pressing need to define EV surface-exposed proteins for the purpose of generating monoclonal antibodies that would allow discrimination of EV class or subtype (*i.e.*, stereotypical markers). Most rapid, one-step approaches for isolating EVs do not take into consideration that they are dealing with a possible mixture of vesicle classes or subtypes and coisolated contaminants such as high relative molecular mass protein oligomer and protein–RNA complexes (*e.g.*, high-density lipoprotein/low-density lipoprotein/AGO2) complexes.

Serial differential centrifugation

Differential centrifugation is the most common and well-known method for the isolation of EVs. Although each group adapts the times and centrifugal speeds depending on their samples, the basic protocol as follows: (1) centrifugation at low speed for the elimination of cells (300 × *g*, 10 minutes), (2) centrifugation at up to 2000 × *g* for 10 minutes to pellet membrane debris and ABs, (3) centrifugation at 10,000 to 20,000 × *g* for 30 minutes to pellet MVs, and (4) a crude EXO preparation is pelleted by ultracentrifugation at 100,000 to 200,000 × *g* for 70 minutes. After steps 1 to 3 of centrifugation, supernatants are transferred to new tubes for the isolation of the subsequent EV type. Pellets (steps 2 to 4) containing different cell populations are washed by resuspension in phosphate-buffered saline (PBS) and recentrifugation under the same conditions. The

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

washing step removes some impurities, but also reduces EV yield.

Apart from vesicle size, centrifugation alone cannot achieve the separation of pure populations for various reasons: sedimentation of other particles in the supernatant depending on density, distance of the particles from the bottom of the tube, and vesicle/particle aggregation (111).

To improve EV population purity, a gradient step can be added to the centrifugation protocol. This system aims to avoid as far as possible the contamination of EV pellets with large protein/protein-RNA aggregates and proteins nonspecifically bound to EVs (4). The essentials of the technique are resuspension of the pellet from the previous serial differential centrifugation in a suitable buffer (*i.e.*, PBS), then loading on either the top or base of a prepared sucrose cushion (112, 113) or a sucrose gradient (114, 115). Following ultracentrifugation, vesicles are recovered either from the bottom of the tube (for cushions) or from a specific fraction of the gradient, depending on their buoyant density. Moreover, substitution of sucrose by a nonionic density gradient medium, called iodixanol (116), offers many advantages: better separation of viral particles from EVs; low toxicity toward biological material; is clinically applicable; and forms isosmotic solutions compatible with the size and shape of EVs in a wide range of densities (117–119).

Size exclusion methods: filtration and chromatography

Filtration for isolation of EVs is often used in combination with ultracentrifugation protocols to improve separation efficiency based on size. Filtration steps using 0.8-, 0.2-, or even 0.1-μm filters can be inserted between the centrifugation steps depending on the size of the desired population (112, 120, 121). Alternatively, ultrafiltration utilizes filtration units of different molecular mass cutoff membranes that are centrifuged at moderate centrifugal forces. They allow concentration of vesicles in the interface of the filters, from which they can be recovered by washing (122–125). All of these methods face several drawbacks. The pressure of the supernatant can cause the EVs to deform or break into smaller vesicles, and the filter membrane may decrease the yield. Gravity filtration has been proposed to cope with the problems associated with elevated pressures (120), but this can be time consuming and filters can become saturated.

Another option for EV isolation in conjunction with ultracentrifugation is based on size exclusion chromatography. In brief, the medium containing the vesicles is loaded into the chromatography device, generally a gel size exclusion column, equilibrated into the column, and eluted with PBS (126–128). The technique is usually coupled with previous low-speed centrifugation to remove larger debris and subsequent ultracentrifugation to wash and concentrate the vesicles from the different chromatography fractions (129, 142). Its advantages are enhanced separation of EVs from proteins and high-density lipoproteins, avoidance of protein and vesicle aggregate formation, reduced sensitivity to the viscosity of the vesicle media, compatibility with the biological properties and functionalities of the isolated vesicles, and preservation of the vesicular structure and conformation (126). Moreover, it offers shorter isolation times and relatively low cost. As a disadvantage, this technique offers reduced EV recovery yields in comparison with others such as ultracentrifugation or polymeric precipitation, although it is susceptible to scale-up (143, 144). Nevertheless, some studies indicate that a combination of size exclusion chromatography and ultrafiltration may produce a yield surpassing that of classical ultracentrifugation (145, 146).

Other approaches

Immunoaffinity uses microbeads coated with specific antibodies for the recognition of specific surface markers of EV populations. In brief, beads are incubated with the sample containing EVs, then beads linked to their epitopes on the EV surface are recovered by magnetism or low-speed centrifugation, depending on the nature of the beads (130, 131). The technique can follow centrifugation and/or filtration to clear large cellular products (96, 99, 132). This method differentiates EV populations based on surface markers regardless of their size. Nevertheless, care should be taken, as population-specific markers are not necessarily available, and the working surface of the beads is limiting, so the EVs may not have access when they are large or present at high concentrations (116).

Aiming for a quicker and simpler method to isolate EVs, a polymeric precipitation system (ExoQuick) was commercially developed. The experimental procedure is as simple as incubating the kit reagents with the EXO-containing media and recovering the resulting polymeric complex by low-speed centrifugation. A study with human ascite samples showed that ExoQuick could provide high concentrations and purity of exosomal RNA and that the high exosomal protein concentrations from the same samples compared well with other isolation methods such as ultracentrifugation, immunoaffinity isolation, and chromatography (133). Even though ultracentrifugation-based protocols are preferable for exosomal protein recovery and purity, ExoQuick obtains better results in terms of exosomal messenger RNA (mRNA) and microRNA (miRNA) yield and quality (134). The method has a series of limitations. Impurities such as lipoproteins are possibly coisolated along with EVs, and the method is unable to provide isolation of different EV subpopulations. It works ideally with small vesicles in the size range of 60 to 180 nm (111).

"Differential centrifugation is the most common and well-known method for the isolation of EVs."

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 1. Classification of the Methods of Isolation of EVs Based on Their Principles

Method	Technique	Isolation Principle	General Workflow	Advantage	Limitation	References
Centrifugation	Serial differential centrifugation	Sedimentation velocity	Serial or differential centrifugation: (1) 300 × *g*, 10 min to remove cells → (2) 2000 × *g*, 10 min to remove cell debris, ABs → (3) 10,000/20,000 × *g*, 30 min to isolate MVs → (4) 100,000/200,000 × *g*, 70 min to isolate EXOs	• Broad application • Standardization • Ease of use • Reproducibility • Yield	• Sedimentation dependent on density, tube length, sample viscosity, concentration, and vesicle aggregation apart from size	(16, 112, 116, 138)
	Density gradient	Buoyant density	Generally introduced to further purify distinct types of EVs (*i.e.*, MVs or EXOs). Various different reagents, including sucrose or iodixanol. Crude EV populations loaded either on top (float down) or at bottom (float up) of gradient. Ultracentrifugation performed under pre-established conditions	• Purification: increases EV population purity from: protein aggregates, RNA–protein complexes, separation of EV subpopulations within the same type • Soft isolation approach • Clinically applicable medium (iodixanol) • EV homogeneisty	• Yield • Reproducibility • Trained user • Time-consuming	(4, 112–115, 117–119,139)
Size exclusion	Filtration	Size/shape	Generally interspersed within centrifugation steps: prior to centrifugation, supernatants are challenged through syringe filters of determined pore size	• Easy to use • Further stringency of the populations based on their canonical sized • Reproducibility	• Yield loss within filtering membrane • Risk of vesicles deformation or fragmentation.	(112, 120, 121)
	Ultrafiltration	Size	Centrifugal filtration units of prefixed molecular size range that selectively retain vesicles Previous studies shown to isolate distinct subtypes of EVs using this strategy	• Easy to use • Quick technique • Reproducible	• Yield loss within filtering membrane. • Risk of vesicles deformation or fragmentation.	(73, 122–125, 140)
	Chromatography	Size/charge	Purification of EVs based on surface charge or size	• High resolving power; improved purification of EVs from proteins and lipid particles • Limits EVs and protein aggregation based on buffer used • Less sensitive to the viscosity of the media • Respectful with EV functionalities and biological properties • Shorter isolation times	• Usually coupled to centrifugation to remove cell debris and recover EV containing fractions • Often issues with volume or buffer associated with elution	(126–129, 142)

(*Continued*)

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 1. *Continued*

Method	Technique	Isolation Principle	General Workflow	Advantage	Limitation	References
Immunoaffinity		Presence of specific EV surface molecules	Microbeads coupled to antibodies are incubated with EVs for specific surface markers recognition (*i.e.*, A33, EpCAM, CD63). Afterward, beads are washed and recovered by precipitation or magnetism.	• Separation based on specific molecules further than by size	• Sometimes coupled to centrifugation and/or filtration to initially remove larger cellular debris	(96, 98, 99, 130–132)
				• Selectivity	• Select surface markers of EVs are not always known or available	
				• Resolution	• Cost	
				• Speed of isolation	• Yield	
Polymeric precipitation		Weight increase to pellet at low centrifugal force	Incubation of polymerization kit reagents with EV solution and recovery by low-speed centrifugation	• High speed	• Possibility of coprecipitating impurities	(111, 133, 134)
				• Simple procedure	• Unable to separate EV fractions	
					• Ideal only for small (60 to 180 nm) EV populations	
Microfluidics		Different possible principles:	(1) EVs are passed through microfluidic system and EV-specific markers are recognized by antibodies in a device surface	• Reduced sample volume needed	• Habitually couple to centrifugation to remove undesired EV populations	(135–137, 141)
		(1) Presence of specific molecules	(2) Still not applicable for EVs	• Smaller processing times and costs, maintaining high sensitivity	• Unable to differentiate EVs populations	
		(2) Physical properties such as size	(3) Combination of microfluidics and polymer filter that allow passing EVs under a certain size	• Possibility to process, quantify. and image the samples within the system itself	• Still under development	
		(3) Microfluidic filtration				

A new technology based on microfluidic devices has recently been developed for the isolation of EVs. It allows the reduction of sample volumes, processing times, and costs while maintaining high sensitivity. The chip technology can be based on different principles. The first developed systems relied on the recognition of EVs by specific antibodies on the surface of the device (147). The surface of the flow system was coated with anti-CD63 antibodies. When EV-containing media was pumped through the system, EXOs were restrained. The system allows scanning electron microscopy (SEM) imaging and lysis of EVs for RNA isolation directly on the chip. However, it does not provide sufficient material for protein or functional analyses. Subsequently, the system was expanded with lipophilic staining of EXOs to allow simultaneous quantitation (135). A third microfluidic scheme used physical properties as the principle for EV isolation, separating microparticles based on their size within the micrometric size range (136). Clearly this method is not applicable to EV population analysis. This technology has also been combined with porous polymers, allowing purification of vesicles in the nanometric size range: the pore size can be modulated so that only EVs under a certain size can be filtered (148). A recent study introduced the concept of using a combination of acoustics and microfluidics for a high-purity degree of EXO isolation. The platform is composed of two sequential modules that remove larger components and other EV groups (MVs and ABs, respectively), allowing the direct use of undiluted body fluid samples (tested in whole blood) or conditioned media from cell cultures in a single step. The system is based on the combination of microfluidic channel conformation and adjusted acoustic pressure, which

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

make it possible to set the cutoff particle diameter (149).

The demands of clinical applications involving diagnostics and therapeutics such as low cost, reliability, and speed can eventually be met with modifications to existing technologies for improved scalability. Isolation of EVs from blood and urine is a challenge due to the presence of abundant and complex protein and lipoprotein networks, which undoubtedly will attenuate intrinsic EV protein/RNA signatures. Distinct clinically relevant strategies to isolate EVs are currently being investigated (75, 150, 151).

Methods for characterization of EVs

Characterization of EVs is fundamental to enable differentiation among the different subpopulations within the same biological sample, between vesicles of distinct cellular origin or even of the same origin in pathological vs physiologically normal conditions (summarized in Table 2).

Microscopy: morphology and size analysis

Electron microscopy techniques are the only method available to provide the appearance of EVs related to their size. Different variants offer different data to the user. TEM was initially used by Raposo *et al.* (152), who described EXOs as cup-shaped vesicles. Although different protocols can be used for TEM visualization, two general schemes offer different views. EVs can be resuspended in fixative media and laid into grids for staining and visualization. Alternatively, EV pellets from centrifugation and ultracentrifugation steps can be fixed, resin embedded, and cut into ultrathin slides, which are then stained and laid in grids. The first method is simpler and less time consuming, and it offers a view of the exterior of the EVs. The second method is more informative, shows the interior of the EVs, and allows immunogold staining of specific markers that are seen as electron-dense spots (153, 154). Cryo-electron microscopy allows direct visualization of frozen EVs without previous fixation and contrast steps. The structures are seen as close as possible to their native states (not dehydrated or fixed) and demonstrate variable EV morphology (155). Indeed, such analysis showed that the classical cup shape attributed to EXOs was an artifact of fixation (2). Finally, SEM offers three-dimensional imaging of the EVs for further morphological description (156, 157).

Atomic force microscopy (AFM) is an alternative for the analysis of size distribution and quantity of EVs within a sample and is based on the scanning of the sample by a mechanical probe, which physically touches the sample, providing topographical information. AFM allows imaging at the subnanometric level. It can be adjusted to air (dry samples) or liquid modes (aqueous samples), and differences in size or number measurements are negligible between them. The possibility of measuring samples in aqueous media is advantageous, as it permits the maintenance of EV physiological properties and structure (158–160). AFM has been efficiently combined with microfluidic isolation devices to provide consecutive isolation and characterization of EVs. Mica-microfluidic chips are also of interest, as they provide a nonconductive flat surface for *in situ* AFM analysis (137, 160).

Size distribution analysis techniques

Nanoparticle tracking analysis (NTA), a light-scattering technique, is now widely used for the assessment of EV size distributions and concentration in the range of 50 to 1000 nm. The principle of the technique is based in the inherent Brownian motion of the particles in a solution: EVs in suspension are irradiated by a laser beam, thus emitting dispersed light. This scattered light is captured by a microscope, and NTA software tracks the movement of each particle in a time lapse. Silica nanospheres have been proposed for standardization, as their refraction index (1.46) is similar to that observed for most EVs (refraction index of ~1.39) (161).

Dynamic light scattering is also used for the assessment of EV size distribution. Although the principle for size determination is also Brownian movement of particles in suspension, the way to attain these data varies from NTA technology. It has limitations when measuring polydisperse samples and those containing big EVs, because the bigger particles scatter more light, masking the smaller ones (162). It is also possible to calculate vesicle concentrations in the samples by direct extrapolation from the distribution representations using mathematical criteria (162).

Tunable resistive pulse sensing (or qNano by its IZON commercial name; Izon Science Ltd., New Zealand) is a novel and less expensive technique for the analysis of particle size distributions. The system is composed of a thermoplastic polyurethane membrane containing nanopores that are selected by size requirements. Currently, the system can measure individual particles in the size range of 30 nm to 10 μm and in the concentration range of 10^5 to 10^{12} particles per milliliter. Because the system analyzes the particles individually, multimodal populations can be studied. Alternatively, a configuration of only one pore type restricts measures to a narrow size range, which is particularly useful for analysis of a specific vesicle population. Combining pores of distinct size and geometry allows widening of this range and analysis of a greater volume of sample (163–165).

Flow cytometry has also been applied to the analysis of size distribution, concentration, and qualitative characteristics of the EVs within a sample. Light scatter flow cytometry allows the analysis of vesicles with a lower size limit of usually between 300 and 500 nm (166, 167), but small EVs, including EXOs,

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

cannot be studied by this method. However, innovative new flow cytometry technology and the use of fluorescent labeling of EVs has reduced the lower limit of detection to ~100 nm, and it is possible to discriminate between vesicles 100 to 200 nm in size (168, 169). Finally, EVs can be coupled via antibodies to their surface markers, to latex beads of greater size. In this way even nano-EVs can be analyzed, but no quantification or differentiation between vesicle populations is possible (170, 171).

Molecular marker characterization

The most effective and well-accepted approach to measure EV purity is the concentration of a specific EV surface marker antigen. Approaches including western immunoblotting, enzyme-linked immunosorbent assay (ELISA) using surface markers that can be used with adaptation for the quantitation of EVs within a sample (172, 173), and ExoScreen have been used (174).

Another approach for the characterization and quantitation of EVs is based on micronuclear magnetic resonance spectrometry (μNMR) (175) EV labeling with specific EV surface molecular marker antibodies coupled to magnetic nanoparticles enables specific detection by microfluidic μNMR. The technique offers a detection sensitivity level that greatly surpasses ELISA or flow cytometry.

Finally, transmission surface plasmon resonance can provide an alternative method for the molecular characterization and quantitation of EXOs in a system called nano-plasmonic EXO assay. This consists of a gold film patterned with a series of nanohole arrays, each of which is coated with specific monoclonal antibodies for the recognition of EXO-specific proteins. Compared with previous systems, the nano-plasmonic EXO assay is label free, easy to miniaturize and scalable for higher throughput detection, and improves detection sensitivity to a magnitude order lower than that for μNMR (176, 177).

During the past decade, recent studies and groups have used developments in proteomic profiling to characterize specific markers for highly purified EV subtypes (EXOs and MVs). Since the emergence of the interest in studying different vesicles populations as isolated entities, many of the classical markers of EXOs have been uncovered as widespread between populations, although with different relative abundances. This is the case of CD9, CD63, HSC70, EpCAM, and flotillin-1, among others (98, 100). Alternatively, some new molecular markers have been identified and ratified as markers of EXOs, including TSG101, syntenin-1, and Alix/PDCD6IP (39, 100). Numerous proteins found exclusively/enriched in MVs [*e.g.*, KIF23, RACGAP1, chromosome segregation 1–like protein, exportin-2 (CSE1L/CAS)] warrant further study as to their potential use as discriminatory markers for MVs. Furthermore, care should be taken when analyzing PS as a marker of ABs, as it has also been reported to be exposed in the surface of EXOs produced by different cell types (92, 97) and also MVs (47, 48). An in-depth review detailing proteomic insights into EV biology and defining markers for EV subtypes and understanding their trafficking and function was provided by Greening *et al.* (183).

EV cargo

Membrane receptors and cargo content are the most important feature of EVs, because they define their cellular selectively, target, uptake, and functionality, respectively. EV cargo includes proteins, bioactive lipids, various RNAs (including fusion gene and splice-variant transcripts), and DNAs (described later), as well as other cell regulatory molecules (1, 4). To date, most studies have focused on their genetic (particularly RNA and miRNA) and protein content, as sensitive methods exist for their comprehensive analysis and detection.

Protein contents in EVs have been widely studied since the application of mass spectrometry–based techniques (184). EVs have been shown as to be enriched in proteins from cytoskeleton, cytosol, plasma membrane, heat-shock proteins, and proteins involved in EVs biogenesis, whereas proteins from cellular organelles are less abundant (1). From initial studies, EVs were shown to carry commonly widespread EV proteins and a specific subset of proteins, depending on the cell, the type of vesicle, and the method of isolation (5). Moreover, it has been observed that EV number, protein content, and protein concentration vary depending on the stimuli for vesiculation, even in the same subpopulation of vesicles (185).

Cytokines have also been described to be carried by EVs (1). Interleukin (IL)-1β is among the examples of theses soluble mediators that are secreted in EVs. Indeed, secretion pathways of EVs may constitute an alternative to exocytosis for proteins that lack leader signal peptide (186). Another interesting example of cytokine cargo is IL-1α, which has been reported to be selectively carried by ABs but not by smaller-in-size vesicles (<1 μm) in endothelial cells (187), thus confirming the cargo sorting into different populations of EVs. Further examples of cytokines released into EVs are IL-18 (188), IL-32 (189), tumor necrosis factor (TNF)-α (190), and IL-6 (191), among many others. During pregnancy, EV cytokine cargo has been shown to be modified toward an increase in comparison with nonpregnancy, maybe contributing to the modulation of maternal immune response against the fetus. Levels of transforming growth factor-β1 and IL-10 were increased in EVs from pregnant women, along with an enhanced ability to induce caspase-3 activity in cytotoxic natural killer (NK) cells, thus promoting an immunosuppressive phenotype through the induction of apoptosis in these cells (192).

"The most effective and well-accepted approach to measure EV purity is the concentration of a specific EV surface marker antigen."

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 2. Classification of the Methods of Characterization of EVs Based on Their Principles

Method	Technique	Principle	Main Features	Quantitative/Qualitative	References
Microscopy	TEM	Negitive staining of EVs with electron-dense molecules (heavy metals)	• Direct imaging of EV size	• Semiquantitative	(139, 152–154, 178)
			• Size distribution	• Dehydrating (fixation)	
			• Can be coupled to immunogold labeling to stain specific structures	• Possibility to take measures within the imaging field	
	SEM	Covering of molecules with microgold particles and electron reflection scanning	• Three-dimensional imaging of EV structures	• Semiquantitative	(156, 157, 179)
				• Possibility to take measuresments within the imaging field	
	Cryo-electron microscopy	Plunge-frozen in liquid ethane/nitrogen	• Avoids fixation and contrasting steps	• Semiquantitative	(2, 73, 155)
			• Allows seeing structures closer to their native states	• Possibility to take measures within the imaging field	
			• Size distribution	• Highly trained user	
	AFM	Use of a cantilever with a free end that touches the surface to obtain topographical information	• Resolution at the nanometric level	• Quantitative	(137, 158–160)
			• Possibility to analyze both dry and aqueous samples	• Size-distribution profile determination	
			• Can be combined with microfluidic isolation devices	• Require homogeneous EV purification	
			• It does not provide direct imaging of EVs		
Size distribution analysis techniques	NTA	Particles are challenged with a laser beam and forward scattered light is real-time captured by a microscope to calculate sizes based on particles using their Brownian motion.	• Size measures in the range of 50 to 1000 nm	• Qualitative: not only size populations but also EV markers can be analyzed by fluorescent labeling	(161, 180, 181)
			• Standardization is not needed but possible (interest for concentration assessments)	• Quantitative: possibility to get precise size distributions and their associated concentrations in 1-nm intervals	
			• Size distribution	• Cost	
			• Low sample use		
			• Compatibility of fluorescence detectors		
	Dynamic light scattering	Particles are challenged with a laser beam and reflected light is captured by a detector in a certain variable angle. The detector converts time-dependent fluctuations in the scattered light intensity into particle size data.	• Size measurements in the range of 1 to 6000 nm for EV concentrations from 10^6 to 10^9 particles/mL	• Mainly qualitative	(162, 182)
			• Samples can be recovered after the analysis	• Semiquantitative if standards are used	

(*Continued*)

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 2. *Continued*

Method	Technique	Principle	Main Features	Quantitative/Qualitative	References
			• Limitations with polydisperse samples and those containing big EVs		
	Tunable resistive pulse sensing	A transmembrane voltage is established in a porous membrane. The crossing of EVs through the pores alters the electrophoretic flow causing a resistance that can be translated into size data.	• Size measurements in the range of 70 nm to 10 μm for EV concentrations from 10^5 to 10^{12} particles/mL	• Qualitative	(163–165)
			• Single EV measures that allow multimodal EV population studies	• Quantitative	
			• By modifying pore configuration, the analyzable EV size and sample volume can be regulated		
	Flow cytometry	EVs are swept along by a liquid stream to align them in single file in the center of the stream until the interrogation point, where they are excited by a laser beam. Laser scattered light is gathered by detectors situated 180° (size data) and 90° (morphology or fluorescently stained structure data) to the laser beam.	• Analysis of EVs with a lower size limit of 250 to 500 nm and ability to distinguish vesicles that differ 200 nm in size	• Qualitative: not only size populations but also EV markers can be analyzed	(166–171)
			• New technological developments have reduced the limit of detection to ~100 nm and the discrimination power to 100 to 200 nm	• Quantitative	
			• Possibility to coupling to latex beads for easy marker analysis		
			• No sorting capacity		
			• Dependent on EV surface markers or use of EV fluorescent labels		
Molecular marker characterization techniques	Western blotting/ ELISA	Both techniques share the same principle: proteins are attached to support (membranes or plates, respectively) and challenge with antibodies carrying a certain label.	• Easy to perform	• Qualitative	(39, 172, 173)
			• Cheap and available	• Semiquantitative in the case of western blot and quantitative for ELISA	
			• Relatively quick		
	ExoScreen	ELISA sandwich-like system with modifications in the detection tandem. The method relies on that all the components of the system must stay closed (~200 nm, within the same vesicle) for laser stimuli transfer and detection.	• Reduced time consumption	• Qualitative	(174)
			• Increased sensitivity	• Quantitative	
			• EV isolation is not mandatory		
			• Little sample volumes are required		
	μNMR	Labeling of specific EV surface molecular markers with antibodies coupled to magnetic nanoparticles and detection by microfluidic μNMR	• Greatly higher sensitivity	• Qualitative	(175)
				• Quantitative	
	Nano-plasmonic EXO assay	A gold film with nanoholes coated with specific antibodies for the recognition of exosomal proteins is light-excited, generating surface plasmons. Joining of EVs to the antibodies causes plasmon intensity changes that are proportional to the amount of joined EVs.	• Label-free	• Qualitative	(176, 177)
			• Easy to miniaturize	• Quantitative	
			• Scalable for higher throughput detection		
			• A magnitude order more detection sensitivity than μNMR		

Abbreviations: ELISA, enzyme-linked immunosorbent assay; μNMR, micronuclear magnetic resonance spectrometry.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Lipid content of EVs has been much less studied. However, some groups have shown that EVs are enriched in certain types of lipids in comparison with their parent cells, demonstrating the sorting of these molecules. Specifically, vesicles are enriched in sphingomyelin, cholesterol, PS (193, 194), ceramide and its derivate, and, in general, saturated fatty acids (195). It is also remarkable that the lipid/protein ratios are higher in vesicles than in parent cells. In contrast, phospatidylinositols, phospatidylglycerols, phosphatidylcholine, and phosphatidylethanolamines are more present in parent cells than in vesicles (193). Recently, when using mass spectrometry, quantitative lipidomic combinations of three lipid species were shown to distinguish cancer patients from healthy controls (196).

RNAs in EVs were first described by Valadi *et al.* (197) in mast cells. They found that EXOs released by these cells contained mRNAs and miRNAs and were able to transfer their content to other cells, where mRNA was functional and could be translated into protein. More recent studies using high-throughput sequencing techniques have shown that EXOs contain various classes of small noncoding RNAs in addition to mRNA, that is, miRNA, small interfering RNA (siRNA), small nucleolar RNA, Y RNA, vault RNA, ribosomal RNA, transfer RNA (tRNA), long noncoding RNA, and piwi-interacting RNA (198–200). Ng *et al.* (171) showed that endometrial epithelial cells cultured *in vitro* produced EVs containing a different miRNA profile from that of parent cells, thus suggesting a sorting mechanism of these miRNAs into EXOs. This could constitute a mechanism for communication between the mother and the embryo with potential implications in embryo implantation. Indeed, bioinformatic studies on the EV miRNAs showed that some of the genes targeted by the miRNAs are involved in implantation. More recently, our investigation group deepened the knowledge of maternal–embryo crosstalk and demonstrated that EXOs containing miR-30d were actively transferred from endometrial epithelial cells to trophoblastic cells, were the miRNA was subsequently internalized (156).

A major problem concerning RNA analysis from EVs is the variability of the results depending on the methodology used for isolation of cells and obtaining data. One of the major factors affecting this variability is the possibility that the RNA present in the medium, for example, from lysed cells, could stick to the external EV walls, thus being isolated along with internal RNA. In this sense, RNase A treatment previous to EV RNA isolation should be conducted (201). Even with this procedure, it has been stated that extravesicular RNAs associated with proteins, such as miRNAs in complex with argonaute proteins, can circumvent RNase A degradation, thus leading to bias in interpretation of results. This protective role of protein complexes has been reported in extravesicular medium (202, 203) and inside EVs (204). To overcome complex protection, treatment with proteinase K has been proposed for dissociation of RNA–protein complexes (205). Nevertheless, negative impact in EV yields should be investigated, as proteases may provoke vesicle lysis.

Less has been reported regarding DNA content in EVs. Some studies have currently reported the presence of double-stranded DNA in EVs (206, 207), even distinguishing a different pattern of content among EV subpopulations (208). A previous study conducted in a similar way in tumor cells, using DNase to cleave extravesicular DNA, showed that EV DNA was more abundant in MVs from tumor cells than from normal cells and that this DNA was mainly single stranded (209). It has been shown that mitochondrial DNA can also be transported between cells inside EXOs, possibly constituting a pathway to transmit altered mitochondrial DNA and associated pathologies (210). This may serve as evidence of a *trans*-acting function of DNA, being able to have functional effects on the recipient cells.

Of note, both the amount and content of EV genetic cargo can be hormonally regulated in EXOs from target cells: this is of particular relevance to reproductive tissues and is further discussed later.

EV mechanism of recognition and uptake

Mechanisms of EV uptake

For EVs to act in cell–cell signaling, they must recognize their specific cellular target, bind to that cell, and undergo internalization (Fig. 2).

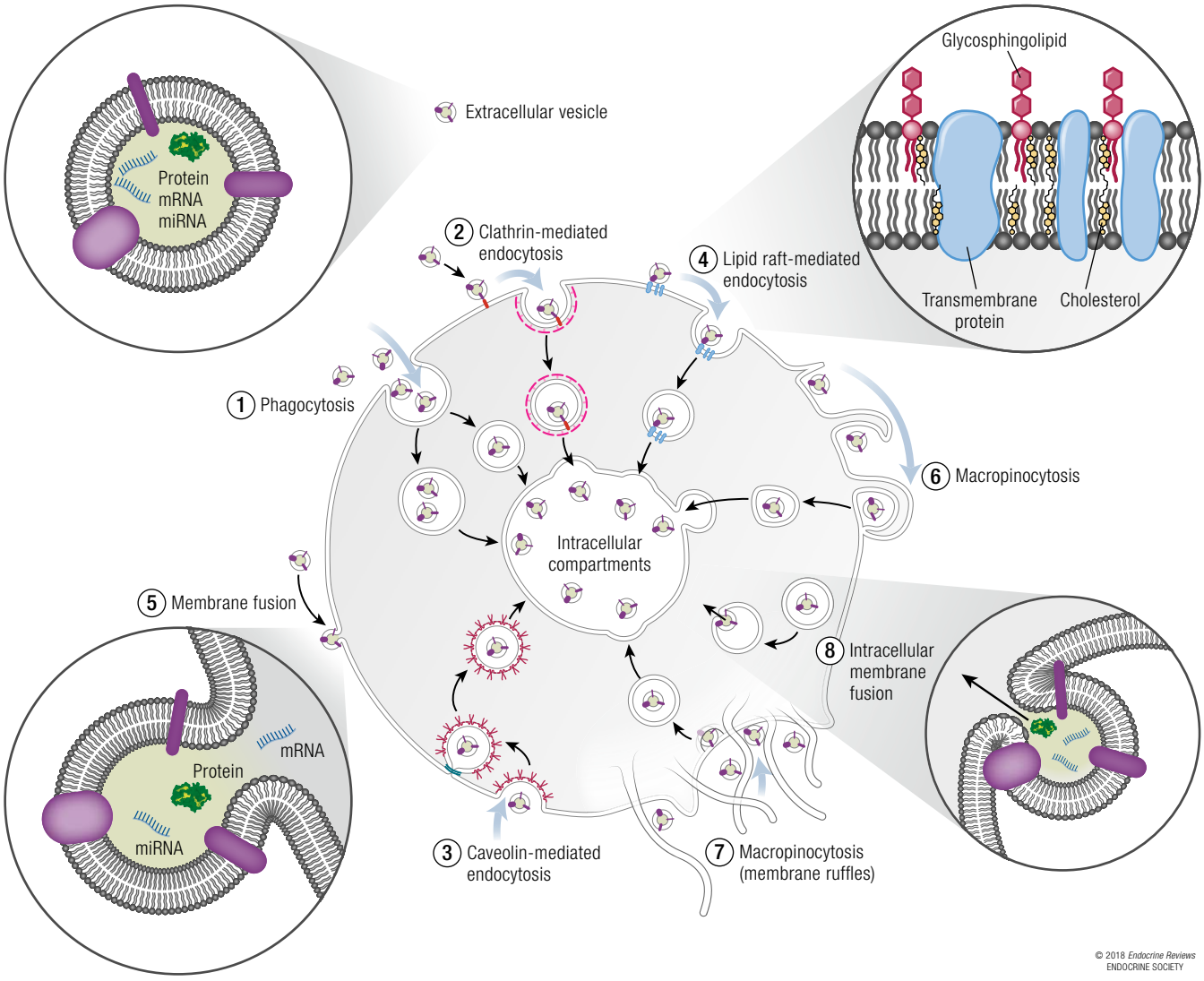
Target cell recognition. EVs may interact with recipient cells by direct signaling through ligand/receptor molecules on their respective surfaces or by direct fusion of EV and recipient cell plasma membranes (211) through lipid raft–, clathrin-, and calveolae-dependent endocytosis, micropinocytosis, and phagocytosis (212–217).

Cell surface and integral membrane/adhesion proteins on distinct EVs are important in mediating associated cell recognition and adhesion. These include integrin pairs: for example, distinct EXO integrin repertoires, specifically integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$, were identified as associated with lung metastasis, whereas EXO integrin $\alpha_v\beta_5$ associated with liver metastasis (218). The integrin profile of each EXO subtype permits selective cellular targeting.

Differences in EXO tetraspanin complexes also appear to influence target cell interaction *in vitro* and *in vivo*, possibly by modulating the functions of associated integrin adhesion molecules (219). EXO capture by dendritic cells was reduced by 5% to 30% following coincubation with blocking antibodies specific for various integrins, adhesion molecules, or tetraspanins (212). Other membrane proteins reported as important in targeting selected EVs to recipient cells

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Figure 2. Pathways shown to participate in EV uptake by target cells. EVs transport signals between cells and facilitate selective reprogramming. EVs have been shown to be internalized by cells through (1) phagocytosis and (2) clathrin- and (3) caveolin-mediated endocytosis. There is also evidence to support their interaction with (4) lipid rafts resulting in EV uptake. Lipid rafts are involved in both clathrin- and caveolin-mediated endocytosis. EVs may also deliver their protein, mRNA, and miRNA content by (5) fusion with the plasma membrane. EVs can be internalized by (6) macropinocytosis where membrane protrusions or blebs extend from the cell, fold backward around the EVs, and enclose them into the lumen of a macropinosome; (7) alternatively, EVs are macropinocytosed after becoming caught in membrane ruffles. On the other hand, (8) intraluminal EVs may fuse with the endosomal limiting membrane following endocytosis to deliver their protein, mRNA, and miRNA cargo and elicit a phenotypic response.



include intercellular adhesion molecule 1 and milk fat globule–epidermal growth factor VIII protein (220, 221). Furthermore, the delivery efficiency of EXOs to cells is reported to be directly related to rigidity of cargo lipids, including sphingomyelin and (*N*-acetylneuraminyl)galactosylglucosylceramide (23).

Recent new data indicate that proteoglycans and lectins can participate in EXO binding and internalization. Proteoglycans are cell surface proteins, whereas lectins, such as galectins 1, 3, and 5, which recognize and bind proteoglycans, are identified on EVs. Indeed, proteoglycan receptors along the plasma membranes of cells and proteoglycans on EXO surfaces have been shown to promote docking (222).

Exosome uptake and release of cargo. EV internalization by recipient cells is reported to occur via multiple processes such as phagocytosis (197, 212, 213), clathrin-mediated endocytosis (223), macropinocytosis (217), receptor interaction (224), and direct fusion (23). However, a better understanding of the underlying mechanisms and, importantly, whether EV subtypes have distinct mechanisms of uptake at their target cell specificity is required (225–229) [reviewed in (230)].

EV uptake is readily demonstrated in cell culture using fluorescently labeled EVs (91). Uptake and cargo

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

release occur very rapidly, within minutes to hours. However, such techniques do not absolutely prove release, as it is possible that the transfer and spread of fluorescence results from the culture conditions and lipid/membrane transfer.

Recent developments in modification of EVs have also facilitated monitoring and tracking their behavior, interaction, and transfer *in vivo* (231). Intracellular probes are used to fluorescently label mRNA within EVs to monitor EV-borne mRNA encoding luciferase. Developments in transgenic mice enable visualization of EV transfer to cells associated with tumor stroma (232) and immune cells (233, 234), whereas EV-mediated transfer of donor genomic DNA to recipient cells supports a mechanism for genetic influence between cells (235). Such *in vivo* approaches have not specifically shown whether transfer involves a direct fusion of EVs with the recipient cells, formation of gap junctions or nanotubes, or phagocytosis of live or apoptotic cell–derived EVs by the recipient cell.

Low pH is important for EXO uptake. There appears to be elevated stability and lipid/cholesterol content of exosomal membranes in an acidic environment (23).

Understanding recipient cell function and regulation by EXOs needs to focus on specific mechanisms of targeting and delivery, uptake, and transfer, including modulation of key signaling pathways in various recipient cells both *in vitro* and *in vivo*. Processes that control target cell recognition and EV uptake are not well understood.

Inhibiting EV recognition and uptake

Although several uptake mechanisms have been proposed for EVs, detailed knowledge regarding the key steps in EV target cell definition and definitive mechanisms of uptake is required (230) particularly because variability is found between cell types in vesicle internalization (236). The use of inhibitors is proving useful in elucidating cell type–specific mechanisms.

As discussed, using fluorescently labeled EVs, internalization can be readily observed *in vitro* within a short period of time (91, 237). Treatment with inhibitory agents such as chlorpromazine to examine clathrin-dependent uptake (214) and specific RGD inhibitory peptides (238) to target integrin-mediated EV uptake allows identification of selective processes of internalization. The efficacy of EV exchange between cells probably depends on their surface antigen repertoires because partial digestion of membrane proteins exposed on EVs with proteinase K can significantly decrease their uptake (214), and blockage of selected integrins or tetraspanins with monoclonal antibodies also has suppressive effects on EV internalization (212). Furthermore, the use of cytochalasin D, which interferes with actin polymerization and endocytosis, significantly reduces the uptake of EVs (214, 215). Similarly, the inhibition or knockout of dynamin, a GTPase responsible for formation of endosomal vesicles, significantly suppresses EV uptake (216). Further research is needed to understand the precise mechanisms that underpin distinct EV entry into selected target cells and importantly how to control this process.

EVs as Messengers in Reproductive Physiology

Normal reproductive processes are highly dynamic, with well-characterized stages. The considerable intercellular interactions involved at each stage have prompted the study of the involvement of EVs in both the male and female reproductive tracts, from preconception to birth. EVs associated with reproductive biology have been specifically identified and studied in different fluids such as prostatic and epididymal fluid (239), seminal fluid (7, 155), follicular fluid (240, 241) oviductal fluid (242, 243), cervical mucus (244), uterine fluid (156, 171), amniotic fluid (115, 245), and breast milk (246), as well as the originating tissues [reviewed in (247)] (summarized Table 3).

There are currently increasing data pointing to EVs as key regulators of different reproduction processes such as sperm and ovum maturation, coordination of capacitation/acrosome reaction, prevention of polyspermy, endometrial embryo crosstalk, and even communication between *in vitro*–cocultured embryos leading to quorum improved development (291). In these initial steps of the reproductive process (*e.g.*, preconception) EVs are widely produced by different organs and show specific functions. Once implantation has taken place, production of EVs continues throughout pregnancy, with the placenta being the main source of EVs. During early pregnancy, EVs are released by the extravillous trophoblast (EVT). Later on, the syncytiotrophoblast (STB) is formed and establishes contact with maternal blood flow. From here on, STBs constitute the main site of EV generation, and these EVs get access to the maternal systemic vasculature, where they show important roles in immune modulation, either for the innate or the adaptive response (32). Of note, EVs are also found in amniotic fluid, where they are attributed to inflammatory and procoagulant activities (292), and in maternal breast milk. In the latter case, an important influence of EV recovery procedure has been detected on subsequent analysis (293). Among attributed roles, milk EVs have been involved in bone formation, immune modulation, and gene expression regulation, with special emphasis for long noncoding RNAs (246, 294).

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

EVs in the male reproductive tract: epididymis and prostate

After leaving the seminiferous tubules, spermatozoa (SPZ) are still immature cells. SPZ are stored in the epididymis where they undergo a series of morphological and biochemical modifications that provide them with motility and fertilization ability in their transit from the caput to the cauda, a process called sperm maturation (295, 296). During ejaculation, SPZ mix with seminal fluid from the seminal vesicles, the prostate, and the bulbourethral gland to form the ejaculated semen, which is ejected into the vaginal cavity. Seminal fluid composition is crucial in promoting sperm motility and genomic stability (295, 297). Moreover, it contributes to the establishment of maternal immune tolerance (298, 299). Subsequently, as SPZ travel through the female reproductive tract to the upper fallopian tube where fertilization occurs, they interact with the endometrial and tubal milieu. Finally, to achieve successful fertilization, SPZ undergo capacitation: sperm head membranes undergo a series of biochemical modifications that enable the acrosome reaction when the spermatozoon reaches the zona pellucida of the oocyte. This leads to the release of enzymes that allow SPZ to penetrate the zona pellucida and fuse with the oocyte plasma membrane (300–302). In this context, secretions from the different components of the male and female reproductive tracts have been proposed to play a sequential role in programming sperm function (303).

Epididymosomes

Epididymosomes (EVs originating from the epididymis) were first described in 1967 by Pikó (304) in the Chinese hamster as having diameters between 20 and 100 nm and being associated with the SPZ acrosomal membrane (305). More recently, it has been shown that epididymosomes are a population of roughly spherical bilayered vesicles that display heterogeneity both in size and content that varies between the different segments of the epididymis. Their sizes range from 50 to 800 nm or even to 2 to 10 μm in the first segments of the caput (239). Their lipidic composition also varies: indeed, an increase in sphingomyelin and a general decrease in the other phospholipids and in the proportion of cholesterol occurs with epididymal progression from the caput to the cauda. This is in contrast to SPZ, where the proportions remain more constant. Epididymosomes also have an increased ratio of saturated/unsaturated fatty acids from the caput to the cauda, whereas the opposite situation is found in SPZ. Taken together, these data indicate that epididymosomes tend to gain membrane rigidity whereas SPZ membranes tend to become more fluid (239).

Two main classes of epididymosomes have been identified: CD9-positive epididymosomes, which preferentially bind live SPZ, and ELSPBP1-enriched epididymosomes, which present higher affinity for dead SPZ (306). CD9-positive epididymosomes are EVs of size ranging from 20 to 150 nm (307). These were recovered by ultracentrifugation from the total epididymal fluid EVs, specifically in the epididymis cauda. CD9-positive epididymal cargo transferred to SPZ includes proteins involved in sperm maturation, namely P25b, GliPr1L1, and MIF (248–250), in contrast to ELSPBP1, which was widespread between all EVs. Moreover, CD9, in cooperation with CD26, plays a role in promoting this transfer (307).

ELSPBP1-enriched epididymosomes constitute a subpopulation of vesicles obtained from the epididymal fluid by high-speed ultracentrifugation (120,000 × *g*) after SPZ and debris removal at 4000 × *g* (308). It had been suggested that ELSPBP1 allowed distinction between dead and viable SPZ, as it was only detectable in the dead SPZ population (309). Later, the same group demonstrated that epididymosomes were the only path for the transmission of molecules including ELSPBP1 to dead SPZ (307, 308). Interestingly, ELSPBP and biliverdin reductase A (BLVRA) can associate and bind in tandem to dead SPZ, concurrently with the epididymal maturation of SPZ, a process during which these cells cease producing BLVRA. Therefore, BLVRA could act as a quencher of reactive oxygen species generated by dead and immature SPZ, protecting viable SPZ from oxidative stress. Moreover, BLVRA may be involved in hemic protein catabolism, changes also important in the SPZ maturation process (251, 306).

"Normal reproductive processes are highly dynamic, with well-characterized stages."

Because the epididymis brings SPZ to functional maturity before they enter the vas deferens, it is not surprising that epididymosomes serve as a means for protein transfer into SPZ during their transit in the epididymal duct. Some epididymosomal proteins have proven roles in sperm maturation: these include P25b, MIF, or sperm adhesion molecule (SPAM1), among others (252, 310). SPAM1 is a hyaluronidase with roles in both fertilization and sperm maturation. It is transferred to SPZ from epididymosomes, increasing their ability for penetrating the oocyte cumulus (253). Another protein transferred to SPZ by this mechanism is ADAM7, which is important for sperm motility, morphology, and establishment of membrane correct protein composition (254, 255). Of note is the transfer of the plasma membrane ATPase 4 (PMCA4), a major Ca^{2+} efflux pump, into epididymosomes: this plays a pivotal role in SPZ maturation and motility (253). Glutathione peroxidase 5 associates with SPZ during its transit through the epididymis, protecting them from lipid peroxidation stress and, independently, is transferred to SPZ via epididymosomes (256). Finally, components of the Notch pathway are described

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 3. Main Functions of EVs in Reproductive Physiology Classified by Their Origin

EV Type	Main Features	Target	Function	References
Epididymosomes	First described by Pikó in 1967	SPZ	Transfer of molecules involved in sperm maturation (P25b, GliPr1L1, MIF, SPAM1, PMCA4)	(248–250, 252, 253)
	Sizes: 50 to 8000 nm or even 2 to 10 μm		Protection from oxidative stress (BLVRA)	(251)
			Protection from lipid peroxidation (glutathione peroxidase 5)	(256)
	Two main classes: CD9-positive (affinity for live SPZ) and ELSPBP1-enriched (affinity for dead SPZ) epididymosomes		Morphology and membrane composition regulation (ADAM7)	(254, 255)
			Sperm motility (ADAM7, PMCA4)	(253–255)
			Small RNA regulation of gene expression	(257, 258, 291)
Prostasomes	First described by Ronquist *et al.* in 1978	SPZ	Enhancement of sperm motility (progesterone receptors, Ca^{2+} cascade signaling components, aminopeptidase N)	(261, 263, 264, 265, 266)
			Protection from acidic female reproductive tract environment	(262)
	Sizes: 30 to 500 nm		Protection from oxidative stress (PMCA4)	(264)
	Unusual lipid composition that provides them with increased ordered structure, rigidity, and viscosity		Prevention of premature capacitation and acrosome reaction (cholesterol)	(260, 267–269)
			Posterior induction of capacitation, SPZ hypermotility, and acrosome reaction at the moment of fertilization (cAMP, progesterone receptors, hydrolases, lipoxygenases)	(270– 274)
			Protection from the hostile female reproductive tract: immunity, oxidative stress, bacteria	(259, 260, 270)
Uterine microenvironment EVs	Wide variety of origins: serum transudates, residues from womb cell apoptosis, endometrial epithelial cells, and conceptus	Endometrium: endometrial origin	Promotion of embryo implantation (specific miRNA cargo)	(171)
	Variations throughout the menstrual cycle	Endometrium: embryo origin	Regulation of endometrial angiogenesis (specific miRNA and protein cargo) and uterine spiral arteries remodeling	(285, 286)
	—	Embryo: endometrial origin	Embryo development (enJSRV *env* gene RNA) and subsequent priming of the endometrium for embryo harboring	(282–284)
			Promotion of embryo implantation (miR-30d, specific protein cargo, influenced by uterine hormones—functional with trophoderm)	(91, 156)
		Embryo: embryo origin	Enhancing of trophoblast cells migratory ability and implantation efficiency (laminin, fibronectin)	(88)
		SPZ	Sperm maturation (SPAM1)	(287)
			Capacitation, acrosome reaction and motility promotion (PMCA4)	(243, 288)
Oviductal EVs	First described for their implications in SPZ final competence acquisition	SPZ	Regulation of SPZ storage and promotion of capacitation, acrosome reaction, and hypermotility (PMCA4a)	(243, 279, 280, 289)
			Regulation of molecule delivery into SPZ (integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$)	(242)
		Embryo	Enhancement of embryo quality and early development	(281)

(*Continued*)

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 3. *Continued*

EV Type	Main Features	Target	Function	References
Follicular EVs	First described by da Silvera *et al.* in 2012	Cumulus–oocyte complex	Follicle development and oocyte growth (specific miRNA cargo, ACVR1, ID2)	(275, 277)
	miRNA cargo variation with female age and reproductive aging		Follicle maturation: proliferation of small follicles and inflammatory response of large developed follicles (specific miRNA signatures)	(276)
			Cumulus–oocyte complex expansion and related genes upregulation	(278)

Abbreviation: SPZ, spermatozoa.

in epididymosomes, suggesting that these vesicles transmit Notch signaling at a distance between epididymal epithelial cells, but also between the epididymis and SPZ with important implications for sperm motility (311).

Epididymosomes also convey miRNAs within the epididymal duct. As with proteins, distinct regions of the epididymis produce EVs with a specific set of miRNA whose profiles differ from those of parent cells, suggesting a sorting mechanism (290). Indeed, it has been proposed that epididymosomes may act as modulators of gene expression between sections of the epididymal duct (290). Recent analysis confirmed that they contain >350 miRNA, showing a different profile from that of parent cells and dependent on the region of the epididymis from which they originate. Finally, it was demonstrated that many of these miRNAs are transported into the SPZ (312).

An emerging concept is the transfer of traits to the offspring by epigenomic modifications. In this respect, tRNA has been attributed a new function as a modulator of genetic expression. It was initially discovered that a respiratory syncytial virus infection of lung and kidney cell lines led to the production of specific tRNA fragments (tRFs) that are able to repress the expression of specific mRNAs in the cytoplasm to favor viral replication and survival (313). Subsequently, further examples of tRFs have been described with potential implications in pathological processes, such as cell proliferation in cancer (314). Mature molecules corresponding to tRNA fragments are highly enriched in mature sperm. Interestingly, these fragments are produced by sequence-specific cleavage, giving place to fragments corresponding to the tRNA 5′ end (257). Recently, the transfer of tRFs to maturing SPZ in epididymosomes was demonstrated in mice (258), providing an explanation for the scarcity of these molecules in testicular SPZ but with an increase with SPZ maturation. A tRF (tRF-Gly-GCC) has been identified as transferred to SPZ by epididymosomes. This tRF represses MERVL, an endogenous retroelement that positively regulates a set of genes that are highly expressed in preimplantation embryos. Interestingly, male mice treated with a low-protein diet have a trend (nonsignificant) to increased tRF-Gly-GCC in mature SPZ and to downregulate tRF-Gly-GCC targets in embryos at the two-cell stage. This evidence supports that parental diet can affect the offspring epigenome; however, these preliminary data require confirmation (258).

Prostasomes

Prostasomes were first described as vesicles recovered from human prostatic fluid by centrifugation that were associated with Mg^{2+}- and Ca^{2+}-dependent ATPase activity (315). They are now considered a population of EVs produced by the prostate epithelial cells that interact with SPZ, epididymal, and seminal secretions at the time of ejaculation. They are EVs that range from 30 to 500 nm and are surrounded by lipoprotein bilaminar or multilaminar membranes (259, 316). It is likely that a population of prostasomes is exosomal, as they originate from structures resembling MVBs and exhibit classical EXO markers (317). The lipid composition of prostasomes is unusual and provides them with a characteristic highly ordered structure, rigidity, and viscosity due to several factors: a high cholesterol/phospholipid ratio reaching values of 2, which greatly surpasses the values for most of biological cholesterol-rich membranes; phospholipid composition domination by sphingomyelin, which accounts for almost a half of the phospholipids found in prostasomes (318); and finally prostasomes show a strongly saturated fatty acid profile in comparison with SPZ membranes (319). It has been reported that prostasome uptake decreases the fluidity of SPZ membranes by transfer of lipids directly dependent on the prostasome/SPZ ratio (316, 320). This decrement is crucial, as it stands as a regulator of the acrosome reaction, preventing a premature response (260).

Different roles have been attributed to prostasomes in sperm maturation and function, either directly or indirectly. These include protection of SPZ from the female acidic environment and immune surveillance modulation of SPZ motility, capacitation, acrosome reaction, and fertilizing ability, among others (259, 260, 316, 317).

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

SPZ motility is vital for a successful fertilizing ability, especially for traversing the cervical mucus and zona pellucida (321). One of the first roles attributed to prostasomes was the enhancement of SPZ motility (261) in a pH-dependent manner, suggesting that prostasomes might alleviate the negative effects of the vaginal acidic microenvironment on SPZ motility, thus showing a protective effect (262). Ca^{2+} has been well known as the major ion promoting SPZ motility and fertility, from initial studies carried out in the hamster (322). Increased SPZ Ca^{2+} levels have been linked to prostasomal delivery, directly depending on the extent of fusion/prostasome concentration and influenced by pH (323). However, it took a decade to identify a mechanism. Park *et al.* (263) showed that a progesterone-triggered long-term sustained Ca^{2+} stimulus is involved in SPZ motility promotion via fusion of (acidic) pH-dependent prostasomes. Specifically, prostasomes transferred progesterone receptors and different Ca^{2+} signaling cascade components to the SPZ neck region where, following progesterone stimulation, they triggered the release of Ca^{2+} from SPZ internal acidic stores to promote SPZ motility (263). Other proteins involved in intracellular Ca^{2+} homeostasis are also transported into SPZ in prostasomes, including PMCA4 (264), which along with nitric oxide synthases (NOSs) are delivered into SPZ by prostasomes. PMCA4 and NOS activity is stimulated by Ca^{2+} ions (324) and, indeed, NOS spatially interacts with PMCA4 to a degree positively related to Ca^{2+} concentration levels. This supports the theory that PMCA4 expels Ca^{2+} from SPZ in the presence of NOS to reduce nitric oxide production and thus oxidative stress, which could reduce SPZ viability, resulting in asthenozoospermia (264). Prostasomes also carry aminopeptidase N, a protein involved in modulating sperm motility, which acts through the regulation of endogenous opioid peptides, such as enkephalins, once in SPZ (265, 266).

Interestingly, EXO-like EVs found in cervical mucus have been reported to carry sialidase activity, which reaches a maximum during the ovulatory phase in healthy women. This is likely involved in modifying the properties of the highly glycoslyated mucus to favor SPZ access to the uterine cavity and tubes (244).

There are scarce data on the nucleic acid cargo of prostasomes and its implications for male reproductive physiology. Prostasomes contain various coding and regulatory RNAs, with potential modulatory functions (199). Interestingly, mRNA and miRNA do not represent most of the prostasomal RNA (317), and it has been postulated that mRNA in semen is predominantly transported inside vesicles whereas miRNA is mostly contained in the vesicle-free fraction of the semen, forming complexes with proteins (204). DNA inside prostasomes apparently represents random regions of the genome and is effectively transported into SPZ (325, 326). Nevertheless, this DNA may be a contaminant from ABs in the semen (327).

Capacitation is a cyclic adenosine monophosphate (cAMP)–regulated process, whose production is in turn promoted by bicarbonate and Ca^{2+} ions and influenced by membrane dynamic changes mainly due to cholesterol composition (259, 317). It has been proposed that prostasomes may act as inhibitors of the capacitation process and acrosomal reaction, mainly through transfer of cholesterol (267, 268). Indeed, this might represent a mechanism to avoid premature capacitation and acrosome reaction (260, 269). A switch between positive and negative regulation exerted by prostasomes may be influenced by the environment or even determined by specific prostasome subpopulations. cAMP promotes capacitation through the protein kinase A (PKA) axis by the simultaneous tyrosine phosphorylation of specific downstream proteins and plasma membrane protein and lipid remodeling. This remodeling breaks down plasma membrane asymmetry, exposing cholesterol molecules to external acceptors to trigger the capacitation process (270). In this context, coincubation of equine SPZ with prostasomes led to increased cAMP levels and tyrosine phosphorylation of PKA cascade proteins, in addition to the prostasome endogenous PKA activity described in previous reports. However, these changes were not correlated with increased capacitation and acrosome reaction rates and reverted after 3 hours of coincubation in capacitating conditions (267). Interestingly, Aalberts *et al.* (270) observed that at least a subpopulation of prostasomes is able to bind to live SPZ only when capacitation-inducing conditions are established, probably to promote hypermotility and acrosome reaction at the precise moment it is needed. Nonetheless, care should be taken when interpolating these results into humans, as they were obtained from a stallion model, a species that deposits its ejaculate directly in the uterus.

Following capacitation, SPZ need to undergo an acrosome reaction to enable penetration of the zona pellucida of the oocyte and fusion of plasma membranes. The zona pellucida glycoprotein ZP3 is mandatory for this process, as it facilitates sperm binding, triggering the acrosome reaction. Nevertheless, the acrosome reaction begins before the SPZ contacts the zona pellucida, probably due to the progesterone-dependent stimulus produced by cumulus cells (328). Conversely, prostasomes have been proposed as inhibitors of the acrosome reaction through the transference of cholesterol to the SPZ (268, 329) or as inducers by facilitating progesterone uptake by the SPZ (271), most likely by the transfer of progesterone receptors (263). Other studies also supporting the promotion of the acrosome reaction via prostasomes include delivery of molecules to the SPZ membrane in a pig model

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

(272), or progesterone priming, acting via the Ca^{2+} signaling axis (330). Other acrosome reaction–promoting molecules in prostasomes include hydrolases (273) and lipoxygenases (274).

In summary, the role of prostasomes in sperm-fertilizing ability in humans is most likely the result of orchestrated actions. Initially, prostasomes would attach to SPZ after mixing during ejaculation, favored by the acidic environment of the vagina, thus transferring cholesterol to stabilize SPZ membranes and prevent premature capacitation and acrosome reaction. This would enable prostasomes to pass the barrier of cervical mucus adhered to SPZ with subsequent fusion and transfer of their content to the SPZ when the SPZ first contacts the oocyte. At this time, the progesterone secreted by the cumulus cells would activate Ca^{2+}-dependent pathways that promote the capacitation process and acrosome reaction (259).

Finally, of note is the role of prostasomes in protecting SPZ from the potentially hostile female genital tract. They appear to exert roles as protectors from female immunity, antioxidants, antibacterial agents, and the process of semen liquefaction [see reviews in (259, 260, 270)].

EVs in the female reproductive tract: follicular fluid, oviduct/tube, and uterine cavity

Contemporarily with sperm maturation, coordinate oocyte development must be taking place so that both gametes can meet at the appropriate location and time inside the female reproductive tract. Developing oocytes are arrested in prophase I of meiosis in primordial follicles from the fetal period until female reproductive maturity. From this moment, cohorts of these oocytes cyclically restart growth, forming the zona pellucida, while granulosa cells proliferate to form the cumulus, which will support posterior egg fertilization. Concomitantly, meiosis is reinitiated, extruding the first polar body and arresting again at metaphase II during ovulation (331). The resumption of meiosis is stimulated by the luteinizing hormone peak, which in turn is initiated by a surge in estradiol-17β levels due to the secretion by granulosa cells from the preovulatory follicle and results in ovulation 36 hours later (332). After ovulation, the extracellular matrix of the cumulus cells serves as an adhesion dock for the fallopian tubes, through which the eggs travel as far as the ampulla where they await SPZ for fertilization (333). Following fertilization, embryo development to the blastocyst stage proceeds as the embryo passages through the fallopian tubes, reaching the uterine cavity ~4 days after ovulation. The blastocyst undergoes final preparation for implantation into the maternal endometrium in the microenvironment of uterine fluid with implantation occurring 6 to 10 days after ovulation (334).

The process of embryo implantation can only occur during a short period of time during the luteal phase of the menstrual cycle, which has been classically regarded as the window of implantation and that typically extends from 5.5 to 9.5 days after ovulation in healthy normal cycling women (334, 335). At this point, different factors affect and limit embryo implantation, namely embryo quality, endometrial receptivity and embryo–endometrial crosstalk (336), where EVs stand as important potential mediators.

During this process, EVs carry out many different supporting actions: they assist follicle and oocyte development and maturation at the initial stages, and they further assist early embryo development and implantation as the conceptus reaches the uterus. Furthermore, female tract EVs contribute in preparing endometrial vascular net, promote embryo implantation, and prime the endometrium for harboring the embryo. Moreover, these EVs also contribute to SPZ maturity, capacitation, and acrosome reaction coordination, support SPZ storage while waiting for the oocyte, and regulate molecule delivery into SPZ during this period. All of these concepts are discussed in the following sections.

Follicular fluid EVs

Oocyte maturation occurs within the microenvironment of follicular fluid (337). The easy availability of this fluid during oocyte retrieval in assisted reproductive techniques makes it attractive in the search of biomarkers for oocyte quality (338). EVs (resembling EXOs and MVs) were first identified in follicular fluid by da Silveira *et al.* (339), who demonstrated follicular fluid EV uptake by granulosa cells, both *in vivo* and *in vitro*, and their protein and miRNA cargo. EV miRNAs were also present in the surrounding granulosa and cumulus cells, thus suggesting EVs as a vehicle for biomolecule transfer within the ovary. Of particular interest, the miRNA signature of follicular EVs varied with the age of the female, suggesting EV miRNA cargo as an indicative and possible predictor of age-related decline in oocyte quality (339). Subsequently, EV miRNAs were further evaluated and a set of four differentially expressed miRNAs based on age (young/old) was defined. However, these age-related miRNAs were studied in complete follicular fluid samples and as such cannot be confidently attributed to EVs (340).

The miRNA of bovine follicular fluid is present both as free miRNAs and in EXOs, each with different composition (275). The EXOs were taken up by granulosa cells *in vitro*, resulting in increased miRNA content and variations in mRNA profiles: some of the affected genes are involved in follicle development. Moreover, some of the miRNA within EXOs may also contribute to oocyte growth, as they were differentially expressed in follicles containing oocytes at different maturation stages (275). A more exhaustive characterization of the EV content of bovine follicular fluid demonstrated variation in number, protein markers,

"Following capacitation, SPZ need to undergo an acrosome reaction to enable penetration of the zona pellucida."

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

and miRNA contents depending on the developmental stage of the follicles. What is more interesting, variation in the miRNA signature suggested a switch in genetic programming concurrent with follicular maturation. As such, EV miRNAs from small follicles preferentially promoted cell proliferation pathways whereas those from large follicles related to inflammatory response pathways (276). A possible role in follicle development through the TGFB/BMP axis by follicular fluid–derived exosomes was demonstrated when granulosa cells were exposed to follicular fluid exosomes *in vitro*. It was proposed that these effects were triggered by the direct delivery of ACVR1 and ACRV1 regulatory miRNA within follicular EXOs to granulosa cells (277).

Cumulus–oocyte complex expansion is a critical process for ovulation. In this context, *in vitro* coculture experiments using bovine follicular fluid–derived EXOs and cumulus–oocyte complexes from mice and cattle revealed that follicular EVs are taken up by cumulus cells, promoting both cumulus expansion and related expansion of genes (278).

Oviduct/tubal EVs

Fertilization of the oocyte by SPZ occurs within the fallopian tubes/oviduct. After capacitation, SPZ must undergo an acrosome reaction and maintain hyperactivated motility to fuse with the oocyte, with both functions being regulated by high intracellular Ca^{2+} concentration levels. In this context, the major murine Ca^{2+} efflux pump PMCA4, and particularly its splicing variant PMCA4a, is predominant in oviductal fluid, compared with uterine and vaginal fluids, and is totally associated with EVs. Moreover, these PMCA4a-carrying vesicles had exosomal characteristics and were taken up by SPZ, where the efflux pump was functionally relocated to their membranes. This was the first study describing the presence of EXOs in the oviducts and introduced the relevance of PMCA4 as a tool for the maintenance of Ca^{2+} homeostasis and SPZ viability during SPZ storage, regulating capacitation and acrosome reaction timing and SPZ motility (242, 243, 279, 280). Subsequently, the same authors discovered that integrins ($\alpha_5\beta_1$ and $\alpha_v\beta_3$) in oviductal EVs were transferred to SPZ and were involved in EV–SPZ fusion for cargo delivery. Although the oviductal EVs include both MVs and EXOs, the former appeared to be more efficient in fusing with SPZ (242).

Bovine oviductal EVs produced *in vitro* by cell lines have beneficial effects on the quality and development of *in vitro*–cocultured bovine embryos, suggesting a functional communication between the oviduct and embryo during the early stages of embryo development (281). However, these results must be treated with caution, as oviductal EVs produced *in vitro* have been observed to carry a different cargo compared with *in vivo*–produced EVs. This is the case, for example, for OVGP and HSPA8, oviductal proteins known to be important in the fertilization process and early pregnancy. Whereas HSPA8 was found in both *in vitro* and *in vivo* EXOs, OVGP was absent in EXOs of *in vitro* origin (341).

Uterine EVs

Endometrial fluid is a viscous liquid secreted by the endometrial epithelial cells from the glands into the uterine cavity. Because the endometrium is a hormonally regulated organ, the molecular composition of the fluid varies depending on the phase of the menstrual cycle (342). Uterine fluid, a biologically and clinically relevant sample source (343), also contains contributions from the oviductal fluid and a large cohort of plasma proteins along with other factors differentially exudated from the blood (344). Importantly, this uterine fluid carries information that mirrors maternal environmental exposure and possibly relays such information to the embryo, subsequently generating long-term epigenetic effects on the offspring via embryonic and placental programming.

To date, EVs have been reported throughout menstrual/estrous cycles in the endometrial fluid of different species, including humans (156, 171) and sheep (282–284, 345), and they are also released by endometrial epithelial cells in culture (156, 171).

Ng *et al.* (171) first described the production of EVs by human endometrial epithelial cells in primary culture and by the endometrial epithelial cell line ECC1. These EVs contained a specific subset of miRNAs, not detectable in the parent cells. Bioinformatic analysis revealed that some of the target genes of the EV miRNAs are relevant to processes involved in embryo implantation. Importantly, they also verified the presence of EVs in human uterine fluid and the associated mucus (171).

Greening *et al.* (91) demonstrated that the proteome of highly purified EXOs derived from human endometrial epithelial cells is regulated by steroid hormones and thus varies with the progression of the menstrual cycle. Under follicular phase hormonal conditions, when estrogen constitutes the main hormonal stimulus, the EXO proteome was enriched in proteins related to cytoskeletal reorganization and signaling cascades, coinciding with the phase of endometrial restoration. Importantly, after ovulation, when progesterone is the dominant hormone driving endometrial receptivity, the proteome altered with changes indicating enrichment in extracellular matrix reorganization and embryo implantation. As in other systems, the exosomal protein profiles were shown to be distinct from parental cells. Importantly, this study demonstrated that endometrial EXOs were transferred and internalized by human HTR-8 trophoblast cells, enhancing their adhesive capacity, partially through focal adhesion kinase (FAK) signaling (91). This was

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

significantly higher when the EXOs were derived from cells subjected to both estrogen and progesterone to mimic the receptive phase of the menstrual cycle.

Embryonic and trophectodermal EVs

Interestingly, murine embryonic stem cells from the inner cell mass generate MVs that reach the trophectodermal layer and enhance the migration ability of trophoblast cells in culture, either as isolated cells or in the whole embryo. The presence of laminin and fibronectin in the cargo of the inner cell mass EVs enabled attachment to the integrins on the trophoblast cell surfaces and stimulated c-Jun N-terminal kinase and FAK cascades, increasing trophoblast migration. Furthermore, injection of these EVs inside the blastocoele cavity of day 3.5 blastocysts increased their implantation efficiency (88). Importantly, note that this mechanism may be particular to the mouse and to other species in which the inner cell mass is distal to the site of trophectoderm attachment to the endometrial surface: in women this is the reverse, with the extracellular matrix tightly aligned with the attaching trophectoderm.

EVs produced by ungulate trophectoderm participate in crosstalk with the maternal endometrium (283). Bidarimath *et al.* (285) observed that EVs from a porcine trophectodermal cell line stimulated the proliferation of endothelial cells *in vitro*, thus being potential regulators of maternal endometrial angiogenesis. These vesicles contained an miRNA and protein cargo likely to annotate functions in the angiogenesis process. Again, care should be taken with these data, as they were retrieved from cell lines cultured *in vitro*. Furthermore, the pig is a species with epitheliochorial placentation, and thus the *in utero* development is very different from that of the human (285). Nevertheless, study of human EVT cell (HTR-8/SVneo and Jeg3)–derived EXOs similarly showed that these vesicles promote vascular smooth muscle cells migration, which is important during human uterine spiral artery remodeling in successful pregnancies (346). Importantly, the two trophoblast cell lines (which are different stages along their differentiation pathway) produced differential migration results, raising the likelihood that cell origin as well as content and bioactivity of the exosomal cargo are of considerable importance, emphasizing the need to keep models as close to the physiological situation as possible.

EVs as vehicles for embryo–maternal crosstalk. The first indication that the endometrium produced EVs with unique cargo was that the human endometrial epithelial cell model ECC1 (which best represents luminal epithelium) released EVs containing a different miRNA profile from that of parent cells (171). These EVs could provide a mechanism for communication between the mother and the embryo with potential implications in embryo implantation. Indeed, bioinformatic analyses on the EV miRNAs showed predominance of the genes targeted by the miRNAs as involved in implantation. Furthermore, interrogation of the proteome of ECC1 EVs, cultured under conditions to represent the proliferative (estrogen-dominant) and secretory (estrogen plus progesterone) phases of the cycle, showed that the protein cargo of EVs is hormone specific, enriched with 254 and 126 proteins, respectively (91). Importantly, 35% of the endometrial EV proteome had not been previously reported, indicating the unique cargo of endometrial EVs. These findings were validated in EVs from primary endometrial epithelial cells. Functionally, the EVs were internalized by human trophoblast cells, inducing increased adhesive capacity, that was at least partially mediated through active FAK signaling, indicating a likely role in promoting embryo implantation (109). Interestingly, among the implantation-related proteome of these endometrial EXOs were the cell surface metalloproteinases ADAM10 and matrix metalloproteinase-14 (a membrane-bound matrix metalloproteinase), for which there are abundant substrates on the trophectoderm.

Another study showed that endometrial epithelial-derived EVs in the uterine fluid contain hsa-miR-30d during the receptive phase of the cycle. This EXO-associated hsa-miR-30d was internalized by mouse embryos via the trophectoderm, resulting in an indirect overexpression of genes encoding for certain molecules involved in the murine embryonic adhesion phenomenon—*Itgb3*, *Itga7*, and *Cdh5*. Functionally, *in vitro* treatment of murine embryos with miR-30d resulted in a notable increase in embryo adhesion, again indicating how maternal endometrial miRNAs might act as transcriptomic modifiers of the preimplantation embryo (156).

"Fertilization of the oocyte by SPZ occurs within the fallopian tubes/oviduct."

Implications of EVs in Reproductive Pathology

Given their seminal functional role and presence in various aspects of reproductive biology, a growing field of evidence is uncovering potential roles for EVs in regulating reproductive pathological conditions, including endometriosis, polycystic ovaries syndrome, erectile dysfunction (ED), early pregnancy loss, hypertension, preeclampsia (PE), and gestational diabetes (GD) mellitus (summarized Table 4). Given this importance of EVs during maternal environment and development, significant efforts are now focused on evaluating prognostic value and applicability of EVs as diagnostic and therapeutic agents (107, 370).

EVs in endometriosis

Endometriosis is an estrogen-dependent inflammatory disease that is characterized by the deposition and growth of endometrial cells outside the uterine cavity,

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Table 4. Involvement of EVs in Reproduction-Related Pathologies

Disease	EV Pathogenic Role			References
Endometriosis	Promotion of endometriotic lesions invasion and progression			(347, 348, 350)
	Enhancement of angiogenic potential			(347, 349)
Polycystic ovaries syndrome	miRNA expression regulation toward PCOS phenotype			(351)
ED	Promotion of endothelial dysfunction, vascular damage, and atherogenesis			(352, 353)
Early pregnancy loss	Induction of an excessive procoagulant activity			(354, 355, 368)
	Promotion of endothelial dysfunction			(356)
PE	Placental origin	Promotion of abnormal remodeling of uterine spiral arteries		(333)
		Enhancement of angiogenic failure and subsequent endothelial dysfunction		(358)
		Stimulation of proinflammatory and procoagulant activities		(357–360, 366)
		Generation of oxidative stress into the placenta and mother vasculature		(361, 362)
	Maternal origin	General	Transportation of PE risk factors	(363)
			Failure to ensure appropriate vascular development	(367)
		Platelet EVs	Unleashing of thrombo-inflammatory placental response	(364)
		Leukocyte EVs	Promotion of proinflammatory cytokines release by the placenta	(365)
GD mellitus	Promotion of proinflammatory cytokines production by endothelial cells			(369)

Abbreviation: PCOS, polycystic ovarian syndrome.

with the pelvic peritoneum and ovaries being the most common sites for ectopic growth (371). For this reason, endometriosis is considered a benign metastasizing disease (372).

Endometriosis is characterized in part by an increase in the expression of angiogenic factors and metalloproteinases. Patients with endometriosis show higher levels of these molecules in endometriotic lesions than in eutopic endometrium, and eutopic endometrium of endometriosis patients shows higher levels than in healthy endometrial controls (347). Indeed, by inhibiting metalloproteinases it is possible to avoid the establishment of ectopic endometriotic cysts (373). In this context, EMMPRIN, a metalloproteinase inducer, is carried in EVs produced by uterine epithelial cells and stimulates the expression of metalloproteinases in stromal fibroblasts. The secretion of both EMMPRIN and metalloproteinases is positively regulated by IL-1β/α, whose secretion is increased in women under endometriosis conditions in whom there is a proinflammatory peritoneal environment. This would allow the increase of metalloproteinase production by fibroblasts to trigger endometriotic lesion invasion (348).

In terms of EV RNA cargo, EVs from endometrial stromal cells from women with endometriosis vs women without the disorder showed different profiles of exosomal miRNA content between EVs derived from eutopic and ectopic endometrium from endometriosis subjects and between eutopic endometrium from women without or with disease (349). Moreover, there was a differential miRNA signature between eutopic endometriotic and control EXOs. Among these miRNAs, miR21 is already known for a role in angiogenesis. It remains to be established whether miR-21 can promote angiogenesis following EV uptake (349).

Ectonucleotidases are enzymes involved in inflammatory processes and previously reported as expressed in the endometrium. Teixidó *et al.* (350) investigated ectonucleotidase activity from endometriotic cysts (endometriomas) on the ovary, one of the common sites for endometriotic lesion development. Ectonucleotidases were highly enriched in endometriomas compared with simple cysts. Interestingly, the ectonucleotidase activity was also contained by EXOs derived from endometriomas and simple cyst fluids, but it was significantly higher for EXOs from endometriomas.

Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is one of the most common hormonal disorders affecting women, characterized by androgen excess and insulin resistance, leading to androgenism, high risk of glucose intolerance, diabetes, and lipid abnormalities (374). Its complex phenotypic manifestation was formally described nearly a century ago as the concurrence in

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

women of amenorrhea, hirsutism, obesity, and typical polycystic appearance of the ovaries (375).

Koiou *et al.* (376) observed that platelet MVs in plasma from PCOS affected women (defined by elevated circulating androgens and insulin resistance markers) were at higher levels than in healthy controls. Moreover, there was a significant positive correlation between MVs and numbers of follicles in the ovaries of these women. Subsequent confirmation of the increase in EV levels (mainly of exosomal size) in PCOS also demonstrated a direct correlation with insulin resistance markers. Furthermore, polycystic ovary–derived EVs showed a higher content in annexin V along with 16 miRNAs that are normally expressed at low levels, being increased with PCOS (377).

Sang *et al.* (351) described EVs in the human follicular fluid and identified 120 miRNAs within their cargo, 11 of which were highly expressed and with target genes in pathways involved in reproduction, endocrine, and metabolic processes. Two of these miRNAs, miR-132 and miR-320, were significantly decreased in the follicular fluid EVs from PCOS patients compared with nonaffected controls (351). Of note, miR-132 and miR-320 have HMGA2 and RAB5B, respectively, as target genes: these were associated with key roles in the etiology of PCOS in a previous genome-wide association study (378).

DENND1A is a PCOS candidate locus, characterized in a number of genome-wide association studies (378, 379). DENND1A variants at two levels, both at the protein and mRNA levels, were increased in theca cells of PCOS patients compared with healthy controls. In agreement with these results, mRNA for this locus was significantly increased in EXOs extracted from the urine of PCOS-affected women in comparison with normal-cycling controls. In this sense, the exosomal miRNA profile is proposed to reflect the physiological status of the source cells, providing a potential biomarker of PCOS (380). Further studies are needed to uncover the roles of EVs in the triggering and development of PCOS.

Erectile dysfunction

ED is the most studied sexual problem worldwide and mainly affects men >40 years of age. It costs up to £7 million in the United Kingdom and $15 billion in the United States. The prevalence of this condition varies greatly throughout the world, highlighting the Middle East (45.1%), United States (37.7%), and especially mainland China (varying from 17.1% to 92.3%), according to a retrospective study carried out on men of different ages (381).

Microparticles have been proposed as involved in endothelial dysfunction and atherogenesis, with special regard to ED. Initially, microparticles defined as membrane vesicles, apoptotic or not, <1.5 μm were recovered from plasma after platelet depletion at 900 × *g* and measured by flow cytometry using specific markers (352). These circulating endothelial-derived microparticles were increased in type 2 diabetic men with ED, compared with controls, and a positive correlation between microparticle counts and ED severity, determined by the International Index of Erectile Function, was shown. However, diabetes risk factors did not influence microparticle levels, and thus these were postulated to be independently linked to ED severity. Finally, microparticles were proposed as possible links between endothelial dysfunction and ED (352). Retrospectively, a molecular signature identified in microparticles enabled discrimination between diabetes and ED. The marker CD31 in microparticles was mainly related to diabetes, whereas CD62E was directly linked to ED, without diabetes. The CD31/CD62 ratio could be used to evaluate endothelial function, with a high ratio being related to endothelial activation and a low ratio associated with apoptosis. In the study, diabetic men with ED showed lower ratios, possibly indicating a cooperative effect of the two disorders. Finally, levels of $CD31^+$ microparticles were directly correlated with ED aggressiveness (382).

La Vignera *et al.* (353) increased the centrifugal force to achieve a better clearance of platelets from serum (13,000 × *g*). They confirmed an increase in endothelial-derived microparticle levels in ED patients with arterial etiology, in comparison with patients with ED of psychogenic origin. Because a positive correlation was observed with typical ED metabolic parameters, they proposed endothelial dysfunction as the cause underlying ED and reasserted microparticles as predictors of the condition. Furthermore, their levels were directly related to the aggressiveness of arterial ED (383): a combination of disorders leading to greater vascular damage was associated with more severe ED and endothelial dysfunction, and correlated with increasing levels of endothelial microparticles (384).

ED is associated with increased endothelial apoptosis, and both can be, in part, reverted by treatment with a type 5 phosphodiesterase inhibitor such as tadalafil (385). Treatment benefits were maintained for 4 weeks after the cessation of a 1-year treatment in almost half of the analyzed cases (386). Subsequently, the effect of tadalafil treatment and discontinuation on the production of apoptotic endothelial-derived microparticles was examined. ED patients had increased levels of apoptotic microparticles compared with controls before the start of the treatment. Ninety days of tadalafil administration improved International Index of Erectile Function score and endothelial parameters and reduced apoptotic microparticle levels, although not to control levels. These improvements reverted by 6 months after treatment discontinuation (387). Interestingly, complementation of tadalafil treatment with an antioxidant maintained the tadalafil effects at least until 6 months after treatment cessation, prolonging the duration of the antiapoptotic effect within the endothelium (388). This is in accord with

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

other studies implicating oxidative stress in endothelial dysfunction (389, 390). Patients with greater severity and duration of ED, associated with the concurrence of high cardiovascular risk profiles, were nonresponders to sildenafil, another type 5 phosphodiesterase inhibitor.

Androgen deficiency has also been proposed to contribute to the development of cardiovascular disease and endothelial function impairment (391). Six months of androgen replacement therapy (testosterone) improved endothelial and ED features and decreased endothelial derived microparticle levels in patients of ED and late onset hypogonadism (a new vascular risk factor) (392). Indeed, late-onset hypogonadism worsened metabolic parameters and increased the already high endothelial microparticle levels in ED patients (393).

Pregnancy complications

EVs from a variety of sources (epididymis, prostate, cervical mucus, ovarian follicle, embryo, and endometrium) have potential roles in both the establishment and development of a successful pregnancy. However, from the sixth week of gestation (394), placenta-derived EVs mainly of STB origin represent the main source of vesicles with potential implication in feto–maternal communication (32, 87). Their concentrations in maternal plasma increase gradually as pregnancy progresses (286). Their release and bioactivity are favored by both low oxygen tensions (395) and high D-glucose concentrations (396). Changes in concentration, composition, and bioactivity of placental and nonplacental EVs have been reported in pregnancy disorders (397). Notably, the secretion of EVs is increased in the two main EV-related pregnancy complications, that is, GD (398) and PE (399).

EVs in early pregnancy loss

Early pregnancy loss is a common complication that affects ~15% of the gestations and shows recurrence rates of 2% to 3%. Importantly, up to 50% of these cases are usually of idiopathic etiology (400). Interestingly, the levels of plasma endothelial microparticles are decreased in pregnancy loss, especially in cases with recurrent miscarriage, compared with controls (401). However, these results should be viewed with caution, as in healthy pregnancy (their controls) there is also an increase in EV levels, mainly due to the contribution of placenta-derived EVs (394).

In pregnancy, the hemostatic balance shifts toward upregulated procoagulant activity, with increased clotting factors and fibrinogen, and concurrently decreased anticoagulant factors and fibrinolytic activity (402). An excessive procoagulant response leading to thrombosis of the uteroplacental vasculature and subsequent hypoxia has been proposed as a factor accounting for an important part of the fetal loss cases (403). In this regard, blood microparticles with procoagulant activity are increased in miscarriage cases, in parallel with the enhanced coagulation-promoting activity. These microparticles may play a role in this outcome by favoring the thrombotic phenomena (354, 355). Furthermore, pregnancy loss–affected women present with lower levels of platelet microparticles and higher levels of endothelial microparticles than do controls; although this could not be directly related to the hypercoagulation phenotype, it was suggested to reflect endothelial dysfunction (356). In contrast, plasma platelet-derived microparticles were increased in women with recurrent miscarriage compared with controls (376). However, these results may be biased by the small size of the study population (404), and the controls may be inappropriate due to the contribution of the placenta to the total EV content.

EVs in gestational vascular complications

Gestational vascular complications, which include hypertension and PE, are prevalent causes of maternal and fetal morbidity and mortality. Hypertension may appear as a consequence of abnormal placentation into the maternal uterus, and it may lead to the development of impaired liver function, progressive renal insufficiency, pulmonary edema, and the new onset of cerebral or visual disturbances that might end in the hemolysis, elevated liver enzymes, and low platelet count syndrome and/or eclampsia (405). PE is a complex disorder causing preterm birth, intrauterine growth restriction, and maternal death (406). In general, different studies point toward an increase in endothelial microparticle shedding within gestational vascular complication conditions, thus suggesting vascular injury (407).

Preeclampsia

PE is a pregnancy-related syndrome affecting between 2% and 8% of pregnancies and is characterized by a variety of systemic symptoms. It is detected by new-onset hypertension and proteinuria after the 20th week of gestation. Its etiology is not well known, but the pathogenesis of PE is conceptualized in a two-stage model with the placental defect precipitating an abnormal vascular maternal response that manifests as the signs of this pathological condition. Early PE appears before 34 weeks of gestation and involves the fetus, showing reduced placental perfusion, possibly due to abnormal trophoblast invasion and/or uterine spiral artery remodeling. Late PE appears after 34 weeks, and the maternal manifestations appear; a series of inflammatory, metabolic, and thrombotic responses compromise vascular function up to the point of producing systemic organ damage (408).

Several published studies have attempted to elucidate the relevance of EVs of both maternal and placental STB origin in the pathophysiology of PE. Changes in EV concentration and cargo affect PE

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

development via proinflammatory and procoagulatory activity enhancement. In this section, we summarize current knowledge of EVs in relationship to PE.

Placenta-derived EVs. The placenta plays a critical role and is undoubtedly the source of PE development. PE can develop even in the absence of a fetus, provided that trophoblast tissues are established, forming the characteristic mass known as a hydatidiform mole, a tissue abnormality formed by the distension of some or all of the chorionic villi (409).

STB-derived EXOs and MVs (STBMs) are increased in PE compared with normal pregnancies (357), perhaps in part owing to the hypoxia that results from abnormal placentation (346). This increase occurs specifically in early-onset PE cases but not in late-onset PE or normotensive intrauterine growth restriction (410, 411). Importantly, early-onset PE is established in the first trimester when trophoblast invasion and vascular remodeling occur (346), emphasizing the importance of STBMs in these processes. Furthermore, variations in protein (399, 412, 413), lipid (414), and miRNA (399) cargo of STBMs may explain the specific roles of STBMs in PE, including immune response, coagulation, oxidative stress, and apoptosis.

One of the main characteristics of PE is abnormal remodeling of the uterine spiral arteries, which in normal pregnancies ensures enough maternal blood flow to support fetal growth and development. Thus, a role for EVT-derived EVs has been proposed in PE development. Variations in concentration, cargo, and bioactivity of EVT-derived EVs as indicated above may result from a proinflammatory environment, inducing these changes, impairing their physiological roles in vascular/smooth muscle tissue remodeling, and thus stimulating the emergence of PE (346, 415). In PE, increased amounts of proinflammatory cytokines (IL-18, IL-12, TNF-α) are released by monocytes and lymphocytes. PE-increased STBMs can bind monocytes to promote the production of more inflammatory cytokines, perpetuating the proinflammatory environment and hence stimulation of EV alterations and endothelial cell damage (357). Furthermore, villous cytotrophoblast-derived EXOs carry syncytin-1 and syncytin-2, which are involved in EXO fusion with the target cells. Importantly, syncytin-2 content was reduced in EXOs derived from serum of PE patients (416).

Antiangiogenic factors, such as sFlt1 and sEng, appear to participate in PE through a series of mechanisms that lead to the imbalance of angiogenic factors and finally to the generation of endothelial dysfunction and the maternal syndrome of PE. Increasing levels of sFLT and sEng can predict PE and directly correlate with the aggressiveness of this syndrome (417). PAI-1 and, to a lesser extent, PAI-2, which is predominant in placenta, are important inhibitors of fibrinolysis. Their overactivation results in the establishment of fibrin deposits that occlude placental vasculature and spiral arteries, leading to hypertension and endothelial damage causing PE. Moreover, increasing levels of PAI-1 in plasma directly correlate with PE severity (418). Eng and PAI-2 are highly expressed and localized to the surface of STBM MVs and EXOs, and thus can readily influence the development of PE (358). Additionally, STBMs from PE patients possess increased tissue factor activity compared with normotensive patients (359), and this could increase fibrin deposition. Coagulation may be enhanced by STBM action directly by direct association with platelets leading to activation: such activity is increased in PE-derived STBMs and correlates with PE-associated thrombotic risk. Moreover, treatment with aspirin, which is usually prescribed for PE women to reduce platelet aggregation, also inhibits STBM-induced platelet aggregation (360).

Cell-free hemoglobin (HbF) is released by the placenta, and increased hemoglobin expression as well as HbF accumulation in the vascular lumen of PE placentas have been reported (419). Indeed, HbF has been proposed as an important factor marking the transition between the first and second stages of PE. HbF causes placental damage similar to that observed in PE by inducing oxidative stress, which affects the blood–placenta barrier integrity (420). Blood–placenta barrier disruption may lead to the release of placental factors, including HbF, which leak into the maternal circulation, contributing to the maternal manifestations of PE. Moreover, levels of HbF correlate with PE severity symptoms (421). Placental HbF can provoke differential alterations in STBM miRNA cargo between EV populations: three miRNAs were specifically downregulated in MV populations under HbF influence. STBMs may also transport HbF itself, although these data may be an artifact of the external HbF perfusion (361). Furthermore, STBMs from PE pregnancies exacerbated the production of superoxide radicals by neutrophils in a dose-dependent manner, also correlating with PE severity. In this way, STBMs display multiple mechanisms to cause vascular damage and dysfunction in women with PE (362).

Maternally derived EVs. Even before pregnancy, maternal risk factors for PE are obesity, diabetes mellitus, hypertension, and systemic lupus erythematous. Pro-PE EVs have altered concentrations and modified molecular contents that may alter the functioning of maternal tissues prior to pregnancy. In particular, changes in endothelial cells and leukocyte- and platelet-derived EVs are associated with the risk of PE. All share the common feature of a general increase in endothelial and platelet-derived EV levels [see review in (363)].

Once pregnancy is established, maternal EVs of different cellular origin interact with embryonic tissues with potential implications in PE pathogenesis. Platelets have crucial roles in PE development, and

"Early pregnancy loss is a common complication that affects ~15% of the gestations and shows recurrence rates of 2% to 3%."

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

several studies report decreased platelet-derived EV levels in pregnancy compared with nonpregnancy, with a further decrease in PE (363). EVs of maternal endothelial and platelet origin appear to unleash a thrombo-inflammatory response in the placenta. EVs cause activated platelet aggregation and inflammasome activation within the placental vascular and trophoblastic cells, triggering a PE-like phenotype. Furthermore, inhibition of inflammasome or platelet activation components within the placenta abrogated the PE-like phenotype (364).

In contrast to platelets, leukocytes and certain derived EVs populations are increased in PE in comparison with normotensive pregnancies, mainly those EVs of granulocyte and monocyte origin (422). Interestingly, low levels of NK cell–derived EVs are observed in PE, linking with PE-associated maternal immune tolerance disorders (NK cell death activity dysfunction) (423). Of interest, Holder *et al.* (365) showed that human placenta is able to internalize EXOs from macrophages via endocytosis. Importantly, macrophage EXO uptake induced the release of proinflammatory cytokines by the placenta. Previously, the same group had reported that EXOs from PE placenta can activate peripheral blood mononuclear cells, inducing a proinflammatory response to a greater extent than EVs from normal placenta, and related to their cytokine content, mainly IL-1β. Moreover, PE-derived EVs stimulated an enhanced response of peripheral blood mononuclear cells to external pathogen-associated molecular patterns such as lipopolysaccharide (365). Such outcomes may be triggered by direct stimulation by EVs of Toll-like receptor, the signal subsequently internalized via nuclear factor κB (366). Taken together, these studies indicate a potential positive feedback loop by which an inflammatory response is overstimulated under PE conditions via EVs. Endothelial-derived EV levels correlate with the increment of the antiangiogenic factor sFLt1 and the sFLt1/P1GF ratio. This combined evidence suggests that apoptosis of endothelia occurs along with inhibition of angiogenesis and correlates with PE-characteristic endothelial damage, which persists between $<$1 week (424) and 72 hours postpartum (425).

Regarding obesity, a link between EXO release and the progression of PE is emerging. A recent study has observed that the levels of EXOs in maternal blood are correlated with maternal body mass index (BMI). A positive correlation of BMI with EXO levels was established, leading to the decrease of placenta-derived EXO proportions throughout gestation. These increased EXO levels contributed to a further exacerbated release of IL-6, IL-8, and TFN-α from endothelial cells, thus leading to worsened systemic inflammation in a BMI-dependent manner (426).

Finally, it has been observed that serum MVs from healthy pregnant women can reduce caspase activity and stimulate migration and tube formation in endothelial cells, whereas this is abrogated when the MVs are derived from patients with gestational vascular complications such as PE and hypertension. Furthermore, similar opposing actions on early-stage trophoblast of these vesicles was observed (367).

EVs in gestational diabetes

GD is defined as a carbohydrate intolerance of variable severity that appears or is first recognized during pregnancy. Along with PE, GD represents the most common metabolic complication of pregnancy, affecting between 1% and 15% of all pregnancies and increasing concurrently with obesity rates. It is characterized by pancreatic β cell–insufficient insulin production, usually due to pregnancy and characteristic insulin resistance, and is associated with maternal and fetal morbidity. Moreover, women with GD have increased risks of developing type II diabetes in the future (427, 428).

To date, little is known about the contribution of EVs in this pathophysiology. Salomon *et al.* (369) showed increased serum placenta-derived EXOs in GD pregnancies compared with control pregnancies, regardless of gestational age. *In vitro*, GD EXOs increased the release of proinflammatory cytokines from endothelial cells contributing to the enhanced proinflammatory state in pregnancy under GD conditions.

Clinical and Therapeutic Applications of EVs

The involvement of EVs in a wide variety of pathophysiological processes has made them appealing players as biomarkers and to carry therapeutic agents. This may also be the case when considering reproductive disorders.

EVs as biomarkers

EVs have been proposed as potential biomarkers of disorders of reproductive organs. The placenta releases EVs from the sixth week of pregnancy with steady increase as pregnancy proceeds, peaking at term (360). Importantly, their release is modulated by a number of factors that arise from the placenta; hence, EVs may provide mirrors of placental/fetal health and evolution (397). Because maternal blood is the primary source of placental EXOs, it will contain both maternal and placenta-specific EV populations and thus placental alkaline phosphatase (PLAP) has been proposed as a marker for the placental EVs, because it is restricted to placental cell lineages (394).

Alterations in both the levels and cargo of placenta-derived EXOs during pregnancy are associated with different pregnancy complications. A proteomic signature of 62 proteins in microparticles was developed from plasma samples of women at 10 to 12 weeks of

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

gestation (363). This signature was able to predict and differentiate SPBs from normal term births. Functional enrichment analyses showed processes related with preterm birth, such as inflammation, fibrinolysis, immune modulation, the coagulation cascade, or steroid metabolism. Currently, the only tool for evaluation of risk of spontaneous preterm birth is measurement of cervical length by ultrasound (364). A retrospective study on plasma samples of women at early gestational age (prior to 18 weeks) demonstrated potential for EXOs in the diagnosis of PE and SPB with higher (but not significant) levels of EXOs in both pathological conditions vs normal pregnancies. More interestingly, a specific exosomal miRNA signature could differentiate between the three conditions, being more similar between normal pregnancy and SPB compared with that of PE. When these miRNA profiles were compared with those from the EVT HTR-8/SVneo cell line cultured under normal and low-oxygen tension (LOT) conditions there was a strong correlation between the SPB and LOT conditions, with a common variation in >45% of the SPB condition miRNA profile. Placental–exosomal miRNA cargo was related to cell migration potential and inflammatory cytokine production. Particularly, LOT EXOs decreased endothelial cell migration potential and increased their TNF-α production, which could negatively impact spiral artery remodeling during placentation. Thus, under circumstances that favor a proinflammatory environment or a reduction of oxygen tension such as advanced gestational age, placental EVs may be negatively altered, impacting spiral artery remodeling and resulting in development of pathologies such as PE or SPB (422). In this sense, placental EVs may be potential early biomarkers of PE/SPB or as targets for directed therapy. Finally, both total and placenta-derived EVs are increased in women delivering low birth weight babies compared with those with normal birth weight deliveries (429).

EVs have been further investigated as biomarkers of PE. Recent publications debate the usefulness of the content of EVs for their predictive value in the diagnosis of PE. As an example, Tan *et al.* (430) analyzed three candidate biomarkers, TIMP-1, PAI-1, and P1GF, for their predictive ability in a large cohort of low-risk PE women from EVs isolated from bank plasma samples. They concluded that measurement of TIMP-1 and PAI-1 reinforced the value of the classical P1GF for PE prediction. Indeed, TIMP-1 and PAI-1 were analyzed in specific subgroups of EVs that can be retrieved thanks to their affinity to cholera toxin B and annexin V, both of which had been described previously in the search for PE biomarkers. In this study, EVs were purified from plasma of women at ~32 weeks of pregnancy, using immunoadsorption to the surface proteins, GM1 ganglioside (binds to cholera toxin B chain), and PS (binds to annexin V). Using these two populations of EVs (one from each marker), a specific protein signature was identified in women with PE compared with healthy pregnant controls. Importantly, note that such biomarker discovery is highly dependent on the selected conditions, providing a possible limitation. Indeed, in this study, large cellular debris was not removed from samples prior to the immunoadsorption step, providing a major potential source of error (431). In another study, different subtypes of MVs were evaluated in plasma, compared with cord blood from normal women and those with PE. Microparticles were more abundant and had altered coagulation-related factors in cord blood in PE compared with no PE (432). Recently, Salomon *et al.* (433) investigated whether EXOs and their miRNA cargo might provide early biomarkers of PE. More than 300 miRNAs were identified in total and placenta-derived EXOs in maternal plasma across gestation with hsa-miR-486-1-5p and hsa-miR-486-2-5 being identified as candidates for further study. Functional analysis showed that these miRNAs are involved in migration, placental development, and angiogenesis. Because PLAP is a marker of serum placenta-derived EXOs, which trend upwards with gestational age, exosomal content of PLAP has been proposed as a potential biomarker of PE in saliva and gingival cervical fluid (434). Finally, reduced EV-associated endothelial nitric oxide synthase expression and activity, a common feature of PE, was elevated in EVs from PE placentas (defined by PLAP) in both serum and placental perfusates, compared with healthy controls (435). Importantly, considering the above information, note that current biomarkers of pregnancy complications, such as PE or gestational diabetes mellitus, allow us to diagnose these states only once the pathologies are established and when the clinical management is limited. In this sense, to advance the field, efforts should focus on discovery of new biomarkers during early gestation.

EVs have also been proposed as biomarkers of peripartum cardiomyopathy (PPCM). PPCM is an idiopathic form of cardiomyopathy characterized by left ventricular systolic dysfunction (the ejaculation fraction is reduced normally <45%) and subsequent heart failure. It usually appears around the end of pregnancy and in the next few months and it is currently only diagnosed by exclusion of other heart failure causes (436), making a search for new biomarkers of considerable importance. Initially, Walenta *et al.* (437) reported increased levels of blood-derived activated endothelial microparticles in PPCM when compared with healthy postpartum, pregnant, and nonpregnant control but also with patients of ischemic cardiomyopathy and stable coronary arterial disease. These microparticles in PPCM were mainly platelet derived and monocyte microparticles. Treatment with bromocriptine, a therapy proven to work in animal models and human patients, significantly reduced endothelial and platelet-derived microparticles in

"EVs have also been proposed as biomarkers of peripartum cardiomyopathy (PPCM)."

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

PPCM compared with patients treated with standard undirected heart failure therapy. Thus, specific microparticle profiles may provide biomarkers that can distinguish PPCM from normal pregnancy, vascular diseases, and heart failure of different origin. miR146a has also been identified as a possible EXO-associated biomarker for PPCM. The 16-kDa N-terminal prolactin fragment, the primary known trigger of PPCM, stimulates the packaging of miR-146a into EXOs from human umbilical vein endothelial cells, which then are able to reach cardiomyocytes and trigger PPCM. Thus, miR-146a may provide a biomarker and therapeutic target for PPCM (438).

Placental EVs may provide indicators of infectious diseases during pregnancy. Both total and placental-derived EVs are increased in plasma from pregnant women with HIV infection compared with noninfected controls. In contrast, there were no changes in the level of plasma EVs due to malaria infection, neither for placental malaria nor for its peripheral variant. Nonetheless, miR-517c was found to be increased in microparticles from plasma of women with active placental malaria compared with noninfected controls (429).

Clinical and therapeutic aspects of EVs in reproductive biology

Intercellular transfer of genetic and protein material mediated by EVs could facilitate new diagnostic and therapeutic tools in the field of reproductive biology. As discussed, EVs are stable, versatile, cell-derived nanovesicles with target-homing specificity and the ability to transfer through *in vivo* biological barriers and they hold promise for the development of new approaches in drug delivery (75). Specifically, bioengineered EVs are being successfully deployed to deliver potent drugs and the capacity for select cellular reprogramming (6, 41). Recently, members of the International Society for Extracellular Vesicles and the Society for Clinical Research and Translation of Extracellular Vesicles presented a framework for challenges associated with development of EV-based therapeutics at the preclinical and clinical levels (439). This discussion addresses development of best practice models and current outlook for EV therapies.

Engineered or modified EVs can be designed for cell-specific targeting and delivery (440, 441). A seminal study has demonstrated that the selective cellular uptake of EVs surpasses that of more traditional carriers such as liposomes or nanoparticles, taking advantage of the natural characteristics of EVs to deliver molecules to target cells (442). Such insights provide future possibilities for clinical applications of EVs based on their ability to circumvent the limitations of various drug delivery systems of mucosal and blood–brain barrier traversal. The physicochemical configuration of EVs can also be modified to enable extended clearance compared with synthetic nanoparticles and spatiotemporal localization (ligand and cell type–specific targeting) and controlled release (238, 443–445). With respect to modifying EV cargo, a recent, comprehensive study compared various passive and active drug-loading methods, including electroporation, saponin treatment, extrusion, and dialysis, and used porphyrins of various hydrophobicities as model drugs (446). A comprehensive overview of EV cargo loading strategies, including electroporation, sonication, direct transfection, and cellular engineering, is provided in the literature (447, 448).

The potential functional roles of EVs in human embryo development have only recently been demonstrated. Embryos may generate their own microenvironment by secreting soluble factors and membrane vesicles, which constitute a secretome with selected autocrine and paracrine signaling (91, 449–453). In reproductive biology, nanoparticles have been used experimentally to load sperm with exogenous genetic material that is subsequently transferred to the oocyte during fertilization (454, 455). EVs have been identified in uterine fluid during the estrous/menstrual cycles, including humans, sheep, and mice (75, 156, 171, 282, 456). Indeed, EVs derived from the maternal endometrium contain multiple subtypes, including mixtures of EVs, EXOs and packaged different proteins, miRNAs and endogenous retrovirus mRNA (91, 156, 282–284, 287, 348). In the broader context of trophectodermal preparation for implantation, EVs have been shown to mediate communication between the inner cell mass and the trophectoderm (88). EV-encapsulated cargo is protected from degradation and is highly stable in biological fluids. Such unique properties may greatly facilitate the translation of EVs and their selected bioactive cargo and surface ligands into clinical applications. The study of EVs in reproduction has the potential for expanding our current understanding of the normal physiology of reproduction and pathological conditions such as implantation failure (452). Recent studies have provided key insights into the functional capacity of maternal EVs and how the protein cargo is directly modulated by uterine hormones during implantation to subsequently modulate trophoblast adhesive capacity (91). This study further validated selected components in primary human endometrial cells under hormonal control.

Recent studies have observed the ability of EVs to undergo cell-selective fusion (457) and tissue-specific tropism (228, 238, 458, 459), as well as their capacity to transverse the blood–brain barrier (460) and penetrate dense structural tissue (461). Importantly, based on their surface composition, EVs may be directed to specific tissues and organs (219, 238, 458, 459). Imaging of EVs in selected targeted organs has indeed demonstrated that the interactions of EVs with target cells are highly dynamic (232, 462). Such unique

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

properties of circulating EVs make them promising applications for the delivery of therapeutic cargo. Several studies support the utility of EVs as a novel path for drug delivery and as new drug targets. Alvarez-Erviti *et al.* (462), in an *in vivo* study, demonstrated that systemically injected neuron-targeted EXOs loaded with BACE1 siRNAs were able to significantly reduce BACE mRNA and protein, specifically in neurons (463). Furthermore, EXOs loaded with artificial siRNA against MAPK efficiently knocked down MAPK1 upon their delivery into monocytes and lymphocytes *in vitro* (464). Similarly, EXOs from induced pluripotent stem cells have been shown to deliver siRNA to attenuate expression of intercellular adhesion molecule 1 and neutrophil adhesion in pulmonary microvascular endothelial cells (465). Exosomes have further been applied for drug delivery to target a small-molecule, anti-inflammatory drug to selected organs and immune cells (466). These studies have demonstrated the capacity for EV-mediated targeted and delivery capacity and importantly the ability for EXOs to deliver and modulate multiple pathways simultaneously in the targeted cells. All of these studies are examples showing how EV cargo can be manipulated in a way that may be useful for target-based drug development for successful *in vivo* drug delivery.

Recent reviews have discussed the rationale to aim for selective silencing of EVs that promote unwanted functional effects. However, this is still an emerging concept in the field. Some of the strategies for specific silencing of EV subtypes (cell specific) are likely to require careful and detailed mechanistic studies. There are inhererent difficulties in avoiding the blocking of all EV types indiscriminately, which may interfere with and perturb physiological intercellular communication. Some examples of systems for abrogating EV formation and targeting/recipient cell uptake [reviews include (222, 230, 448, 467)]: (1) inhibition of EXO formation, including treatment with dimethyl amiloride; (2) inhibition of the endolysosomal compartment functions, including proton pump inhibitors, (3) blocking of EXO release (for example, silencing GTPase Rab11/27A/35 using siRNA or targeting ESCRT proteins and/or GTPases involved in trafficking of EXOs); and (4) prevention of fusion or uptake of EXOs by target cells, which can be done using a variety of reagents that block phosphatidyl serine such as diannexin, heparin to inhibit endocytosis (heparan sulfate proteoglycans), cytochalasin D to inhibit endocytosis and micropinocytosis, chlorpromazine to inhibit clathrin-dependent endocytosis, EIPA and LY294002 to block micropinocytosis, annexin V to inhibit phagocytosis and macropinocytosis, methyl-β-cyclodextrin, simvastatin, and filipin III to target lipid raft–mediated endocytosis, nystatin to target caveolae-mediated endocytosis, dynasore to inhibit clathrin-independent endocytosis (calveolae), and nystatin to perturb lipid raft–mediated endocytosis.

Future studies are required toward investigating EVs from primary tissues and biofluids and incorporating state-of-the-art quantitative analyses, including quantitative proteomics (183, 468) and sequencing technology that could be exploited to study protein and gene regulation during pregnancy. These would enable identification and monitoring of functional or low-abundant EV cargo, as well as cellular drivers of implantation and signaling, that hitherto have been unreported or functionally masked. Unlike small-molecule pharmaceutical compounds, there are no defined parameters or assays for current safety testing of EV-based therapeutics (469). Understanding biodistribution patterns and circulating timeframe of locally and systemically administered EVs is important to assessing safety, in addition to techniques that enable reproducible monitoring and safety testing of selected EV marker cargo. Targeted studies using EVs (modified or engineered) will hold the potential to develop novel nanodiagnostics and nanotherapeutics to increase the success of pregnancy rates during assisted reproductive technologies or *in vitro* fertilization. Recent work on targetable biodegradable delivery platforms for transporting biological cargo into gametes and embryos [reviewed in (470)] emphasizes the need to understand how EVs enter cells. We anticipate that future investigations into the use of EVs for the intentional targeted delivery of molecular compounds will provide new horizons for reproductive science and clinical assisted reproductive technologies, ultimately leading to improvements in pregnancy success.

"Recent reviews have discussed the rationale to aim for selective silencing of EVs that promote unwanted functional effects."

Concluding Remarks

Considering the body of evidence treated in the present review, there is no doubt that the field of EVs and its implication in reproduction is rapidly evolving and promises a further understanding of the processes that lead to a successful pregnancy, as well as markers of correct or compromised reproductive function. Nonetheless, there is still a difficult path to negotiate. First, there is an unavoidable need to firmly define standard methods for EVs isolation, because these define the fractions considered as different EV populations and, as such, may lead to ambiguous results that cannot be compared among studies. New challenges associated with standardization of methods for isolation, quantification, and analysis of EVs from complex tissues such as blood, as well as the stability of EVs within such biofluid samples, need to be overcome before the EV field can provide reliable tools for diagnosis and therapy.

It is also necessary to define the extent to which EVs are important participants in the reproductive events that lead to the delivery of healthy normal newborns, as this knowledge will lead to new therapies and clinical tests to ensure good pregnancy outcomes. Sample availability is maybe one of the main limiting factors that hinders such progress. In this sense, much more is known about epididymal and prostatic EV regulation of sperm compared with embryo maternal crosstalk through EVs. Nevertheless, EV communication may provide a cornerstone to enable better understanding of the conception and implantation

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

processes. This is important as it paves the way to deal with those patients in which the present assisted reproductive techniques fail.

Finally, data regarding the involvement of EVs in the triggering, maintenance, and progression of reproductive and obstetric-related disorders is still in its infancy and further key investigations utilizing homogeneous and human-specific material are needed. The use of EVs as disease biomarkers provides the opportunity for diagnostic potential with reduced invasiveness, as they can be retrieved from body fluid instead of tissue biopsies. This is vital for embryo diagnoses, where the possibility of getting STBMs from mother blood flow appears as an interesting alternative to invasive amniocentesis and chorionic villi sampling, further offering the possibility of an earlier diagnosis. Regarding EVs used as therapeutic agents, many different variants could be exploited. EVs could be used as vectors to deliver drugs and biological compounds in a targeted manner. Nevertheless, they could potentially be used as therapeutic targets if they are produced by affected cells and present disease-promoting characteristics. This may be achieved by inhibiting EV biosynthesis, by capturing them once produced, or by blocking their uptake by target cells, and this may be applicable in diseases such as PE. Furthermore, they could be used as natural therapeutic agents when experimental strategies rely on their natural features. Understanding cell type specificity and the long-term effects of EV remodeling, the potential of EVs to impart transgenerational consequences on the offspring's health, ranging from metabolism to sex determination, and potential epigenetic changes affecting the mother's fertility and altering the offspring's fertility are key factors to be addressed as the field moves forward. EVs derived from the immune cells including dendritic cells within the reproductive tissues also need examination, since such cells, once stimulated, may trigger detrimental immune responses. Advances in research on noncoding RNAs contained in EVs must also be considered (471). Understanding all these molecular signaling networks, utilizing advances in quantitative proteomics and sequencing technology, and mediated by EVs that coordinate strategies for successful implantation, may lead to approaches to improve the outcomes of natural pregnancy and pregnancy achieved using reproductive technologies.

References

1. Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borràs FE, Buzás EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E, Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostenfeld MS, Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MHM, De Wever O. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;**4**(1):27066.
2. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013; **200**(4):373–383.
3. Hardie DG. *Biochemical Messengers: Hormones, Neurotransmitters, and Growth Factors*. New York, NY: Chapman & Hall; 1991.
4. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular vesicles: composition, biological relevance, and methods of study. *Bioscience*. 2015;**65**(8): 783–797.
5. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;**30**(1):255–289.
6. EL Andaloussi S, Mäger I, Breakefield XO, Wood MJA. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*. 2013;**12**(5):347–357.
7. Ronquist G, Brody I. The prostasome: its secretion and function in man. *Biochim Biophys Acta*. 1985; **822**(2):203–218.
8. Ogawa Y, Kanai-Azuma M, Akimoto Y, Kawakami H, Yanoshita R. Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. *Biol Pharm Bull*. 2008;**31**(6):1059–1062.
9. Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005; **17**(7):879–887.
10. Lässer C, Alikhani VS, Ekström K, Eldh M, Paredes PT, Bossios A, Sjöstrand M, Gabrielsson S, Lötvall J, Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med*. 2011;**9**(1):9.
11. Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci USA*. 2004;**101**(36): 13368–13373.
12. Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D, Witkin SS. Heat shock protein-containing exosomes in mid-trimester amniotic fluids. *J Reprod Immunol*. 2008;**79**(1):12–17.
13. Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*. 2013;**113**(1):1–11.
14. György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B, László V, Pállinger E, Pap E, Kittel A, Nagy G, Falus A, Buzás EI. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci*. 2011;**68**(16):2667–2688.
15. Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, Vandesompele J, Bracke M, De Wever O, Hendrix A. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles*. 2014;**3**(1):24858.
16. Szatanek R, Baran J, Siedlar M, Baj-Krzyworzeka M. Isolation of extracellular vesicles: determining the correct approach. [Review] *Int J Mol Med*. 2015; **36**(1):11–17.
17. Tkach M, Théry C. Communication by extracellular vesicles: where we are and where we need to go. *Cell*. 2016;**164**(6):1226–1232.
18. Simpson RJ, Kalra H, Mathivanan S. ExoCarta as a resource for exosomal research. *J Extracell Vesicles*. 2012;**1**(1):18374.
19. Kim D-K, Kang B, Kim OY, Choi D-S, Lee J, Kim SR, Go G, Yoon YJ, Kim JH, Jang SC, Park K-S, Choi E-J, Kim KP, Desiderio DM, Kim Y-K, Lötvall J, Hwang D, Gho YS. EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *J Extracell Vesicles*. 2013;**2**(1):20384.
20. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borràs FE, Breakefield X, Budnik V, Buzas E, Camussi G, Clayton A, Cocucci E, Falcon-Perez JM, Gabrielsson S, Gho YS, Gupta D, Harsha HC, Hendrix A, Hill AF, Inal JM, Jenster G, Krämer-Albers E-M, Lim SK, Llorente A, Lötvall J, Marcilla A, Mincheva-Nilsson L, Nazarenko I, Nieuwland R, Nolte-'t Hoen ENM, Pandey A, Patel T, Piper MG, Pluchino S, Prasad TSK, Rajendran L, Raposo G, Record M, Reid GE, Sánchez-Madrid F, Schiffelers RM, Siljander P, Stensballe A, Stoorvogel W, Taylor D, Théry C, Valadi H, van Balkom BWM, Vázquez J, Vidal M, Wauben MHM, Yáñez-Mó M, Zoeller M, Mathivanan S. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol*. 2012;**10**(12):e1001450.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

21. Budnik V, Ruiz-Cañada C, Wendler F. Extracellular vesicles round off communication in the nervous system. *Nat Rev Neurosci*. 2016;**17**(3):160–172.
22. Maas SLN, Breakefield XO, Weaver AM. Extracellular vesicles: unique intercellular delivery vehicles. *Trends Cell Biol*. 2017;**27**(3):172–188.
23. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C, Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem*. 2009;**284**(49):34211–34222.
24. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ, Bernad A, Sánchez-Madrid F. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun*. 2011;**2**:282.
25. Kucharzewska P, Belting M. Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress. *J Extracell Vesicles*. 2013;**2**(1):20304.
26. An Q, van Bel AJ, Hückelhoven R. Do plant cells secrete exosomes derived from multivesicular bodies? *Plant Signal Behav*. 2007;**2**(1):4–7.
27. Rutter BD, Innes RW. Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. *Plant Physiol*. 2017;**173**(1):728–741.
28. Silverman JM, Clos J, de'Oliveira CC, Shirvani O, Fang Y, Wang C, Foster LJ, Reiner NE. An exosome-based secretion pathway is responsible for protein export from *Leishmania* and communication with macrophages. *J Cell Sci*. 2010;**123**(Pt 6):842–852.
29. Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, Bursac D, Angrisano F, Gee M, Hill AF, Baum J, Cowman AF. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell*. 2013;**153**(5):1120–1133.
30. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol*. 2015;**13**(10):620–630.
31. Deatherage BL, Cookson BT. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect Immun*. 2012;**80**(6):1948–1957.
32. Tannetta D, Dragovic R, Alyahyaei Z, Southcombe J. Extracellular vesicles and reproduction-promotion of successful pregnancy. *Cell Mol Immunol*. 2014;**11**(6):548–563.
33. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972;**26**(4):239–257.
34. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 2008;**9**(3):231–241.
35. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;**35**(4):495–516.
36. Atkin-Smith GK, Tixeira R, Paone S, Mathivanan S, Collins C, Liem M, Goodall KJ, Ravichandran KS, Hulett MD, Poon IKH. A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. *Nat Commun*. 2015;**6**(1):7439.
37. Hristov M, Erl W, Linder S, Weber PC. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood*. 2004;**104**(9):2761–2766.
38. Willms E, Johansson HJ, Mäger I, Lee Y, Blomberg KEM, Sadik M, Alaarg A, Smith CIE, Lehtiö J, El Andaloussi S, Wood MJA, Vader P. Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci Rep*. 2016;**6**(1):22519.
39. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B, Dyrskjøt L, Orntoft TF, Howard KA, Ostenfeld MS. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J Extracell Vesicles*. 2014;**3**(1):25011.
40. Osteikoetxea X, Németh A, Sódar BW, Vukman KV, Buzás EI. Extracellular vesicles in cardiovascular disease: are they Jedi or Sith? *J Physiol*. 2016;**594**(11):2881–2894.
41. van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;**64**(3):676–705.
42. van Engeland M, Kuijpers HJ, Ramaekers FC, Reutelingsperger CP, Schutte B. Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp Cell Res*. 1997;**235**(2):421–430.
43. Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. *Cold Spring Harb Perspect Biol*. 2013;**5**(1):a008748.
44. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol*. 2006;**16**(4):189–197.
45. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest*. 2001;**108**(7):957–962.
46. Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: cytometry in studies of programmed cell death. *Methods Cell Biol*. 2011;**103**:55–98.
47. Bailey RW, Nguyen T, Robertson L, Gibbons E, Nelson J, Christensen RE, Bell JP, Judd AM, Bell JD. Sequence of physical changes to the cell membrane during glucocorticoid-induced apoptosis in S49 lymphoma cells. *Biophys J*. 2009;**96**(7):2709–2718.
48. Hugel B, Martínez MC, Kunzelmann C, Freyssinet J-M. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)*. 2005;**20**:22–27.
49. Friedl P, Vischer P, Freyberg MA. The role of thrombospondin-1 in apoptosis. *Cell Mol Life Sci*. 2002;**59**(8):1347–1357.
50. Takizawa F, Tsuji S, Nagasawa S. Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. *FEBS Lett*. 1996;**397**(2–3):269–272.
51. Abas L, Luschnig C. Maximum yields of microsomal-type membranes from small amounts of plant material without requiring ultracentrifugation. *Anal Biochem*. 2010;**401**(2):217–227.
52. Lavoie C, Lanoix J, Kan FW, Paiement J. Cell-free assembly of rough and smooth endoplasmic reticulum. *J Cell Sci*. 1996;**109**(Pt 6):1415–1425.
53. Tong M, Kleffmann T, Pradhan S, Johansson CL, DeSousa J, Stone PR, James JL, Chen Q, Chamley LW. Proteomic characterization of macro-, micro- and nano-extracellular vesicles derived from the same first trimester placenta: relevance for feto-maternal communication. *Hum Reprod*. 2016;**31**(4):687–699.
54. Pantham P, Viall CA, Chen Q, Kleffmann T, Print CG, Chamley LW. Antiphospholipid antibodies bind syncytiotrophoblast mitochondria and alter the proteome of extruded syncytial nuclear aggregates. *Placenta*. 2015;**36**(12):1463–1473.
55. Holmgren L, Szeles A, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, Falk KI. Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood*. 1999;**93**(11):3956–3963.
56. Ehnfors J, Kost-Alimova M, Persson NL, Bergsmedh A, Castro J, Levchenko-Tegnebratt T, Yang L, Panaretakis T, Holmgren L. Horizontal transfer of tumor DNA to endothelial cellsin vivo. *Cell Death Differ*. 2009;**16**(5):749–757.
57. Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc Natl Acad Sci USA*. 2001;**98**(11):6407–6411.
58. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol*. 2002;**2**(12):965–975.
59. Bellone M, Iezzi G, Rovere P, Galati G, Ronchetti A, Protti MP, Davoust J, Rugarli C, Manfredi AA. Processing of engulfed apoptotic bodies yields T cell epitopes. *J Immunol*. 1997;**159**(11):5391–5399.
60. Cocca BA, Cline AM, Radic MZ. Blebs and apoptotic bodies are B cell autoantigens. *J Immunol*. 2002;**169**(1):159–166.
61. Bellone M. Apoptosis, cross-presentation, and the fate of the antigen specific immune response. *Apoptosis*. 2000;**5**(4):307–314.
62. Chargaff E, West R. The biological significance of the thromboplastic protein of blood. *J Biol Chem*. 1946;**166**(1):189–197.
63. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. *J Cell Sci*. 2010;**123**(Pt 10):1603–1611.
64. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 2009;**19**(2):43–51.
65. Tricarico C, Clancy J, D'Souza-Schorey C. Tricarico C, Clancy J, D'Souza-Schorey C. Biology and biogenesis of shed microvesicles. *Small GTPases*. 2017;**8**(4):220–232.
66. D'Souza-Schorey C, Clancy JW. Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes Dev*. 2012;**26**(12):1287–1299.
67. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol*. 2009;**9**(8):581–593.
68. Antonyak MA, Cerione RA. Emerging picture of the distinct traits and functions of microvesicles and exosomes. *Proc Natl Acad Sci USA*. 2015;**112**(12):3589–3590.
69. Lawson C, Vicencio JM, Yellon DM, Davidson SM. Microvesicles and exosomes: new players in metabolic and cardiovascular disease. *J Endocrinol*. 2016;**228**(2):R57–R71.
70. Vader P, Breakefield XO, Wood MJA. Extracellular vesicles: emerging targets for cancer therapy. *Trends Mol Med*. 2014;**20**(7):385–393.
71. Kreimer S, Belov AM, Ghiran I, Murthy SK, Frank DA, Ivanov AR. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. *J Proteome Res*. 2015;**14**(6):2367–2384.
72. Sluijter JPG, Verhage V, Deddens JC, van den Akker F, Doevendans PA. Microvesicles and exosomes for intracardiac communication. *Cardiovasc Res*. 2014;**102**(2):302–311.
73. Xu R, Greening DW, Rai A, Ji H, Simpson RJ. Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct. *Methods*. 2015;**87**:11–25.
74. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G, D'Souza-Schorey C. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr Biol*. 2009;**19**(22):1875–1885.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

75. Xu R, Greening DW, Zhu H-J, Takahashi N, Simpson RJ. Extracellular vesicle isolation and characterization: toward clinical application. *J Clin Invest*. 2016; **126**(4):1152–1162.
76. Clancy JW, Sedgwick A, Rosse C, Muralidharan-Chari V, Raposo G, Method M, Chavrier P, D'Souza-Schorey C. Regulated delivery of molecular cargo to invasive tumour-derived microvesicles. *Nat Commun*. 2015;**6**(1):6919.
77. Menck K, Scharf C, Bleckmann A, Dyck L, Rost U, Wenzel D, Dhople VM, Siam L, Pukrop T, Binder C, Klemm F. Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN. *J Mol Cell Biol*. 2015;**7**(2): 143–153.
78. Antonyak MA, Li B, Boroughs LK, Johnson JL, Druso JE, Bryant KL, Holowka DA, Cerione RA. Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc Natl Acad Sci USA*. 2011; **108**(12):4852–4857.
79. Arshad Malik MF. Influence of microvesicles in breast cancer metastasis and their therapeutic implications. *Arch Iran Med*. 2015;**18**(3):189–192.
80. McDaniel K, Correa R, Zhou T, Johnson C, Francis H, Glaser S, Venter J, Alpini G, Meng F. Functional role of microvesicles in gastrointestinal malignancies. *Ann Transl Med*. 2013;**1**(1):4.
81. Jorfi S, Inal JM. The role of microvesicles in cancer progression and drug resistance. *Biochem Soc Trans*. 2013;**41**(1):293–298.
82. Dye JR, Ullal AJ, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. *Scand J Immunol*. 2013;**78**(2):140–148.
83. Lo Cicero A, Majkowska I, Nagase H, Di Liegro I, Troeberg L. Microvesicles shed by oligodendroglioma cells and rheumatoid synovial fibroblasts contain aggrecanase activity. *Matrix Biol*. 2012;**31**(4): 229–233.
84. Sellam J, Proulle V, Jüngel A, Ittah M, Miceli Richard C, Gottenberg J-E, Toti F, Benessiano J, Gay S, Freyssinet J-M, Mariette X. Increased levels of circulating microparticles in primary Sjögren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis Res Ther*. 2009;**11**(5):R156.
85. Nomura S, Shimizu M. Clinical significance of procoagulant microparticles. *J Intensive Care*. 2015; **3**(1):2.
86. Xiong J, Miller VM, Li Y, Jayachandran M. Microvesicles at the crossroads between infection and cardiovascular diseases. *J Cardiovasc Pharmacol*. 2012;**59**(2):124–132.
87. Tong M, Chamley LW. Placental extracellular vesicles and feto-maternal communication. *Cold Spring Harb Perspect Med*. 2015;**5**(3):a023028.
88. Desrochers LM, Bordeleau F, Reinhart-King CA, Cerione RA, Antonyak MA. Microvesicles provide a mechanism for intercellular communication by embryonic stem cells during embryo implantation. *Nat Commun*. 2016;**7**:11958.
89. Trams EG, Lauter CJ, Salem N Jr, Heine U. Exfoliation of membrane ecto-enzymes in the form of microvesicles. *Biochim Biophys Acta*. 1981;**645**(1):63–70.
90. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol*. 1983;**97**(2):329–339.
91. Greening DW, Nguyen HPT, Elgass K, Simpson RJ, Salamonsen LA. Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: insights into endometrial-embryo interactions. *Biol Reprod*. 2016; **94**(2):38.
92. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol*. 2002;**2**(8):569–579.
93. Lane RE, Korbie D, Anderson W, Vaidyanathan R, Trau M. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. *Sci Rep*. 2015;**5**(1):7639.
94. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, Manel N, Moita LF, Théry C, Raposo G. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013; **126**(Pt 24):5553–5565.
95. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics*. 2010;**73**(10):1907–1920.
96. Mathivanan S, Lim JWE, Tauro BJ, Ji H, Moritz RL, Simpson RJ. Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature. *Mol Cell Proteomics*. 2010;**9**(2): 197–208.
97. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. *Nature*. 2007; **450**(7168):435–439.
98. Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ. Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. *Mol Cell Proteomics*. 2013;**12**(3):587–598.
99. Ji H, Chen M, Greening DW, He W, Rai A, Zhang W, Simpson RJ. Deep sequencing of RNA from three different extracellular vesicle (EV) subtypes released from the human LIM1863 colon cancer cell line uncovers distinct miRNA-enrichment signatures. *PLoS One*. 2014;**9**(10):e110314.
100. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Théry C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci USA*. 2016;**113**(8):E968–E977.
101. Lai RC, Tan SS, Yeo RWY, Choo ABH, Reiner AT, Su Y, Shen Y, Fu Z, Alexander L, Sze SK, Lim SK. MSC secretes at least 3 EV types each with a unique permutation of membrane lipid, protein and RNA. *J Extracell Vesicles*. 2016;**5**(1):29828.
102. Tkach M, Kowal J, Zucchetti AE, Enserink L, Jouve M, Lankar D, Saitakis M, Martin-Jaular L, Théry C. Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes. *EMBO J*. 2017;**36**(20):3012–3028.
103. Ogawa Y, Miura Y, Harazono A, Kanai-Azuma M, Akimoto Y, Kawakami H, Yamaguchi T, Toda T, Endo T, Tsubuki M, Yanoshita R. Proteomic analysis of two types of exosomes in human whole saliva. *Biol Pharm Bull*. 2011;**34**(1):13–23.
104. Aalberts M, van Dissel-Emiliani FMF, van Adrichem NPH, van Wijnen M, Wauben MHM, Stout TAE, Stoorvogel W. Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. *Biol Reprod*. 2012;**86**(3):82.
105. Laulagnier K, Javalet C, Hemming FJ, Chivet M, Lachenal G, Blot B, Chatellard C, Sadoul R. Amyloid precursor protein products concentrate in a subset of exosomes specifically endocytosed by neurons. *Cell Mol Life Sci*. 2018;**75**(4):757–773.
106. Chen M, Xu R, Ji H, Greening DW, Rai A, Izumikawa K, Ishikawa H, Takahashi N, Simpson RJ. Transcriptome and long noncoding RNA sequencing of three extracellular vesicle subtypes released from the human colon cancer LIM1863 cell line. *Sci Rep*. 2016;**6**(1):38397.
107. De Toro J, Herschlik L, Waldner C, Mongini C. Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. *Front Immunol*. 2015;**6**:203.
108. Suchorska WM, Lach MS. The role of exosomes in tumor progression and metastasis. [Review] *Oncol Rep*. 2016;**35**(3):1237–1244.
109. Greening DW, Gopal SK, Xu R, Simpson RJ, Chen W. Exosomes and their roles in immune regulation and cancer. *Semin Cell Dev Biol*. 2015;**40**:72–81.
110. Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL. Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. *Sci Rep*. 2016;**6**(1):20254.
111. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J, Théry C, Wauben MH, Hochberg F. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;**2**(1):20360.
112. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. 2006;Chapter 3:Unit 3.22. doi: https://doi.org/10.1002/0471143030.cb0322s30.
113. Lamparski HG, Metha-Damani A, Yao J-Y, Patel S, Hsu D-H, Ruegg C, Le Pecq J-B. Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods*. 2002; **270**(2):211–226.
114. Chiou N-T, Ansel KM. Improved exosome isolation by sucrose gradient fractionation of ultracentrifuged crude exosome pellets. Protocol Exchange. Available at: www.nature.com/protocolexchange/protocols/5035.
115. Keller S, Ridinger J, Rupp A-K, Janssen JWG, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. *J Transl Med*. 2011;**9**(1):86.
116. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol*. 2015;**1295**:179–209.
117. Cantin R, Diou J, Bélanger D, Tremblay AM, Gilbert C. Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants. *J Immunol Methods*. 2008;**338**(1–2):21–30.
118. Klimentová J, Stulík J. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol Res*. 2015;**170**:1–9.
119. Ford T, Graham J, Rickwood D. Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. *Anal Biochem*. 1994;**220**(2):360–366.
120. György B, Módos K, Pállinger E, Pálóczi K, Pásztói M, Misják P, Deli MA, Sipos A, Szalai A, Voszka I, Polgár A, Tóth K, Csete M, Nagy G, Gay S, Falus A, Kittel A, Buzás EI. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood*. 2011;**117**(4):e39–e48.
121. Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigor'eva AE, Zaporozhchenko IA, Morozkin ES, Ryabchikova EI, Yurchenko YB, Voitsitskiy VE, Laktionov PP. Comparative study of extracellular vesicles from the urine of healthy individuals and prostate cancer patients. *PLoS One*. 2016;**11**(6):e0157566.
122. Lobb RJ, Becker M, Wen SW, Wong CSF, Wiegmans AP, Leimgruber A, Möller A. Optimized exosome

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles*. 2015;**4**(1):27031.
123. Klein-Scory S, Tehrani MM, Eilert-Micus C, Adamczyk KA, Wojtalewicz N, Schnölzer M, Hahn SA, Schmiegel W, Schwarte-Waldhoff I. New insights in the composition of extracellular vesicles from pancreatic cancer cells: implications for biomarkers and functions. *Proteome Sci*. 2014;**12**(1):50.
124. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PST, Star RA. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *Am J Physiol Renal Physiol*. 2007;**292**(5):F1657–F1661.
125. Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM, McAfee KJ, Fleischer C, Klein E, Klein JB. Microfiltration isolation of human urinary exosomes for characterization by MS. *Proteomics Clin Appl*. 2010;**4**(1):84–96.
126. Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles*. 2014;**3**(1):23430.
127. de Menezes-Neto A, Sáez MJF, Lozano-Ramos I, Segui-Barber J, Martin-Jaular L, Ullate JME, Fernandez-Becerra C, Borrás FE, Del Portillo HA. Size-exclusion chromatography as a stand-alone methodology identifies novel markers in mass spectrometry analyses of plasma-derived vesicles from healthy individuals. *J Extracell Vesicles*. 2015;**4**(1):27378.
128. Lozano-Ramos I, Bancu I, Oliveira-Tercero A, Armengol MP, Menezes-Neto A, Del Portillo HA, Lauzurica-Valdemoros R, Borràs FE. Size-exclusion chromatography-based enrichment of extracellular vesicles from urine samples. *J Extracell Vesicles*. 2015; **4**(1):27369.
129. Taylor DD, Lyons KS, Gerçel-Taylor Ç. Shed membrane fragment-associated markers for endometrial and ovarian cancers. *Gynecol Oncol*. 2002; **84**(3):443–448.
130. Hong CS, Muller L, Boyiadzis M, Whiteside TL. Isolation and characterization of $CD34^+$ blast-derived exosomes in acute myeloid leukemia. *PLoS One*. 2014;**9**(8):e103310.
131. Yoo CE, Kim G, Kim M, Park D, Kang HJ, Lee M, Huh N. A direct extraction method for microRNAs from exosomes captured by immunoaffinity beads. *Anal Biochem*. 2012;**431**(2):96–98.
132. Clayton A, Court J, Navabi H, Adams M. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J Immunol Methods*. 2001;**247**(1–2):163–174.
133. Taylor DD, Zacharias W, Gercel-Taylor C. Exosome isolation for proteomic analyses and RNA profiling. *Methods Mol Biol*. 2011;**728**:235–246.
134. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int*. 2012;**82**(9):1024–1032.
135. Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S. Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. *Lab Chip*. 2014;**14**(11):1891–1900.
136. Bhagat AAS, Kuntaegowdanahalli SS, Papautsky I. Continuous particle separation in spiral microchannels using Dean flows and differential migration. *Lab Chip*. 2008;**8**(11):1906–1914.
137. Ashcroft BA, de Sonneville J, Yuana Y, Osanto S, Bertina R, Kuil ME, Oosterkamp TH. Determination of the size distribution of blood microparticles directly in plasma using atomic force microscopy and microfluidics. *Biomed Microdevices*. 2012;**14**(4): 641–649.
138. Crescitelli R, Lässer C, Szabó TG, Kittel A, Eldh M, Dianzani I, Buzás EI, Lötvall J. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles*. 2013;**2**(1):20677.
139. Ji H, Greening DW, Barnes TW, Lim JW, Tauro BJ, Rai A, Xu R, Adda C, Mathivanan S, Zhao W, Xue Y, Xu T, Zhu H-J, Simpson RJ. Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *Proteomics*. 2013; **13**(10–11):1672–1686.
140. Xu R, Simpson RJ, Greening DW. A protocol for isolation and proteomic characterization of distinct extracellular vesicle subtypes by sequential centrifugal ultrafiltration. *Methods Mol Biol*. 2017;**1545**: 91–116.
141. Chen X, Gao C, Li H, Huang L, Sun Q, Dong Y, Tian C, Gao S, Dong H, Guan D, Hu X, Zhao S, Li L, Zhu L, Yan Q, Zhang J, Zen K, Zhang C-Y. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. *Cell Res*. 2010; **20**(10):1128–1137.
142. Müller G. Novel tools for the study of cell type-specific exosomes and microvesicles. *J Bioanal Biomed*. 2012;**4**(4):40–60.
143. Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa M, Beyer K, Borràs FE. Size-exclusion chromatography-based isolation minimally alters extracellular vesicles' characteristics compared to precipitating agents. *Sci Rep*. 2016;**6**(1): 33641.
144. Corso G, Mäger I, Lee Y, Görgens A, Bultema J, Giebel B, Wood MJA, Nordin JZ, Andaloussi SE. Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography. *Sci Rep*. 2017;**7**(1):11561.
145. Benedikter BJ, Bouwman FG, Vajen T, Heinzmann ACA, Grauls G, Mariman EC, Wouters EFM, Savelkoul PH, Lopez-Iglesias C, Koenen RR, Rohde GGU, Stassen FRM. Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies. *Sci Rep*. 2017; **7**(1):15297.
146. Nordin JZ, Lee Y, Vader P, Mäger I, Johansson HJ, Heusermann W, Wiklander OPB, Hällbrink M, Seow Y, Bultema JJ, Gilthorpe J, Davies T, Fairchild PJ, Gabrielsson S, Meisner-Kober NC, Lehtiö J, Smith CIE, Wood MJA, El Andaloussi S. Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. *Nanomedicine (Lond)*. 2015;**11**(4):879–883.
147. Chen C, Skog J, Hsu C-H, Lessard RT, Balaj L, Wurdinger T, Carter BS, Breakefield XO, Toner M, Irimia D. Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab Chip*. 2010; **10**(4):505–511.
148. Davies RT, Kim J, Jang SC, Choi E-J, Gho YS, Park J. Microfluidic filtration system to isolate extracellular vesicles from blood. *Lab Chip*. 2012;**12**(24): 5202–5210.
149. Wu M, Ouyang Y, Wang Z, Zhang R, Huang P-H, Chen C, Li H, Li P, Quinn D, Dao M, Suresh S, Sadovsky Y, Huang TJ. Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proc Natl Acad Sci USA*. 2017;**114**(40): 10584–10589.
150. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics*. 2017; **7**(3):789–804.
151. Willis GR, Kourembanas S, Mitsialis SA. Toward exosome-based therapeutics: isolation, heterogeneity, and fit-for-purpose potency. *Front Cardiovasc Med*. 2017;**4**:63.
152. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996; **183**(3):1161–1172.
153. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, LeBleu VS, Mittendorf EA, Weitz J, Rahbari N, Reissfelder C, Pilarsky C, Fraga MF, Piwnica-Worms D, Kalluri R. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*. 2015;**523**(7559):177–182.
154. Roccaro AM, Sacco A, Maiso P, Azab AK, Tai Y-T, Reagan M, Azab F, Flores LM, Campigotto F, Weller E, Anderson KC, Scadden DT, Ghobrial IM. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest*. 2013;**123**(4):1542–1555.
155. Höög JL, Lötvall J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *J Extracell Vesicles*. 2015;**4**(1):28680.
156. Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martínez S, Marcilla A, Simón C. Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development*. 2015; **142**(18):3210–3221.
157. Wu Y, Deng W, Klinke DJ II. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst (Lond)*. 2015;**140**(19):6631–6642.
158. Iwai K, Minamisawa T, Suga K, Yajima Y, Shiba K. Isolation of human salivary extracellular vesicles by iodixanol density gradient ultracentrifugation and their characterizations. *J Extracell Vesicles*. 2016;**5**(1): 30829.
159. Hardij J, Cecchet F, Berquand A, Gheldof D, Chatelain C, Mullier F, Chatelain B, Dogné J-M. Characterisation of tissue factor-bearing extracellular vesicles with AFM: comparison of air-tapping-mode AFM and liquid peak force AFM. *J Extracell Vesicles*. 2013;**2**(1):21045.
160. Yuana Y, Oosterkamp TH, Bahatyrova S, Ashcroft B, Garcia Rodriguez P, Bertina RM, Osanto S. Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost*. 2010;**8**(2):315–323.
161. Gardiner C, Ferreira YJ, Dragovic RA, Redman CWG, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles*. 2013;**2**(1):19671.
162. Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res*. 2010;**27**(5):796–810.
163. van der Pol E, Coumans FAW, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost*. 2014;**12**(7):1182–1192.
164. Momen-Heravi F, Balaj L, Alian S, Tigges J, Toxavidis V, Ericsson M, Distel RJ, Ivanov AR, Skog J, Kuo WP. Alternative methods for characterization of extracellular vesicles. *Front Physiol*. 2012;**3**:354.
165. Garza-Licudine E, Deo D, Yu S, Uz-Zaman A, Dunbar WB. Portable nanoparticle quantization using a resizable nanopore instrument—the IZON qNano™. *Conf Proc IEEE Eng Med Biol Soc*. 2010; **2010**:5736–5739.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

166. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *J Thromb Haemost*. 2010;**8**(12):2596–2607.
167. Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming limitations of microparticle measurement by flow cytometry. *Semin Thromb Hemost*. 2010;**36**(8):807–818.
168. van der Vlist EJ, Nolte-'t Hoen ENM, Stoorvogel W, Arkesteijn GJA, Wauben MHM. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. *Nat Protoc*. 2012;**7**(7):1311–1326.
169. Nolte-'t Hoen ENM, van der Vlist EJ, Aalberts M, Mertens HCH, Bosch BJ, Bartelink W, Mastrobattista E, van Gaal EVB, Stoorvogel W, Arkesteijn GJA, Wauben MHM. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. *Nanomedicine (Lond)*. 2012;**8**(5):712–720.
170. Osteikoetxea X, Balogh A, Szabó-Taylor K, Németh A, Szabó TG, Pálóczi K, Sódar B, Kittel Á, György B, Pállinger É, Matkó J, Buzás EI. Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. *PLoS One*. 2015;**10**(3):e0121184.
171. Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, Salamonsen LA. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. *PLoS One*. 2013;**8**(3):e58502.
172. Nakai W, Yoshida T, Diez D, Miyatake Y, Nishibu T, Imawaka N, Naruse K, Sadamura Y, Hanayama R. A novel affinity-based method for the isolation of highly purified extracellular vesicles. *Sci Rep*. 2016;**6**(1):33935.
173. Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, Perdicchio M, Marino ML, Federici C, Iessi E, Brambilla D, Venturi G, Lozupone F, Santinami M, Huber V, Maio M, Rivoltini L, Fais S. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*. 2009;**4**(4):e5219.
174. Yoshioka Y, Kosaka N, Konishi Y, Ohta H, Okamoto H, Sonoda H, Nonaka R, Yamamoto H, Ishii H, Mori M, Furuta K, Nakajima T, Hayashi H, Sugisaki H, Higashimoto H, Kato T, Takeshita F, Ochiya T. Ultrasensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. *Nat Commun*. 2014;**5**:3591.
175. Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, Hochberg FH, Breakefield XO, Weissleder R, Lee H. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med*. 2012;**18**(12):1835–1840.
176. Im H, Shao H, Weissleder R, Castro CM, Lee H. Nano-plasmonic exosome diagnostics. *Expert Rev Mol Diagn*. 2015;**15**(6):725–733.
177. Im H, Shao H, Park YI, Peterson VM, Castro CM, Weissleder R, Lee H. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol*. 2014;**32**(5):490–495.
178. Carrasco-Ramírez P, Greening DW, Andrés G, Gopal SK, Martín-Villar E, Renart J, Simpson RJ, Quintanilla M. Podoplanin is a component of extracellular vesicles that reprograms cell-derived exosomal proteins and modulates lymphatic vessel formation. *Oncotarget*. 2016;**7**(13):16070–16089.
179. Gopal SK, Greening DW, Hanssen EG, Zhu H-J, Simpson RJ, Mathias RA. Oncogenic epithelial cell-derived exosomes containing Rac1 and PAK2 induce angiogenesis in recipient endothelial cells. *Oncotarget*. 2016;**7**(15):19709–19722.
180. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJP, Hole P, Carr B, Redman CWG, Harris AL, Dobson PJ, Harrison P, Sargent IL. Sizing and phenotyping of cellular vesicles using nanoparticle tracking analysis. *Nanomedicine (Lond)*. 2011;**7**(6):780–788.
181. Gardiner C, Shaw M, Hole P, Smith J, Tannetta D, Redman CW, Sargent IL. Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles. *J Extracell Vesicles*. 2014;**3**(1):25361.
182. Palmieri V, Lucchetti D, Gatto I, Maiorana A, Marcantoni M, Maulucci G, Papi M, Pola R, De Spirito M, Sgambato A. Dynamic light scattering for the characterization and counting of extracellular vesicles: a powerful noninvasive tool. *J Nanopart Res*. 2014;**16**(9):2583.
183. Greening DW, Xu R, Gopal SK, Rai A, Simpson RJ. Proteomic insights into extracellular vesicle biology—defining exosomes and shed microvesicles. *Expert Rev Proteomics*. 2017;**14**(1):69–95.
184. Théry C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol*. 1999;**147**(3):599–610.
185. Aatonen MT, Ohman T, Nyman TA, Laitinen S, Grönholm M, Siljander PRM. Isolation and characterization of platelet-derived extracellular vesicles. *J Extracell Vesicles*. 2014;**3**(1):24692.
186. MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, Surprenant A. Rapid secretion of interleukin-1β by microvesicle shedding. *Immunity*. 2001;**15**(5):825–835.
187. Berda-Haddad Y, Robert S, Salers P, Zekraoui L, Farnarier C, Dinarello CA, Dignat-George F, Kaplanski G. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1α. *Proc Natl Acad Sci USA*. 2011;**108**(51):20684–20689.
188. Gulinelli S, Salaro E, Vuerich M, Bozzato D, Pizzirani C, Bolognesi G, Idzko M, Di Virgilio F, Ferrari D. IL-18 associates to microvesicles shed from human macrophages by a LPS/TLR-4 independent mechanism in response to P2X receptor stimulation. *Eur J Immunol*. 2012;**42**(12):3334–3345.
189. Hasegawa H, Thomas HJ, Schooley K, Born TL. Native IL-32 is released from intestinal epithelial cells via a non-classical secretory pathway as a membrane-associated protein. *Cytokine*. 2011;**53**(1):74–83.
190. Zhang H-G, Liu C, Su K, Yu S, Zhang L, Zhang S, Wang J, Cao X, Grizzle W, Kimberly RP. A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death. *J Immunol*. 2006;**176**(12):7385–7393.
191. Kandere-Grzybowska K, Letourneau R, Kempuraj D, Donelan J, Poplawski S, Boucher W, Athanassiou A, Theoharides TC. IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells. *J Immunol*. 2003;**171**(9):4830–4836.
192. Nardi FS, Michelon TF, Neumann J, Manvailer LFS, Wagner B, Horn PA, Bicalho MG, Rebmann V. High levels of circulating extracellular vesicles with altered expression and function during pregnancy. *Immunobiology*. 2016;**221**(7):753–760.
193. Llorente A, Skotland T, Sylvänne T, Kauhanen D, Róg T, Orłowski A, Vattulainen I, Ekroos K, Sandvig K. Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. *Biochim Biophys Acta*. 2013;**1831**(7):1302–1309.
194. Brouwers JF, Aalberts M, Jansen JWA, van Niel G, Wauben MH, Stout TAE, Helms JB, Stoorvogel W. Distinct lipid compositions of two types of human prostasomes. *Proteomics*. 2013;**13**(10–11):1660–1666.
195. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brügger B, Simons M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science*. 2008;**319**(5867):1244–1247.
196. Skotland T, Ekroos K, Kauhanen D, Simolin H, Seierstad T, Berge V, Sandvig K, Llorente A. Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. *Eur J Cancer*. 2017;**70**:122–132.
197. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;**9**(6):654–659.
198. Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M, Kohli M, Thibodeau SN, Boardman L, Wang L. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics*. 2013;**14**(1):319.
199. Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, Strobl J, Westerberg K, Gottardo R, Tewari M, Hladik F. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res*. 2014;**42**(11):7290–7304.
200. van Balkom BWM, Eisele AS, Pegtel DM, Bervoets S, Verhaar MC. Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting. *J Extracell Vesicles*. 2015;**4**(1):26760.
201. Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *J Extracell Vesicles*. 2014;**3**(1):23743.
202. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci USA*. 2011;**108**(12):5003–5008.
203. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res*. 2011;**39**(16):7223–7233.
204. Li H, Huang S, Guo C, Guan H, Xiong C. Cell-free seminal mRNA and microRNA exist in different forms. *PLoS One*. 2012;**7**(4):e34566.
205. Shelke GV, Lässer C, Gho YS, Lötvall J. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. *J Extracell Vesicles*. 2014;**3**(1):24783.
206. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, Zheng Y, Hoshino A, Brazier H, Xiang J, Williams C, Rodriguez-Barrueco R, Silva JM, Zhang W, Hearn S, Elemento O, Paknejad N, Manova-Todorova K, Welte K, Bromberg J, Peinado H, Lyden D. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res*. 2014;**24**(6):766–769.
207. Waldenström A, Gennebäck N, Hellman U, Ronquist G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS One*. 2012;**7**(4):e34653.
208. Lázaro-Ibáñez E, Sanz-Garcia A, Visakorpi T, Escobedo-Lucea C, Siljander P, Ayuso-Sacido A, Yliperttula M.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: apoptotic bodies, microvesicles, and exosomes. *Prostate*. 2014; **74**(14):1379–1390.
209. Balaj L, Lessard R, Dai L, Cho Y-J, Pomeroy SL, Breakefield XO, Skog J. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun*. 2011;**2**:180.
210. Guescini M, Genedani S, Stocchi V, Agnati LF. Astrocytes and glioblastoma cells release exosomes carrying mtDNA. *J Neural Transm (Vienna)*. 2010; **117**(1):1–4.
211. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood*. 2012;**119**(3):756–766.
212. Morelli AE, Larregina AT, Shufesky WJ, Sullivan MLG, Stolz DB, Papworth GD, Zahorchak AF, Logar AJ, Wang Z, Watkins SC, Falo LD Jr, Thomson AW. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood*. 2004;**104**(10): 3257–3266.
213. Feng D, Zhao W-L, Ye Y-Y, Bai X-C, Liu R-Q, Chang L-F, Zhou Q, Sui S-F. Cellular internalization of exosomes occurs through phagocytosis. *Traffic*. 2010;**11**(5):675–687.
214. Escrevente C, Keller S, Altevogt P, Costa J. Interaction and uptake of exosomes by ovarian cancer cells. *BMC Cancer*. 2011;**11**(1):108.
215. Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, Mörgelin M, Belting M. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem*. 2013;**288**(24):17713–17724.
216. Nanbo A, Kawanishi E, Yoshida R, Yoshiyama H. Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. *J Virol*. 2013;**87**(18):10334–10347.
217. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, Regen T, Hanisch U-K, Simons M. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *J Cell Sci*. 2011; **124**(Pt 3):447–458.
218. Paolillo M, Schinelli S. Integrins and exosomes, a dangerous liaison in cancer progression. *Cancers (Basel)*. 2017;**9**(8):E95.
219. Rana S, Yue S, Stadel D, Zöller M. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *Int J Biochem Cell Biol*. 2012;**44**(9):1574–1584.
220. Segura E, Guérin C, Hogg N, Amigorena S, Théry C. $CD8^+$ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. *J Immunol*. 2007;**179**(3):1489–1496.
221. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 2002; **417**(6885):182–187.
222. French KC, Antonyak MA, Cerione RA. Extracellular vesicle docking at the cellular port: extracellular vesicle binding and uptake. *Semin Cell Dev Biol*. 2017; **67**:48–55.
223. Tian T, Zhu Y-L, Zhou Y-Y, Liang G-F, Wang Y-Y, Hu F-H, Xiao Z-D. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem*. 2014; **289**(32):22258–22267.
224. Christianson HC, Svensson KJ, van Kuppevelt TH, Li J-P, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci USA*. 2013;**110**(43):17380–17385.
225. Camussi G, Deregibus M-C, Bruno S, Grange C, Fonsato V, Tetta C. Exosome/microvesicle-mediated epigenetic reprogramming of cells. *Am J Cancer Res*. 2011;**1**(1):98–110.
226. Hong BS, Cho J-H, Kim H, Choi E-J, Rho S, Kim J, Kim JH, Choi D-S, Kim Y-K, Hwang D, Gho YS. Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics*. 2009;**10**(1):556.
227. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. *Cancer Res*. 2011;**71**(11): 3792–3801.
228. Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar C, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;**18**(6): 883–891.
229. Sheldon H, Heikamp E, Turley H, Dragovic R, Thomas P, Oon CE, Leek R, Edelmann M, Kessler B, Sainson RCA, Sargent I, Li J-L, Harris AL. New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood*. 2010;**116**(13):2385–2394.
230. Mulcahy LA, Pink RC, Carter DRF. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles*. 2014;**3**(1):24641.
231. Lai CP, Kim EY, Badr CE, Weissleder R, Mempel TR, Tannous BA, Breakefield XO. Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. *Nat Commun*. 2015;**6**(1):7029.
232. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schäfer R, Beerling E, Schiffelers RM, de Wit E, Berenguer J, Ellenbroek SIJ, Wurdinger T, Pegtel DM, van Rheenen J. In vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell*. 2015;**161**(5):1046–1057.
233. Ridder K, Sevko A, Heide J, Dams M, Rupp A-K, Macas J, Starmann J, Tjwa M, Plate KH, Sültmann H, Altevogt P, Umansky V, Momma S. Extracellular vesicle-mediated transfer of functional RNA in the tumor microenvironment. *OncoImmunology*. 2015; **4**(6):e1008371.
234. Ridder K, Keller S, Dams M, Rupp A-K, Schlaudraff J, Del Turco D, Starmann J, Macas J, Karpova D, Devraj K, Depboylu C, Landfried B, Arnold B, Plate KH, Höglinger G, Sültmann H, Altevogt P, Momma S. Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. *PLoS Biol*. 2014;**12**(6):e1001874.
235. Cai J, Han Y, Ren H, Chen C, He D, Zhou L, Eisner GM, Asico LD, Jose PA, Zeng C. Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells. *J Mol Cell Biol*. 2013;**5**(4): 227–238.
236. Keller S, König A-K, Marmé F, Runz S, Wolterink S, Koensgen D, Mustea A, Sehouli J, Altevogt P. Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett*. 2009;**278**(1):73–81.
237. Christianson HC, Belting M. Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. *Matrix Biol*. 2014;**35**:51–55.
238. Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Müller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015;**527**(7578): 329–335.
239. Rejraji H, Sion B, Prensier G, Carreras M, Motta C, Frenoux J-M, Vericel E, Grizard G, Vernet P, Drevet JR. Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod*. 2006;**74**(6):1104–1113.
240. Franz C, Böing AN, Montag M, Strowitzki T, Markert UR, Mastenbroek S, Nieuwland R, Toth B. Extracellular vesicles in human follicular fluid do not promote coagulation. *Reprod Biomed Online*. 2016; **33**(5):652–655.
241. Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzì P, Rizzari S, Maugeri M, Scollo P, Tatone C, Valadi H, Purrello M, Di Pietro C. Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. *Fertil Steril*. 2014; **102**(6):1751–1761.e1.
242. Al-Dossary AA, Bathala P, Caplan JL, Martin-DeLeon PA. Oviductosome-sperm membrane interaction in cargo delivery: detection of fusion and underlying molecular players using three-dimensional super-resolution structured illumination microscopy (SR-SIM). *J Biol Chem*. 2015;**290**(29):17710–17723.
243. Al-Dossary AA, Strehler EE, Martin-DeLeon PA. Expression and secretion of plasma membrane Ca2+-ATPase 4a (PMCA4a) during murine estrus: association with oviductal exosomes and uptake in sperm. *PLoS ONE*. 2013;**8**(11):e80181.
244. Flori F, Secciani F, Capone A, Paccagnini E, Caruso S, Ricci MG, Focarelli R. Menstrual cycle-related sialidase activity of the female cervical mucus is associated with exosome-like vesicles. *Fertil Steril*. 2007;**88**(4, Suppl):1212–1219.
245. Uszyński W, Zekanowska E, Uszyński M, Zyliński A, Kuczyński J. New observations on procoagulant properties of amniotic fluid: microparticles (MPs) and tissue factor-bearing MPs (MPs-TF), comparison with maternal blood plasma. *Thromb Res*. 2013; **132**(6):757–760.
246. Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, Neve EPA, Scheynius A, Gabrielsson S. Exosomes with immune modulatory features are present in human breast milk. *J Immunol*. 2007;**179**(3):1969–1978.
247. Foster BP, Balassa T, Benen TD, Dominovic M, Elmadjian GK, Florova V, Fransolet MD, Kestlerova A, Kmiecik G, Kostadinova IA, Kyvelidou C, Meggyes M, Mincheva MN, Moro L, Pastuschek J, Spoldi V, Wandernoth P, Weber M, Toth B, Markert UR. Extracellular vesicles in blood, milk and body fluids of the female and male urogenital tract and with special regard to reproduction. *Crit Rev Clin Lab Sci*. 2016;**53**(6):379–395.
248. Frenette G, Sullivan R. Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. *Mol Reprod Dev*. 2001;**59**(1):115–121.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

249. Caballero J, Frenette G, D'Amours O, Belleannée C, Lacroix-Pépin N, Robert C, Sullivan R. Bovine sperm raft membrane associated glioma pathogenesis-related 1-like protein 1 (GliPr1L1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida. *J Cell Physiol*. 2012;**227**(12):3876–3886.
250. Frenette G, Lessard C, Madore E, Fortier MA, Sullivan R. Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis. *Biol Reprod*. 2003;**69**(5):1586–1592.
251. D'Amours O, Frenette G, Caron P, Belleannée C, Guillemette C, Sullivan R. Evidences of biological functions of biliverdin reductase A in the bovine epididymis. *J Cell Physiol*. 2016;**231**(5):1077–1089.
252. Sullivan R, Frenette G, Girouard J. Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J Androl*. 2007;**9**(4):483–491.
253. Martin-DeLeon PA. Epididymosomes: transfer of fertility-modulating proteins to the sperm surface. *Asian J Androl*. 2015;**17**(5):720–725.
254. Oh JS, Han C, Cho C. ADAM7 is associated with epididymosomes and integrated into sperm plasma membrane. *Mol Cells*. 2009;**28**(5):441–446.
255. Choi H, Han C, Jin S, Kwon JT, Kim J, Jeong J, Kim J, Ham S, Jeon S, Yoo YJ, Cho C. Reduced fertility and altered epididymal and sperm integrity in mice lacking ADAM7. *Biol Reprod*. 2015;**93**(3):70.
256. Taylor A, Robson A, Houghton BC, Jepson CA, Ford WCL, Frayne J. Epididymal specific, selenium-independent GPX5 protects cells from oxidative stress-induced lipid peroxidation and DNA mutation. *Hum Reprod*. 2013;**28**(9):2332–2342.
257. Peng H, Shi J, Zhang Y, Zhang H, Liao S, Li W, Lei L, Han C, Ning L, Cao Y, Zhou Q, Chen Q, Duan E. A novel class of tRNA-derived small RNAs extremely enriched in mature mouse sperm. *Cell Res*. 2012; **22**(11):1609–1612.
258. Sharma U, Conine CC, Shea JM, Boskovic A, Derr AG, Bing XY, Belleannée C, Kucukural A, Serra RW, Sun F, Song L, Carone BR, Ricci EP, Li XZ, Fauquier L, Moore MJ, Sullivan R, Mello CC, Garber M, Rando OJ. Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science*. 2016;**351**(6271):391–396.
259. Saez F, Sullivan R. Prostasomes, post-testicular sperm maturation and fertility. *Front Biosci*. 2016; **21**(7):1464–1473.
260. Burden HP, Holmes CH, Persad R, Whittington K. Prostasomes—their effects on human male reproduction and fertility. *Hum Reprod Update*. 2006; **12**(3):283–292.
261. Stegmayr B, Ronquist G. Promotive effect on human sperm progressive motility by prostasomes. *Urol Res*. 1982;**10**(5):253–257.
262. Arienti G, Carlini E, Nicolucci A, Cosmi EV, Santi F, Palmerini CA. The motility of human spermatozoa as influenced by prostasomes at various pH levels. *Biol Cell*. 1999;**91**(1):51–54.
263. Park K-H, Kim B-J, Kang J, Nam T-S, Lim JM, Kim HT, Park JK, Kim YG, Chae S-W, Kim U-H. Ca^{2+} signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. *Sci Signal*. 2011;**4**(173):ra31.
264. Andrews RE, Galileo DS, Martin-DeLeon PA. Plasma membrane Ca^{2+}-ATPase 4: interaction with constitutive nitric oxide synthases in human sperm and prostasomes which carry Ca^{2+}/CaM-dependent serine kinase. *Mol Hum Reprod*. 2015;**21**(11): 832–843.
265. Subirán N, Agirregoitia E, Valdivia A, Ochoa C, Casis L, Irazusta J. Expression of enkephalin-degrading enzymes in human semen and implications for sperm motility. *Fertil Steril*. 2008;**89**(5, Suppl): 1571–1577.
266. Arienti G, Carlini E, Verdacchi R, Cosmi EV, Palmerini CA. Prostasome to sperm transfer of CD13/aminopeptidase N (EC 3.4.11.2). *Biochim Biophys Acta*. 1997;**1336**(3):533–538.
267. Pons-Rejraji H, Artonne C, Sion B, Brugnon F, Canis M, Janny L, Grizard G. Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. *Int J Androl*. 2011;**34**(6 Pt 1):568–580.
268. Cross NL, Mahasreshti P. Prostasome fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. *Arch Androl*. 1997;**39**(1):39–44.
269. Bechoua S, Rieu I, Sion B, Grizard G. Prostasomes as potential modulators of tyrosine phosphorylation in human spermatozoa. *Syst Biol Reprod Med*. 2011; **57**(3):139–148.
270. Aalberts M, Sostaric E, Wubbolts R, Wauben MWM, Nolte-'t Hoen ENM, Gadella BM, Stout TAE, Stoorvogel W. Spermatozoa recruit prostasomes in response to capacitation induction. *Biochim Biophys Acta*. 2013;**1834**(11):2326–2335.
271. Palmerini CA, Saccardi C, Carlini E, Fabiani R, Arienti G. Fusion of prostasomes to human spermatozoa stimulates the acrosome reaction. *Fertil Steril*. 2003; **80**(5):1181–1184.
272. Siciliano L, Marcianò V, Carpino A. Prostasome-like vesicles stimulate acrosome reaction of pig spermatozoa. *Reprod Biol Endocrinol*. 2008;**6**(1):5.
273. Minelli A, Allegrucci C, Liguori L, Ronquist G. Ectodiadenosine polyphosphates hydrolase activity on human prostasomes. *Prostate*. 2002;**51**(1):1–9.
274. Oliw EH, Fabiani R, Johansson L, Ronquist G. Arachidonic acid 15-lipoxygenase and traces of E prostaglandins in purified human prostasomes. *J Reprod Fertil*. 1993;**99**(1):195–199.
275. Sohel MMH, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D. Exosomal and non-exosomal transport of extra-cellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. *PLoS One*. 2013; **8**(11):e78505.
276. Navakanitworakul R, Hung W-T, Gunewardena S, Davis JS, Chotigeat W, Christenson LK. Characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. *Sci Rep*. 2016; **6**(1):25486.
277. da Silveira JC, Carnevale EM, Winger QA, Bouma GJ. Regulation of ACVR1 and ID2 by cell-secreted exosomes during follicle maturation in the mare. *Reprod Biol Endocrinol*. 2014;**12**(1):44.
278. Hung W-T, Hong X, Christenson LK, McGinnis LK. Extracellular vesicles from bovine follicular fluid support cumulus expansion. *Biol Reprod*. 2015;**93**(5): 117.
279. Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch K-P, Neyses L. Plasma membrane Ca^{2+} ATPase 4 is required for sperm motility and male fertility. *J Biol Chem*. 2004;**279**(27): 28220–28226.
280. Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE. Targeted ablation of plasma membrane Ca^{2+}-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *J Biol Chem*. 2004;**279**(32):33742–33750.
281. Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Calle A, Redruello A, López-Martín S, Gutierrez-Adán A, Yañez-Mó M, Ramírez MÁ, Rizos D. Extracellular vesicles from BOEC in in vitro embryo development and quality. *PLoS One*. 2016;**11**(2):e0148083.
282. Burns GW, Brooks KE, Spencer TE. Extracellular vesicles originate from the conceptus and uterus during early pregnancy in sheep. *Biol Reprod*. 2016; **94**(3):56.
283. Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE. Extracellular vesicles in luminal fluid of the ovine uterus. *PLoS ONE*. 2014; **9**(3):e90913.
284. Ruiz-González I, Xu J, Wang X, Burghardt RC, Dunlap KA, Bazer FW. Exosomes, endogenous retroviruses and Toll-like receptors: pregnancy recognition in ewes. *Reproduction*. 2015;**149**(3):281–291.
285. Bidarimath M, Khalaj K, Kridli RT, Kan FWK, Koti M, Tayade C. Extracellular vesicle mediated intercellular communication at the porcine maternal-fetal interface: A new paradigm for conceptus-endometrial cross-talk. *Sci Rep*. 2017;**7**:40476.
286. Salomon C, Torres MJ, Kobayashi M. A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration. *PLoS ONE*. 2014;**9**(6):e98667.
287. Griffiths GS, Galileo DS, Reese K, Martin-Deleon PA. Investigating the role of murine epididymosomes and uterosomes in GPI-linked protein transfer to sperm using SPAM1 as a model. *Mol Reprod Dev*. 2008;**75**(11):1627–1636.
288. Franchi A, Cubilla M, Guidobaldi HA, Bravo AA, Giojalas LC. Uterosome-like vesicles prompt human sperm fertilizing capability. *Mol Hum Reprod*. 2016; **22**(12):833–841.
289. Al-Dossary AA, Martin-Deleon PA. Role of exosomes in the reproductive tract Oviductosomes mediate interactions of oviductal secretion with gametes/early embryo. *Front Biosci*. 2016;**21**(6): 1278–1285.
290. Belleannée C, Calvo É, Caballero J, Sullivan R. Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. *Biol Reprod*. 2013;**89**(2):30.
291. Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Hum Reprod Update*. 2016;**22**(2):182–193.
292. Hell L, Wisgrill L, Ay C, Spittler A, Schwameis M, Jilma B, Pabinger I, Altevogt P, Thaler J. Procoagulant extracellular vesicles in amniotic fluid. *Transl Res*. 2017;**184**:12–20.e1.
293. Wang X. Isolation of extracellular vesicles from breast milk. *Methods Mol Biol*. 2017;**1660**: 351–353.
294. Karlsson O, Rodosthenous RS, Jara C, Brennan KJ, Wright RO, Baccarelli AA, Wright RJ. Detection of long non-coding RNAs in human breastmilk extracellular vesicles: implications for early child development. *Epigenetics*. 2016:0. doi:10.1080/15592294.2016.1216285.
295. Sullivan R, Saez F. Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction*. 2013;**146**(1): R21–R35.
296. Schoysman RJ, Bedford JM. The role of the human epididymis in sperm maturation and sperm storage as reflected in the consequences of epididymovasostomy. *Fertil Steril*. 1986;**46**(2):293–299.
297. Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. *J Androl*. 2005; **26**(4):459–469.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

298. Sisti G, Kanninen TT, Witkin SS. Maternal immunity and pregnancy outcome: focus on preconception and autophagy. *Genes Immun*. 2016;**17**(1):1–7.
299. Robertson SA, Sharkey DJ. The role of semen in induction of maternal immune tolerance to pregnancy. *Semin Immunol*. 2001;**13**(4):243–254.
300. Ickowicz D, Finkelstein M, Breitbart H. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. *Asian J Androl*. 2012;**14**(6): 816–821.
301. Fraser LR. Sperm capacitation and the acrosome reaction. *Hum Reprod*. 1998;**13**(Suppl 1):9–19.
302. Zaneveld LJD, De Jonge CJ, Anderson RA, Mack SR. Human sperm capacitation and the acrosome reaction. *Hum Reprod*. 1991;**6**(9):1265–1274.
303. Zhu J, Barratt CL, Lippes J, Pacey AA, Cooke ID. The sequential effects of human cervical mucus, oviductal fluid, and follicular fluid on sperm function. *Fertil Steril*. 1994;**61**(6):1129–1135.
304. Pikó L. Immunological phenomena in the reproductive process. *Int J Fertil*. 1967;**12**(4):377–383.
305. Yanagimachi R, Kamiguchi Y, Mikamo K, Suzuki F, Yanagimachi H. Maturation of spermatozoa in the epididymis of the Chinese hamster. *Am J Anat*. 1985; **172**(4):317–330.
306. Sullivan R. Epididymosomes: a heterogeneous population of microvesicles with multiple functions in sperm maturation and storage. *Asian J Androl*. 2015;**17**(5):726–729.
307. Caballero JN, Frenette G, Belleannée C, Sullivan R. CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. *PLoS One*. 2013;**8**(6):e65364.
308. D'Amours O, Frenette G, Bordeleau L-J, Allard N, Leclerc P, Blondin P, Sullivan R. Epididymosomes transfer epididymal sperm binding protein 1 (ELSPBP1) to dead spermatozoa during epididymal transit in bovine. *Biol Reprod*. 2012;**87**(4):94.
309. D'Amours O, Bordeleau L-J, Frenette G, Blondin P, Leclerc P, Sullivan R. Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. *Reproduction*. 2012;**143**(6):759–771.
310. Thimon V, Frenette G, Saez F, Thabet M, Sullivan R. Protein composition of human epididymosomes collected during surgical vasectomy reversal: a proteomic and genomic approach. *Hum Reprod*. 2008;**23**(8):1698–1707.
311. Murta D, Batista M, Silva E, Trindade A, Henrique D, Duarte A, Lopes-da-Costa L. Notch signaling in the epididymal epithelium regulates sperm motility and is transferred at a distance within epididymosomes. *Andrology*. 2016;**4**(2):314–327.
312. Reilly JN, McLaughlin EA, Stanger SJ, Anderson AL, Hutcheon K, Church K, Mihalas BP, Tyagi S, Holt JE, Eamens AL, Nixon B. Characterisation of mouse epididymosomes reveals a complex profile of microRNAs and a potential mechanism for modification of the sperm epigenome. *Sci Rep*. 2016;**6**(1): 31794.
313. Wang Q, Lee I, Ren J, Ajay SS, Lee YS, Bao X. Identification and functional characterization of tRNA-derived RNA fragments (tRFs) in respiratory syncytial virus infection. *Mol Ther*. 2013;**21**(2): 368–379.
314. Lee YS, Shibata Y, Malhotra A, Dutta A. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev*. 2009;**23**(22):2639–2649.
315. Ronquist G, Brody I, Gottfries A, Stegmayr B. An Mg^{2+} and Ca^{2+}-stimulated adenosine triphosphatase in human prostatic fluid: part I. *Andrologia*. 1978;**10**(4):261–272.
316. Kravets FG, Lee J, Singh B, Trocchia A, Pentyala SN, Khan SA. Prostasomes: current concepts. *Prostate*. 2000;**43**(3):169–174.
317. Aalberts M, Stout TAE, Stoorvogel W. Prostasomes: extracellular vesicles from the prostate. *Reproduction*. 2013;**147**(1):R1–R14.
318. Arvidson G, Ronquist G, Wikander G, Ojteg AC. Human prostasome membranes exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering. *Biochim Biophys Acta*. 1989;**984**(2): 167–173.
319. Arienti G, Carlini E, Polci A, Cosmi EV, Palmerini CA. Fatty acid pattern of human prostasome lipid. *Arch Biochem Biophys*. 1998;**358**(2):391–395.
320. Carlini E, Palmerini CA, Cosmi EV, Arienti G. Fusion of sperm with prostasomes: effects on membrane fluidity. *Arch Biochem Biophys*. 1997;**343**(1):6–12.
321. Saez F, Frenette G, Sullivan R. Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. *J Androl*. 2003;**24**(2):149–154.
322. Suarez SS, Dai X. Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. *Mol Reprod Dev*. 1995;**42**(3):325–333.
323. Palmerini CA, Carlini E, Nicolucci A, Arienti G. Increase of human spermatozoa intracellular Ca^{2+} concentration after fusion with prostasomes. *Cell Calcium*. 1999;**25**(4):291–296.
324. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J*. 1994;**298**(Pt 2):249–258.
325. Ronquist GK, Larsson A, Ronquist G, Isaksson A, Hreinsson J, Carlsson L, Stavreus-Evers A. Prostasomal DNA characterization and transfer into human sperm. *Mol Reprod Dev*. 2011;**78**(7):467–476.
326. Ronquist KG, Ronquist G, Carlsson L, Larsson A. Human prostasomes contain chromosomal DNA. *Prostate*. 2009;**69**(7):737–743.
327. Ronquist G. Prostasomes: their characterisation: implications for human reproduction: prostasomes and human reproduction. *Adv Exp Med Biol*. 2015; **868**:191–209.
328. Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA*. 2011; **108**(12):4892–4896.
329. Cross NL. Effect of cholesterol and other sterols on human sperm acrosomal responsiveness. *Mol Reprod Dev*. 1996;**45**(2):212–217.
330. Arienti G, Carlini E, Saccardi C, Palmerini CA. Nitric oxide and fusion with prostasomes increase cytosolic calcium in progesterone-stimulated sperm. *Arch Biochem Biophys*. 2002;**402**(2):255–258.
331. Cha K-Y, Chian RC. Maturation in vitro of immature human oocytes for clinical use. *Hum Reprod Update*. 1998;**4**(2):103–120.
332. Reed BG, Carr BR. The normal menstrual cycle and the control of ovulation. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, Koch C, Korbonits M, McLachlan R, New M, Purnell J, Rebar R, Singer F, Vinik A, eds. Endotext [Internet]. South Dartmouth, MA: MDText.com, Inc. Available at: https://www.ncbi.nlm.nih.gov/books/NBK279054/. Accessed 22 May 2015.
333. Okabe M. The cell biology of mammalian fertilization. *Development*. 2013;**140**(22):4471–4479.
334. Hertig AT, Rock J, Adams EC. A description of 34 human ova within the first 17 days of development. *Am J Anat*. 1956;**98**(3):435–493.
335. Aplin JD. The cell biological basis of human implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol*. 2000;**14**(5):457–464.
336. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*. 2006; **7**(3):185–199.
337. Zamah AM, Hassis ME, Albertolle ME, Williams KE. Proteomic analysis of human follicular fluid from fertile women. *Clin Proteomics*. 2015;**12**(1):5.
338. Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol*. 2009;**7**(1): 40.
339. da Silveira JC, Veeramachaneni DNR, Winger QA, Carnevale EM, Bouma GJ. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. *Biol Reprod*. 2012; **86**(3):71.
340. Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter P, Benoit Y, Espeel M, D'Herde K. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. *Hum Fertil (Camb)*. 2014;**17**(2): 90–98.
341. Almiñana C. Snooping on a private conversation between the oviduct and gametes/embryos. *Anim Reprod*. 2015;**12**(3):366–374.
342. Li M-Q, Jin L-P. Ovarian stimulation for in vitro fertilization alters the protein profile expression in endometrial secretion. *Int J Clin Exp Pathol*. 2013; **6**(10):1964–1971.
343. Zhang Y, Wang Q, Wang H, Duan E. Uterine fluid in pregnancy: a biological and clinical outlook. *Trends Mol Med*. 2017;**23**(7):604–614.
344. Salamonsen LA, Nie G, Hannan NJ, Dimitriadis E. Society for Reproductive Biology Founders' Lecture 2009. Preparing fertile soil: the importance of endometrial receptivity. *Reprod Fertil Dev*. 2009;**21**(7): 923–934.
345. Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Pérez-Benavente A, Garcia A, Borrós S, Santamaria A, Ponce J, Matias-Guiu X, Reventós J, Gil-Moreno A, Rigau M, Colás E. Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. *J Transl Med*. 2016;**14**(1):180.
346. Salomon C, Yee SW, Mitchell MD, Rice GE. The possible role of extravillous trophoblast-derived exosomes on the uterine spiral arterial remodeling under both normal and pathological conditions. *Biomed Res Int*. 2014;**2014**:693157.
347. Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G, Nappi C. Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. *Fertil Steril*. 2009;**91**(6):2315–2323.
348. Braundmeier AG, Dayger CA, Mehrotra P, Belton RJ Jr, Nowak RA. EMMPRIN is secreted by human uterine epithelial cells in microvesicles and stimulates metalloproteinase production by human uterine fibroblast cells. *Reprod Sci*. 2012;**19**(12): 1292–1301.
349. Harp D, Driss A, Mehrabi S, Chowdhury I, Xu W, Liu D, Garcia-Barrio M, Taylor RN, Gold B, Jefferson S, Sidell N, Thompson W. Exosomes derived from endometriotic stromal cells have enhanced angiogenic effects in vitro. *Cell Tissue Res*. 2016;**365**(1): 187–196.
350. Texidó L, Romero C, Vidal A, García-Valero J, Fernández Montoli ME, Baixeras N, Condom E, Ponce J, García-Tejedor A, Martín-Satué M. Ecto-nucleotidases activities in the contents of ovarian endometriomas: potential biomarkers of endometriosis. *Mediators Inflamm*. 2014;**2014**:120673.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

351. Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, Xing Q, Jin L, He L, Wu L, Wang L. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. *J Clin Endocrinol Metab*. 2013;**98**(7):3068–3079.
352. Esposito K, Ciotola M, Giugliano F, Schisano B, Improta L, Improta MR, Beneduce F, Rispoli M, De Sio M, Giugliano D. Endothelial microparticles correlate with erectile dysfunction in diabetic men. *Int J Impot Res*. 2007;**19**(2):161–166.
353. La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE. Arterial erectile dysfunction: reliability of new markers of endothelial dysfunction. *J Endocrinol Invest*. 2011;**34**(10):e314–e320.
354. Patil R, Ghosh K, Satoskar P, Shetty S. Elevated procoagulant endothelial and tissue factor expressing microparticles in women with recurrent pregnancy loss. *PLoS One*. 2013;**8**(11):e81407.
355. Laude I, Rongières-Bertrand C, Boyer-Neumann C, Wolf M, Mairovitz V, Hugel B, Freyssinet JM, Frydman R, Meyer D, Eschwège V. Circulating procoagulant microparticles in women with unexplained pregnancy loss: a new insight. *Thromb Haemost*. 2001;**85**(1):18–21.
356. Pasquier E, De Saint Martin L, Bohec C, Collet M. Unexplained pregnancy loss: a marker of basal endothelial dysfunction?. *Fertil Steril*. 2013;**100**(4):1013–1017.
357. Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol*. 2007;**178**(9):5949–5956.
358. Guller S, Tang Z, Ma YY, Di Santo S, Sager R, Schneider H. Protein composition of microparticles shed from human placenta during placental perfusion: potential role in angiogenesis and fibrinolysis in preeclampsia. *Placenta*. 2011;**32**(1):63–69.
359. Gardiner C, Tannetta DS, Simms CA, Harrison P, Redman CWG, Sargent IL. Syncytiotrophoblast microvesicles released from pre-eclampsia placentae exhibit increased tissue factor activity. *PLoS One*. 2011;**6**(10):e26313.
360. Tannetta DS, Hunt K, Jones CI, Davidson N, Coxon CH, Ferguson D, Redman CW, Gibbins JM, Sargent IL, Tucker KL. Syncytiotrophoblast extracellular vesicles from pre-eclampsia placentas differentially affect platelet function. *PLoS One*. 2015;**10**(11):e0142538.
361. Cronqvist T, Saljé K, Familari M, Guller S, Schneider H, Gardiner C, Sargent IL, Redman CW, Mörgelin M, Åkerström B, Gram M, Hansson SR. Syncytiotrophoblast vesicles show altered micro-RNA and haemoglobin content after ex-vivo perfusion of placentas with haemoglobin to mimic pre-eclampsia. *PLoS One*. 2014;**9**(2):e90020.
362. Aly AS, Khandelwal M, Zhao J, Mehmet AH, Sammel MD, Parry S. Neutrophils are stimulated by syncytiotrophoblast microvillous membranes to generate superoxide radicals in women with pre-eclampsia. *Am J Obstet Gynecol*. 2004;**190**(1):252–258.
363. Gilani SI, Weissgerber TL, Garovic VD, Jayachandran M. Preeclampsia and extracellular vesicles. *Curr Hypertens Rep*. 2016;**18**(9):68.
364. Kohli S, Ranjan S, Hoffmann J, Kashif M, Daniel EA, Al-Dabet MM, Bock F, Nazir S, Huebner H, Mertens PR, Fischer K-D, Zenclussen AC, Offermanns S, Aharon A, Brenner B, Shahzad K, Ruebner M, Isermann B. Maternal extracellular vesicles and platelets promote preeclampsia via inflammasome activation in trophoblasts. *Blood*. 2016;**128**(17):2153–2164.
365. Holder B, Jones T, Sancho Shimizu V, Rice TF, Donaldson B, Bouqueau M, Forbes K, Kampmann B. Macrophage exosomes induce placental inflammatory cytokines: a novel mode of maternal–placental messaging. *Traffic*. 2016;**17**(2):168–178.
366. Holder BS, Tower CL, Jones CJP, Aplin JD, Abrahams VM. Heightened pro-inflammatory effect of preeclamptic placental microvesicles on peripheral blood immune cells in humans. *Biol Reprod*. 2012;**86**(4):103.
367. Shomer E, Katzenell S, Zipori Y, Sammour RN, Isermann B, Brenner B, Aharon A. Microvesicles of women with gestational hypertension and preeclampsia affect human trophoblast fate and endothelial function. *Hypertension*. 2013;**62**(5):893–898.
368. Kaptan K, Beyan C, Ifran A, Pekel A. Platelet-derived microparticle levels in women with recurrent spontaneous abortion. *Int J GYnecol Obstet*. 2008;**102**:271–274.
369. Salomon C, Scholz-Romero K, Sarker S, Sweeney E, Kobayashi M, Correa P, Longo S, Duncombe G, Mitchell MD, Rice GE, Illanes SE. Gestational diabetes mellitus is associated with changes in the concentration and bioactivity of placenta-derived exosomes in maternal circulation across gestation. *Diabetes*. 2016;**65**(3):598–609.
370. Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological conditions. *Blood Rev*. 2013;**27**(1):31–39.
371. Bulun SE. Endometriosis. *N Engl J Med*. 2009;**360**(3):268–279.
372. Tarin D. Cell and tissue interactions in carcinogenesis and metastasis and their clinical significance. *Semin Cancer Biol*. 2011;**21**(2):72–82.
373. Bruner-Tran KL, Eisenberg E, Yeaman GR, Anderson TA, McBean J, Osteen KG. Steroid and cytokine regulation of matrix metalloproteinase expression in endometriosis and the establishment of experimental endometriosis in nude mice. *J Clin Endocrinol Metab*. 2002;**87**(10):4782–4791.
374. Ehrmann DA. Polycystic ovary syndrome. *N Engl J Med*. 2005;**352**(12):1223–1236.
375. Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. *Am J Obstet Gynecol*. 1935;**29**(2):181–191.
376. Koiou E, Tziomalos K, Katsikis I, Papadakis E, Kandaraki EA, Panidis D. Platelet-derived microparticles in overweight/obese women with the polycystic ovary syndrome. *Gynecol Endocrinol*. 2013;**29**(3):250–253.
377. Willis GR, Connolly K, Ladell K, Davies TS, Guschina IA, Ramji D, Miners K, Price DA, Clayton A, James PE, Rees DA. Young women with polycystic ovary syndrome have raised levels of circulating annexin V–positive platelet microparticles. *Hum Reprod*. 2014;**29**(12):2756–2763.
378. Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, Zhang B, Liang X, Li T, Chen J, Shen J, Zhao J, You L, Gao X, Zhu D, Zhao X, Yan Y, Qin Y, Li W, Yan J, Wang Q, Zhao J, Geng L, Ma J, Zhao Y, He G, Zhang A, Zou S, Yang A, Liu J, Li W, Li B, Wan C, Qin Y, Shi J, Yang J, Jiang H, Xu J-E, Qi X, Sun Y, Zhang Y, Hao C, Ju X, Zhao D, Ren C-E, Li X, Zhang W, Zhang Y, Zhang J, Wu D, Zhang C, He L, Chen Z-J. Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. *Nat Genet*. 2012;**44**(9):1020–1025.
379. Chen Z-J, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, Liang X, Zhao X, Zhao J, Sun Y, Zhang B, Jiang H, Zhao D, Bian Y, Gao X, Geng L, Li Y, Zhu D, Sun X, Xu J-E, Hao C, Ren C-E, Zhang Y, Chen S, Zhang W, Yang A, Yan J, Li Y, Ma J, Zhao Y. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet*. 2011;**43**(1):55–59.
380. McAllister JM, Modi B, Miller BA, Biegler J, Bruggeman R, Legro RS, Strauss JF III. Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype. *Proc Natl Acad Sci USA*. 2014;**111**(15):E1519–E1527.
381. Wang W, Fan J, Huang G, Zhu X, Tian Y, Tan H, Su L. Meta-analysis of prevalence of erectile dysfunction in mainland China: evidence based on epidemiological surveys. *Sex Med*. 2017;**5**(1):e19–e30.
382. Esposito K, Ciotola M, Giugliano F, Sardelli L, Giugliano F, Maiorino MI, Beneduce F, De Sio M, Giugliano D. Phenotypic assessment of endothelial microparticles in diabetic and nondiabetic men with erectile dysfunction. *J Sex Med*. 2008;**5**(6):1436–1442.
383. La Vignera S, Vicari E, Condorelli RA, Di Pino L, Calogero AE. Arterial erectile dysfunction: reliability of penile Doppler evaluation integrated with serum concentrations of late endothelial progenitor cells and endothelial microparticles. *J Androl*. 2012;**33**(3):412–419.
384. Condorelli RA, Calogero AE, Vicari E, di Pino L, Giacone F, Mongioì L, la Vignera S. Arterial erectile dysfunction and peripheral arterial disease: reliability of a new phenotype of endothelial progenitor cells and endothelial microparticles. *J Androl*. 2012;**33**(6):1268–1275.
385. Park K, Ryu KS, Li WJ, Kim SW, Paick J-S. Chronic treatment with a type 5 phosphodiesterase inhibitor suppresses apoptosis of corporal smooth muscle by potentiating Akt signalling in a rat model of diabetic erectile dysfunction. *Eur Urol*. 2008;**53**(6):1282–1289.
386. Porst H, Glina S, Ralph D, Zeigler H, Wong DG, Woodward B. Durability of response following cessation of tadalafil taken once daily as treatment for erectile dysfunction. *J Sex Med*. 2010;**7**(10):3487–3494.
387. La Vignera S, Condorelli RA, Vicari E, D'Agata R, Calogero AE. Endothelial apoptosis decrease following tadalafil administration in patients with arterial ED does not last after its discontinuation. *Int J Impot Res*. 2011;**23**(5):200–205.
388. La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE. Endothelial antioxidant compound prolonged the endothelial antiapoptotic effects registered after tadalafil treatment in patients with arterial erectile dysfunction. *J Androl*. 2012;**33**(2):170–175.
389. Schulz E, Gori T, Münzel T. Oxidative stress and endothelial dysfunction in hypertension. *Hypertens Res*. 2011;**34**(6):665–673.
390. Higashi Y, Noma K, Yoshizumi M, Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. *Circ J*. 2009;**73**(3):411–418.
391. Francomano D, Bruzziches R, Natali M, Aversa A, Spera G. Cardiovascular effect of testosterone replacement therapy in aging male. *Acta Biomed*. 2010;**81**(Suppl 1):101–106.
392. La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero A. Original immunophenotype of blood endothelial progenitor cells and microparticles in patients with isolated arterial erectile dysfunction and late onset hypogonadism: effects of androgen replacement therapy. *Aging Male*. 2011;**14**(3):183–189.
393. La Vignera S, Condorelli RA, Vicari E, D'Agata R, Calogero AE. New immunophenotype of blood endothelial progenitor cells and endothelial microparticles in patients with arterial erectile

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

dysfunction and late-onset hypogonadism. *J Androl*. 2011;**32**(5):509–517.
394. Sarker S, Scholz-Romero K. Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy. *J Transl Med*. 2014;**12**:204.
395. Salomon C, Kobayashi M, Ashman K, Sobrevia L. Hypoxia-induced changes in the bioactivity of cytotrophoblast-derived exosomes. *PLoS ONE*. 2013; **8**(11):e79636.
396. Rice GE, Scholz-Romero K, Sweeney E, Peiris H, Kobayashi M, Duncombe G, Mitchell MD, Salomon C. The effect of glucose on the release and bioactivity of exosomes from first trimester trophoblast cells. *J Clin Endocrinol Metab*. 2015;**100**(10): E1280–E1288.
397. Mitchell MD, Peiris HN, Kobayashi M, Koh YQ. Placental exosomes in normal and complicated pregnancy. *Am J Obstet Gynecol*. 2015;**213**(4 Suppl): S173–S181.
398. Salomon C, Sobrevia L, Ashman K, Illanes S, Mitchell MD, Rice GE. The role of placental exosomes in gestational diabetes mellitus. In: Sobrevia L, ed. *Gestational Diabetes–Causes, Diagnosis and Treatment*. Available at: https://doi.org/10.5772/55298. Accessed 24 April 2017.
399. Redman C, Tannetta DS, Dragovic RA, Gardiner C. Review: does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta*. 2012; **33**:S48–S54.
400. Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol*. 2009;**2**(2):76–83.
401. Alijotas-Reig J, Palacio-Garcia C, Farran-Codina I, Zarzoso C, Cabero-Roura L, Vilardell-Tarres M. Circulating cell-derived microparticles in women with pregnancy loss. *Am J Reprod Immunol*. 2011; **66**(3):199–208.
402. Katz D, Beilin Y. Disorders of coagulation in pregnancy. *Br J Anaesth*. 2015;**115**(Suppl 2):ii75–ii88.
403. Rai R. Is miscarriage a coagulopathy? *Curr Opin Obstet Gynecol*. 2003;**15**(3):265–268.
404. Katzenell S, Shomer E, Zipori Y, Zylberfisz A, Brenner B, Aharon A. Characterization of negatively charged phospholipids and cell origin of microparticles in women with gestational vascular complications. *Thromb Res*. 2012;**130**(3):479–484.
405. Karthikeyan VJ, Lip GYH. Endothelial damage/dysfunction and hypertension in pregnancy. *Front Biosci (Elite Ed)*. 2011;**3**:1100–1108.
406. Uzan J, Carbonnel M, Piconne O, Asmar R, Ayoubi JM. Pre-eclampsia: pathophysiology, diagnosis, and management. *Vasc Health Risk Manag*. 2011;**7**: 467–474.
407. Aharon A. The role of extracellular vesicles in placental vascular complications. *Thromb Res*. 2015; **135**(Suppl 1):S23–S25.
408. Jeyabalan A. Epidemiology of preeclampsia: impact of obesity. *Nutr Rev*. 2013;**71**(Suppl 1):S18–S25.
409. Roberts JM, Escudero C. The placenta in preeclampsia. *ScienceDirect. Pregnancy Hypertens*. 2012; **2**(2):72–83.
410. Chen Y, Huang Y, Jiang R, Teng Y. Syncytiotrophoblast-derived microparticle shedding in early-onset and late-onset severe pre-eclampsia. *Int J Gynaecol Obstet*. 2012; **119**(3):234–238.
411. Goswami D, Tannetta DS, Magee LA, Fuchisawa A, Redman CWG, Sargent IL, von Dadelszen P. Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. *Placenta*. 2006;**27**(1):56–61.
412. Li H, Han L, Yang Z, Huang W, Zhang X, Gu Y, Li Y, Liu X, Zhou L, Hu J, Yu M, Yang J, Li Y, Zheng Y, Guo J, Han J, Li L. Differential proteomic analysis of syncytiotrophoblast extracellular vesicles from early-onset severe preeclampsia, using 8-plex iTRAQ labeling coupled with 2D nano LC-MS/MS. *Cell Physiol Biochem*. 2015;**36**(3):1116–1130.
413. Baig S, Kothandaraman N, Manikandan J, Rong L, Ee KH, Hill J, Lai CW, Tan WY, Yeoh F, Kale A, Su LL, Biswas A, Vasoo S, Choolani M. Proteomic analysis of human placental syncytiotrophoblast microvesicles in preeclampsia. *Clin Proteomics*. 2014;**11**(1):40.
414. Baig S, Lim JY, Fernandis AZ, Wenk MR, Kale A, Su LL, Biswas A, Vasoo S, Shui G, Choolani M. Lipidomic analysis of human placental syncytiotrophoblast microvesicles in adverse pregnancy outcomes. *Placenta*. 2013;**34**(5):436–442.
415. Truong G, Guanzon D, Kinhal V, Elfeky O, Lai A, Longo S, Nuzhat Z, Palma C, Scholz-Romero K, Menon R, Mol BW, Rice GE, Salomon C. Oxygen tension regulates the miRNA profile and bioactivity of exosomes released from extravillous trophoblast cells—liquid biopsies for monitoring complications of pregnancy. *PLoS ONE*. 2017;**12**(3): e0174514.
416. Vargas A, Zhou S, Éthier-Chiasson M, Flipo D, Lafond J, Gilbert C, Barbeau B. Syncytin proteins incorporated in placenta exosomes are important for cell uptake and show variation in abundance in serum exosomes from patients with preeclampsia. *FASEB J*. 2014;**28**(8):3703–3719.
417. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA; CPEP Study Group. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med*. 2006;**355**(10): 992–1005.
418. Estellés A, Gilabert J, Keeton M, Eguchi Y, Aznar J, Grancha S, Espña F, Loskutoff DJ, Schleef RR. Altered expression of plasminogen activator inhibitor type 1 in placentas from pregnant women with preeclampsia and/or intrauterine fetal growth retardation. *Blood*. 1994;**84**(1):143–150.
419. Centlow M, Carninci P, Nemeth K, Mezey E, Brownstein M, Hansson SR. Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes. *Fertil Steril*. 2008;**90**(5):1834–1843.
420. May K, Rosenlöf L, Olsson MG, Centlow M, Mörgelin M, Larsson I, Cederlund M, Rutardottir S, Siegmund W, Schneider H, Akerström B, Hansson SR. Perfusion of human placenta with hemoglobin introduces preeclampsia-like injuries that are prevented by α_1-microglobulin. *Placenta*. 2011;**32**(4): 323–332.
421. Olsson MG, Centlow M, Rutardóttir S, Stenfors I, Larsson J, Hosseini-Maaf B, Olsson ML, Hansson SR, Akerström B. Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger α_1-microglobulin in preeclampsia. *Free Radic Biol Med*. 2010;**48**(2):284–291.
422. Lok CAR, Jebbink J, Nieuwland R, Faas MM, Boer K, Sturk A, Van Der Post JAM. Leukocyte activation and circulating leukocyte-derived microparticles in preeclampsia. *Am J Reprod Immunol*. 2009;**61**(5): 346–359.
423. Mikhailova VA, Ovchinnikova OM, Zainulina MS, Sokolov DI, Sel'kov SA. Detection of microparticles of leukocytic origin in the peripheral blood in normal pregnancy and preeclampsia. *Bull Exp Biol Med*. 2014;**157**(6):751–756.
424. Joerger-Messerli MS, Hoesli IM, Rusterholz C, Lapaire O. Stimulation of monocytes by placental microparticles involves Toll-like receptors and nuclear factor κ-light-chain-enhancer of activated B cells. *Front Immunol*. 2014;**5**:173.
425. Ling L, Huang H, Zhu L, Mao T, Shen Q, Zhang H. Evaluation of plasma endothelial microparticles in pre-eclampsia. *J Int Med Res*. 2014;**42**(1): 42–51.
426. Elfeky O, Longo S, Lai A, Rice GE, Salomon C. Influence of maternal BMI on the exosomal profile during gestation and their role on maternal systemic inflammation. *Placenta*. 2017;**50**:60–69.
427. Chen L, Mayo R, Chatry A, Hu G. Gestational diabetes mellitus: its epidemiology and implication beyond pregnancy. *Curr Epidemiol Rep*. 2016;**3**(1):1–11.
428. Erem C, Kuzu UB, Deger O, Can G. Prevalence of gestational diabetes mellitus and associated risk factors in Turkish women: the Trabzon GDM Study. *Arch Med Sci*. 2015;**11**(4):724–735.
429. Moro L, Bardají A, Macete E, Barrios D, Morales-Prieto DM, España C, Mandomando I, Sigaúque B, Dobaño C, Markert UR, Benitez-Ribas D, Alonso PL, Menéndez C, Mayor A. Placental microparticles and microRNAs in pregnant women with *Plasmodium falciparum* or HIV infection. *PLoS One*. 2016;**11**(1): e0146361.
430. Tan KH, Tan SS, Ng MJ, Tey WS, Sim WK, Allen JC, Lim SK. Extracellular vesicles yield predictive pre-eclampsia biomarkers. *J Extracell Vesicles*. 2017;**6**(1): 1408390.
431. Tan KH, Tan SS, Sze SK, Lee WKR, Ng MJ, Lim SK. Plasma biomarker discovery in preeclampsia using a novel differential isolation technology for circulating extracellular vesicles. *Am J Obstet Gynecol*. 2014;**211**(4):380.e1–380.e13.
432. Campello E, Spiezia L, Radu CM, Dhima S, Visentin S, Valle FD, Tormene D, Woodhams B, Cosmi E, Simioni P. Circulating microparticles in umbilical cord blood in normal pregnancy and pregnancy with pre-eclampsia. *Thromb Res*. 2015;**136**(2):427–431.
433. Chaparro A, Gaedechens D, Ramírez V, Zuñiga E, Kusanovic JP, Inostroza C, Varas-Godoy M, Silva K, Salomon C, Rice G, Illanes SE. Placental biomarkers and angiogenic factors in oral fluids of patients with preeclampsia. *Prenat Diagn*. 2016;**36**(5):476–482.
434. Salomon C, Guanzon D, Scholz-Romero K, Longo S, Correa P, Illanes SE, Rice GE. Placental exosomes as early biomarker of preeclampsia: potential role of exosomal microRNAs across gestation. *J Clin Endocrinol Metab*. 2017;**102**(9):3182–3194.
435. Motta-Mejia C, Kandzija N, Zhang W, Mhlomi V, Cerdeira AS, Burdujan A, Tannetta D, Dragovic R, Sargent IL, Redman CW, Kishore U, Vatish M. Placental vesicles carry active endothelial nitric oxide synthase and their activity is reduced in preeclampsia. *Hypertension*. 2017;**70**(2):372–381.
436. Hilfiker-Kleiner D, Sliwa K. Pathophysiology and epidemiology of peripartum cardiomyopathy. *Nat Rev Cardiol*. 2014;**11**(6):364–370.
437. Walenta K, Schwarz V, Schirmer SH, Kindermann I, Friedrich EB, Solomayer EF, Sliwa K, Labidi S, Hilfiker-Kleiner D, Böhm M. Circulating microparticles as indicators of peripartum cardiomyopathy. *Eur Heart J*. 2012;**33**(12):1469–1479.
438. Halkein J, Tabruyn SP, Ricke-Hoch M, Haghikia A, Nguyen N-Q-N, Scherr M, Castermans K, Malvaux L, Lambert V, Thiry M, Sliwa K, Noel A, Martial JA, Hilfiker-Kleiner D, Struman I. MicroRNA-146a is a therapeutic target and biomarker for peripartum cardiomyopathy. *J Clin Invest*. 2013;**123**(5):2143–2154.
439. Reiner AT, Witwer KW, van Balkom BWM, de Beer J, Brodie C, Corteling RL, Gabrielsson S, Gimona M, Ibrahim AG, de Kleijn D, Lai CP, Lötvall J, Del Portillo HA, Reischl IG, Riazifar M, Salomon C, Tahara H, Toh WS, Wauben MHM, Yang VK, Yang Y, Yeo RWY, Yin H, Giebel B, Rohde E, Lim SK. Concise review: developing best-practice models for the therapeutic

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022

use of extracellular vesicles. *Stem Cells Transl Med*. 2017;**6**(8):1730–1739.
440. Jang SC, Kim OY, Yoon CM, Choi D-S, Roh T-Y, Park J, Nilsson J, Lötvall J, Kim Y-K, Gho YS. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. *ACS Nano*. 2013;**7**(9):7698–7710.
441. Tan S, Li X, Guo Y, Zhang Z. Lipid-enveloped hybrid nanoparticles for drug delivery. *Nanoscale*. 2013;**5**(3): 860–872.
442. Haney MJ, Klyachko NL, Zhao Y, Gupta R, Plotnikova EG, He Z, Patel T, Piroyan A, Sokolsky M, Kabanov AV, Batrakova EV. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release*. 2015;**207**: 18–30.
443. Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, Wei J, Nie G. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials*. 2014;**35**(7):2383–2390.
444. Ohno S, Takanashi M, Sudo K, Ueda S, Ishikawa A, Matsuyama N, Fujita K, Mizutani T, Ohgi T, Ochiya T, Gotoh N, Kuroda M. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol Ther*. 2013;**21**(1): 185–191.
445. Kooijmans SAA, Aleza CG, Roffler SR, van Solinge WW, Vader P, Schiffelers RM. Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *J Extracell Vesicles*. 2016;**5**(1):31053.
446. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM. Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *J Control Release*. 2015; **205**:35–44.
447. Ohno S, Drummen GPC, Kuroda M. Focus on extracellular vesicles: development of extracellular vesicle-based therapeutic systems. *Int J Mol Sci*. 2016;**17**(2):172.
448. Syn NL, Wang L, Chow EK-H, Lim CT, Goh B-C. Exosomes in cancer nanomedicine and immunotherapy: prospects and challenges. *Trends Biotechnol*. 2017;**35**(7):665–676.
449. Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertil Steril*. 2006;**86**(3):678–685.
450. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*. 2006;**20**(5):847–856.
451. Saadeldin IM, Kim SJ, Choi YB, Lee BC. Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. *Cell Reprogram*. 2014;**16**(3):223–234.
452. Nguyen HPT, Simpson RJ, Salamonsen LA, Greening DW. Extracellular vesicles in the intrauterine environment: challenges and potential functions. *Biol Reprod*. 2016;**95**(5):109.
453. Greening DW, Nguyen HPT, Evans J, Simpson RJ, Salamonsen LA. Modulating the endometrial epithelial proteome and secretome in preparation for pregnancy: the role of ovarian steroid and pregnancy hormones. *J Proteomics*. 2016;**144**:99–112.
454. Kim TS, Lee SH, Gang GT, Lee YS, Kim SU, Koo DB, Shin MY, Park CK, Lee DS. Exogenous DNA uptake of boar spermatozoa by a magnetic nanoparticle vector system. *Reprod Domest Anim*. 2010;**45**(5):e201–e206.
455. Campos VF, de Leon PMM, Komninou ER, Dellagostin OA, Deschamps JC, Seixas FK, Collares T. NanoSMGT: transgene transmission into bovine embryos using halloysite clay nanotubes or nanopolymer to improve transfection efficiency. *Theriogenology*. 2011;**76**(8):1552–1560.
456. Liang X, Zhang L, Wang S, Han Q, Zhao RC. Exosomes secreted by mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-125a. *J Cell Sci*. 2016;**129**(11):2182–2189.
457. Lösche W, Scholz T, Temmler U, Oberle V, Claus RA. Platelet-derived microvesicles transfer tissue factor to monocytes but not to neutrophils. *Platelets*. 2004; **15**(2):109–115.
458. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, Becker A, Hoshino A, Mark MT, Molina H, Xiang J, Zhang T, Theilen T-M, García-Santos G, Williams C, Ararso Y, Huang Y, Rodrigues G, Shen T-L, Labori KJ, Lothe IMB, Kure EH, Hernandez J, Doussot A, Ebbesen SH, Grandgenett PM, Hollingsworth MA, Jain M, Mallya K, Batra SK, Jarnagin WR, Schwartz RE, Matei I, Peinado H, Stanger BZ, Bromberg J, Lyden D. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol*. 2015;**17**(6):816–826.
459. Fong MY, Zhou W, Liu L, Alontaga AY, Chandra M, Ashby J, Chow A, O'Connor STF, Li S, Chin AR, Somlo G, Palomares M, Li Z, Tremblay JR, Tsuyada A, Sun G, Reid MA, Wu X, Swiderski P, Ren X, Shi Y, Kong M, Zhong W, Chen Y, Wang SE. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat Cell Biol*. 2015;**17**(2):183–194.
460. Wood MJ, O'Loughlin AJ, Samira L. Exosomes and the blood–brain barrier: implications for neurological diseases. *Ther Deliv*. 2011;**2**(9):1095–1099.
461. Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, Gil CD, Nerviani A, Dell'Accio F, Pitzalis C, Oliani SM, Jan LY, Perretti M. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. *Sci Transl Med*. 2015;**7**(315): 315ra190.
462. Suetsugu A, Honma K, Saji S, Moriwaki H, Ochiya T, Hoffman RM. Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models. *Adv Drug Deliv Rev*. 2013;**65**(3):383–390.
463. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol*. 2011;**29**(4):341–345.
464. Wahlgren J, De L Karlson T, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen P, Valadi H. Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Res*. 2012;**40**(17):e130.
465. Ju Z, Ma J, Wang C, Yu J, Qiao Y, Hei F. Exosomes from iPSCs delivering siRNA attenuate intracellular adhesion molecule-1 expression and neutrophils adhesion in pulmonary microvascular endothelial cells. *Inflammation*. 2017;**40**(2):486–496.
466. Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, Barnes S, Grizzle W, Miller D, Zhang H-G. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther*. 2010; **18**(9):1606–1614.
467. Luan X, Sansanaphongpricha K, Myers I, Chen H, Yuan H, Sun D. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol Sin*. 2017;**38**(6):754–763.
468. Gopal SK, Greening DW, Rai A, Chen M, Xu R, Shafiq A, Mathias RA, Zhu H-J, Simpson RJ. Extracellular vesicles: their role in cancer biology and epithelial-mesenchymal transition. *Biochem J*. 2017;**474**(1): 21–45.
469. Lener T, Gimona M, Aigner L, Börger V, Buzas E, Camussi G, Chaput N, Chatterjee D, Court FA, Del Portillo HA, O'Driscoll L, Fais S, Falcon-Perez JM, Felderhoff-Mueser U, Fraile L, Gho YS, Görgens A, Gupta RC, Hendrix A, Hermann DM, Hill AF, Hochberg F, Horn PA, de Kleijn D, Kordelas L, Kramer BW, Krämer-Albers E-M, Laner-Plamberger S, Laitinen S, Leonardi T, Lorenowicz MJ, Lim SK, Lötvall J, Maguire CA, Marcilla A, Nazarenko I, Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl IG, Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D, Tonn T, Vader P, van Balkom BWM, Wauben M, Andaloussi SE, Théry C, Rohde E, Giebel B. Applying extracellular vesicles based therapeutics in clinical trials—an ISEV position paper. *J Extracell Vesicles*. 2015;**4**(1):30087.
470. Barkalina N, Jones C, Wood MJA, Coward K. Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature. *Hum Reprod Update*. 2015;**21**(5):627–639.
471. Sousa C, Pereira I, Santos AC, Carbone C, Kovačević AB, Silva AM, Souto EB. Targeting dendritic cells for the treatment of autoimmune disorders. *Colloids Surf B Biointerfaces*. 2017;**158**:237–248.

Acknowledgments

We thank Fernando Ibañez from Contextos Culturales for help in the final edition of the figures for this review.

Financial Support: This work was supported by MINECO/FEDER Grant SAF2015-67154-R to C.S; La Trobe Institute for Molecular Science Fellowship, La Trobe University Leadership Research Focus grant, and La Trobe University Start-up Fund to D.W.G; MECD Grant FPU15/02248 to D.B; Atracció de Talent Program ultraviolet-INV-PREDOC14-178329 to N.B; National Health and Medical Research Council of Australia Project Grant 1002028 and the Victorian Government's infrastructure support funding to the Hudson Institute to L.A.S.; and by Miguel Servet Program Type I of ISCIII (CP13/00038) and FIS project (PI14/00545) to F.V.

Correspondence and Reprint Requests: Felipe Vilella, PhD, Igenomix Foundation/INCLIVA, Narcis de Monturiol Estarriol 11, Parcela B, Paterna, 46980 Valencia, Spain. E-mail: felipe.vilella@igenomix.com.

Disclosure Summary: The authors have nothing to disclose.

*These authors contributed equally to this work.

Abbreviations

AB, apoptotic body; AFM, atomic force microscopy; BLVRA, biliverdin reductase A; BMI, body mass index; cAMP, cyclic adenosine monophosphate; ED, erectile dysfunction; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; EVT, extravillous trophoblast; EXO, exosome; FAK, focal adhesion kinase; GD, gestational diabetes; GTPase, guanosine triphosphatase; HbF, cell-free hemoglobin; IL, interleukin; ILV, intraluminal vesicle; LOT, low-oxygen tension; miRNA, microRNA; mRNA, messenger RNA; MV, microvesicle; MVB, multivesicular body; NK, natural killer; NOS, nitric oxide synthase; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; PCOS, polycystic ovarian syndrome; PE, preeclampsia; PKA, protein kinase A; PLAP, placental alkaline phosphatase; PPCM, peripartum cardiomyopathy; PS, phosphatidylserine; SEM, scanning electron microscopy; siRNA, small interfering RNA; SPAM1, sperm adhesion molecule 1; SPB, spontaneous preterm birth; SPZ, spermatozoa; STB, syncytiotrophoblast; STBM, syncytiotrophoblast-derived exosome and microvesicle; TEM, transmission electron microscopy; TNF, tumor necrosis factor; tRF, transfer RNA fragment; tRNA, transfer RNA; μNMR, micronuclear magnetic resonance spectrometry.

Downloaded from https://academic.oup.com/edrv/article/39/3/292/4828195 by U.S. Department of Justice user on 17 August 2022