Chapter

Roles of Extracellular Vesicles in Human Reproduction

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Abstract

Extracellular vesicles (EVs) are newly identified as cell-to-cell communication mediators that carry and transfer various regulatory molecules. Recent studies have shown that EVs play important roles in normal physiology and pathological conditions of human reproduction. In the female reproductive system, EVs in follicular fluid, oviduct fluid, and uterine luminal fluid are considered as vehicles to regulate follicular development, oocyte maturation and mediate embryo–maternal crosstalk to affect embryo implantation and pregnancy. In the male reproductive system, prostasomes and epididymosomes are involved in regulating sperm maturation, motility, capacitation, acrosome reaction, and fertilization. EVs transmitted cargos also play important roles in reproduction-related pathologies, such as polycystic ovarian syndrome, endometriosis, pregnancy complications, male infertility, and gynecological malignant tumors. In view of the important roles in the reproductive system, EVs may be used as biomarkers or therapeutic targets for reproductive abnormalities and related diseases. In this chapter, we sorted EVs in human reproduction through their physical/pathological functions and mechanisms, and listed several EVs as biomarkers and clinical therapeutic applications in the future.

Keywords: extracellular vesicles, exosome, reproductive system, polycystic ovarian syndrome, endometriosis, pregnancy disorders, male infertility

1. Introduction

Cell communication is vital for all living organisms, whether between environment or host with single-celled organisms, or between cells in multicellular organisms [1]. As new-found mediators, extracellular vesicles (EVs) mediate a cell communication mechanism that is different from classic ways as long/short range of secretory signaling, receptor-dependent contact signaling, gap junction, etc. According to cell origin, biogenetic mechanisms, and physical features (size and density), EVs can be classified into three major categories: apoptotic body (ABs), microvesicles (MVs), and exosomes (EXOs) [2]. These vesicles act as shuttles, transporting "cargo" like protein, lipids, nucleic acids, and other regulatory chemicals from one tissue or cell to another through body fluid [3]. When EVs and their targets fuse, their cargos can influence the functioning of target cells, allowing them to engage in a variety of physiological

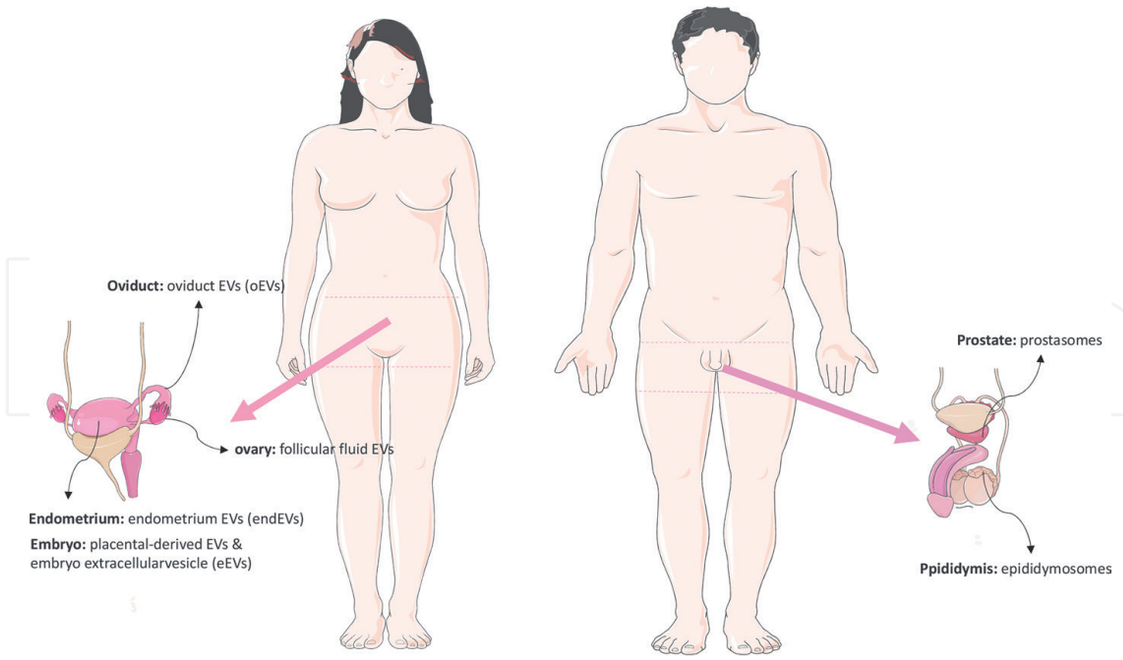

Figure 1.
EVs from human reproductive systems.

and pathological processes. That is why EVs have a significant role in multiple biological processes and diseases, such as immune function, tissue homeostasis, cancer, and neurodegenerative diseases. Recently, EVs have also been described in human female and male reproductive systems, including the oviduct, ovary, endometrium, embryo, prostate, and epididymis (**Figure 1**). EVs are key mediators in human reproduction and take part in physiological processes like gametogenesis (spermatogenesis and oogenesis), fertilization (coordination of sperm capacitation and acrosome reaction), embryogenesis, and implantation (crosstalk between mother and embryo). EVs and trigger and/or maintenance some conditions such as infertility, impotence, polycystic ovary syndrome (PCOS), endometriosis (EMs), premature ovarian failure (POF), and preeclampsia (toxemia) [2, 4].

2. EVs in the female reproductive system

The female reproductive system contains external and internal parts. Internal genital organs are mainly composed of the gonad (ovary), oviduct, and uterus (namely womb). As an organ whose basic functions are ovulation and the production of hormones, ovary influences a woman's feminine physical characteristics and affects the process of reproduction. In fertilization preparation, an ovulated oocyte enters the oviduct to meet spermatozoa (SPZ). Then fertilization occurs in oviduct when a sperm and an oocyte combine and fuse to form a zygote, which develops into a blastocyte and then implant uterus endometrium to further embryonic development. Finally, in the perinatal period, the fetus is delivered from the dilated cervix [5, 6]. Among these progresses, EVs coordinate the maturation of female gamete and mediate crosstalk to help successful embryo implantation (known as blastocyst implantation) and pregnancy in follicular fluid, oviduct, endometrium, vagina, semen, and embryo.

2.1 EVs derived from the ovary

The ovarian follicle is the basic unit of female ovary, which contains an oocyte that is surrounded by two-layer granulosa cells (GCs, including membrane/mural granulosa and cumulus granulosa), stromal cells (theca cells), and a liquid called follicular fluid (FF). FF offers a suitable micro-environment for follicle growth and oocyte development (oogenesis) in the ovary, so their relative stability of composition is indispensable. Similar to gap junctions between oocytes and somatic cells which mediated cell-to-cell communication, EVs in GCs, somatic cells, and FF mediate signal transmission and cell communication [7, 8], suggesting that EVs are carriers for molecular transfer in the ovary.

The first EVs in FF was reported in equine, which contains microRNAs (miRNAs) and proteins, and they could be taken by surrounding GCs *in vitro* and *in vivo* [9]. MiRNAs are involved in RNA silence as post-transcriptional regulators [10]. There are numerous miRNAs indirectly regulating follicular growth and oocyte maturation in human FF [7]: (1) miR-10b, miR-21-5p, miR-31, miR-95, miR-99b-3p, miR-134, miR-135b, miR-140-3p, and miR-190b participate in WNT signaling through glycoprotein signaling molecular WNTs potentially regulate follicle formation, growth, and ovulation/luteinization [11]. (2) miR-203, miR-218 regulate MAPK (ERK1/2) pathway which activation is crucial for oocyte meiotic resumption, GCs proliferation and cumulus expansion [12]. (3) miR-489, miR-493, miR-503, miR-542-5p, miR-654-3p, miR-874, miR-886-5p, miR-887 as mediators of TGFβ. (4) miR-337-5p, miR-339-3p, miR-370, miR-449a, miR-455-5p, miR-483-5p as mediators of ErbB, etc. In addition, as we know, the expansion of cumulus-oocyte complex (COC) is a key process of ovulation. A study showed that bovine FF EVs taken in cumulus could increase gene expression of cumulus expansion (PTGS2, PTX3, and TNFAIP6) and support the expansion process *in vitro* [13].

2.2 EVs derived from the oviduct

Oviduct is the place where occur oocyte transportation, fertilization, and initial embryo development. Oviductal extracellular vesicles (oEVs), as the main components of oviduct fluid, can orchestrate gamete/embryo-oviduct and embryo-maternal interactions, hence supporting oocyte mature (in canine), embryonic development, and motility, capacitation, and fertilizing ability of sperm [14, 15]. The contents of oEVs (mRNAs, miRNAs, and proteins) are dynamic at different stages of the estrous cycle, suggesting that cargos of oEVs are under hormonal control [16]. The first oEVs in the oviductal fluid was described in mouse, and then they were identified in other mammals including human. In humans, oEVs (oviductosomes) arising from the apocrine pathway carry and deliver plasma membrane Ca^2 + -ATPase (PMCA) which are fertility-modulating proteins. PMCA delivered to sperm can prevent premature capacitation and maintain Ca^2 + levels homeostasis; lack of PMCA leads to sperm motility loss and male infertility in mice [17]. Bovine oEVs can pass through the zona pellucida and then be internalized by embryonic cells to increase blastocyst rate and improve the quality of embryo. The possible mechanism may involve: (1) The proteins of bovine oEVs involved in cell communication, cell metabolism, localization, and reproduction [18]; (2) The miRNAs of bovine oEVs contribute to a successful pregnancy through inducing changes of embryonic transcriptome [15]; (3) Exosomes derived from bovine oviduct epithelial cells (BOECs) improve the mitochondrial health of bovine embryo because they re-establish the tricarboxylic acid cycle (TCA-cycle) flux [19].

2.3 EVs derived from the endometrium

Successful pregnancy not only needs intercommunication in ovary and oviduct, but also the crosstalk between endometrium and blastocyst especially the trophoblasts. Indeed, several studies have found that the secretions in endometrium affect the embryo. The first reported EVs of the female reproductive system are endometrium EVs (endEVs) in mouse uterine cavity [20]. EndEVs were found in endometrial fluid during the menstrual/estrous cycle, and were also released from endometrial epithelial cells cultured *in vitro* [21]. Several studies reported that the EVs in the endometrium have autocrine/paracrine effects on regulation of receptivity and implantation of uterine [22].

In EndEVs of primary endometrial epithelial cells (ECCs), 35% of the proteins had not been reported before, indicating that the contents of EndEVs are unique [23]. EndEVs influence blastocyst implantation through their hormone-specific protein cargos to modulate trophoblast capacity of adhesion, migration, invasion, and so on. Among these protein cargos, metalloproteinases (MMP-14 and ADAM10) regulated by the endometrial receptivity-related hormone are essential to trophoblast invasion and pregnancy outcome because they regulate (activate and degrade) other factors in endEVs. *In vitro*, when EndEVs are internalized by trophoblast cells, they can activate Focal Adhesion Kinase (FAK) signaling, thereby enhance the adhesive capacity of trophoblast cells [24–26]. The proteins of bovine endEVs in ECCs change during the peri-implantation period: endEVs enhance the expression of cell apoptosis genes in the preimplantation stage, but cell adhesion genes in the post-implantation stage [27].

MiRNAs of EndEVs act as modifiers for implantation. EndEVs contain specific miRNAs cargo which targets predictably genes involved in blastocyst implantation and crucial signaling pathways such as the VEGF, the Jak–STAT, and the Toll-like receptor in primary ECC and ECC1 (an endometrial epithelial cell line) [21]. During the window of implantation (the period allows blastocyte invasion), maternal miRNAs are differently expressed in human EndEVs. One of the miRNAs, miR-30d upregulates Itgb3, Itga7, and Cdh5, which are involved in embryo adhesion in murine; treating miR-30d *in vitro* to murine embryo can increase embryo adhesion [28].

Sperm adhesion molecule 1(SPAM1)/PH-20 is a hyaluronidase which enhances sperm fertility. EVs in murine uterine luminal fluid can deliver SPAM1 to sperm membranes to increase sperm penetration through cumulus cell layers around oocyte, and adhesive to zona pellucida, same as SPAM1 transferred from epididymosome to SPZ play a significant role in sperm maturation and motility [2, 20]. While as mentioned earlier, in oEVs SPAM1 interaction with sperm to inhibit premature acrosomal reaction, showing that the EVs with the same cargo in different organs have multiple functions [17].

2.4 EVs derived from the placenta and embryo

Pregnancy is a unique immunomodulatory state in which the maternal immune system temporarily tolerates the paternal antigen so as to protect the fetus from allogeneic rejection, at the same time maintaining immune surveillance to protect the mother from external pathogens. EVs have been identified in trophoblastic cells, placenta, and maternal circulation [22]. Maternal immune response is effected by the EVs from endometrium, embryo, and trophoblast cells in early pregnancy, while by the EVs from the human placenta in late pregnancy [29].

Placenta is an important organ and performs a variety of functions to support pregnancy. Placental-derived EVs are important media for intercellular communication, considered to be potential mediators in regulating maternal immune response to achieve a successful pregnancy and maternal and infant health outcomes [30]. These EVs inhibit the immune response to the developing fetus and establish and maintain a systemic inflammatory response against infectious invaders [31]. The underlying mechanisms include: (1) Compared with non-pregnancy, during pregnancy, the cytokines such as transforming growth factor-β 1 and IL-10 increase, and the ability to induce caspase-3 activity in cytotoxic natural killer (NK) cells enhance in peripheral blood EVs, which promoting immunosuppressive phenotype by inducing apoptosis to help regulate the maternal immune response to the fetus [32]. (2) In amnion (the innermost layer of placenta), miR-21 helps in embryo growth; histone 3 (H3), heat shock protein 70 (HSP70), activated form of prosenescenceP-p38, MAPK participate in stress response and are related to pregnancy outcome [33].

In addition to the placenta, embryos can also secrete EVs (embryo extracellular vesicle, eEVs) to participate in the regulation of implantation and other pregnancy processes. Embryos transferred to uterine are known as blastocyst, which consists of a fluid-filled cavity, inner cell mass (ICM), and trophectoderm/trophoblast (T) cells. Mouse embryonic stem cells from the ICM can produce MVs which reach the trophoblast ectoderm, thereupon enhancing the migration ability of trophoblast cells, both as isolated cells and in the whole embryo. Laminin and fibronectin exist in the eEVs of ICM orchestrate ICM to attach to the integrin on the surface of trophoblast cells and stimulates the cascade of c-Jun N-terminal kinase and FAK, increasing the migration of trophoblast cells. Injecting these eEVs into the blastocyst cavity of day 3.5 blastocysts can improve successful implantation rate [34]. EEVs-derived PIBF alters the maternal immune system by increasing IL-3, IL-4, and IL-10 to achieve a Th2-dominant cytokine balance. The aberrant expressions of PIBF may lead to pregnancy failure [35]. In eEVs of trophoblast also carry a variety of factors regulating maternal immunity to protect the fetus, including: tissue factors (TFs), soluble vascular endothelial growth factor receptor1 (sFlt-1), immunosuppressive factors, and so on [33].

3. EVs in the male reproductive system

The male reproductive system consists of internal organs - gonads (testis), reproductive ducts (epididymis, vas deferens, ejaculatory ducts, male urethra), and accessory glands (seminal vesicle, prostate, urethral bulbar gland) and external organs. Testes produce sperm and secrete male sex hormones. SPZ/sperm produced by the testis are first stored in the epididymis; during ejaculation, SPZ are excreted through the vas deferens, ejaculatory ducts, and urethra. Semen is the protect fluid around SPZ containing seminal plasma (SP), which is derived from testis (5%), epididymis and prostate (20%), seminal vesicles (65%), and seminal vesicles (65%). Seminal plasma lipids and/or EVs are rich in lipids, sugars, growth factors, TF and proteins, which play important roles in sperm survival, membrane integrity, maturation, motility, capacitation, acrosome reaction, and immune surveillance regulation [2, 33, 36]. Seminal plasma extracellular vesicles (Seminal plasma EVs, spEVs) are mainly derived from epididymis and prostate in the male reproductive tract, and a large number of them exist in the seminal plasma. Proteins in the seminal plasma are provided by spEVs [37]. According to the source, there are two main types of

spEVs: prostasomes and epididymosomes. A proteomic analysis showed that there are a total of 1474 proteins in seminal plasma-derived exosomes; bioinformatics analysis revealed that these proteins are involved in a variety of biological processes, such as cell growth and maintenance, metabolism, transport, energy pathways, and so on [38]. Human semen exosomes also contain a unique non-coding small RNA library, which may have a potential regulatory function to mediate fertilization by transmitting regulatory signals to the receptors to regulate the female reproductive tract [39].

3.1 EVs derived from the prostate

The first EVs of the reproductive system found in humans are prostasomes, which were observed in human prostatic fluid and seminal plasma in 1978 [40]. Prostasomes are exosomes with a diameter of 30–500 nm, which are released from prostatic epithelial cells and are then transferred to the prostate duct. Prostasomes are characterized by a cholesterol-to-phospholipid ratio of 2:1, of which nearly 50% phospholipid is sphingomyelin, making its bilayer or multilayer lipoprotein plasma membrane very hard. This unique prostate membrane component allows the prostate to fuse with and transfer its contents to other cells [41, 42]. Prostasomes can transport substances such as sphingomyelin, cholesterol, and saturated glycolipids to sperm to reduce their membrane fluidity in order to prevent premature or spontaneous acrosome reaction and premature capacitation [43]. Previous studies have shown that prostasomes may inhibit capacitation and acrosome reaction mainly through cholesterol transfer [44, 45]. Once the appropriate time for acrosome reaction occurs, prostasomes also play important roles in the capacitation and induction of acrosome reactions as mediators of signal transmission to regulate the tyrosine phosphorylation pattern, which is necessary for sperm-oocyte interaction [46].

Sperm motility is affected by intracellular pH and Ca^{2+} concentration. Prostasomes were determined to be associated with Mg^{2+} and Ca^{2+} dependent ATPase activity when they were first identified, suggesting that they are related to sperm energy metabolism [47]. Annexins of prostasome can activate Ca^{2+} channel and increase the level of Ca^{2+} in sperm through carrying CD38 and RyR to sperm to stimulate the production of cyclic adenylate diphosphate, thus affecting sperm motility. After ejaculation, prostasomes interact with sperm and protect them from female reproductive tract acidic environment and regulate sperm motility through PH- dependent manner to maintain the ability of sperm fertilization and prepare for an encounter with oocytes [48, 49]. Prostasomes transfer proteins (such as galactose lectin 3 and CD48) can protect sperm from immune reaction by regulating immune response pathways, such as inhibition of complement pathway, lymphocyte proliferation, and phagocytosis of monocytes and neutrophils in the female reproductive tract [50].

Additionally, prostasomes have antioxidant properties. By interacting with polymorphonuclear neutrophils that produce ROS, prostasomes can reduce the production of ROS, protect sperm and improve sperm survival rate. Prostasomes also carry aminopeptidase N, a protein involved in regulating sperm motility which acts by regulating endogenous opioid peptides (such as enkephalin) [51, 52]. Prostasomes are known to contain a variety of coding and non-coding regulatory RNAs with potential regulatory functions [39]. Currently, there are few studies on nucleic acid cargos of prostasomes and their effects on the male reproductive system.

3.2 EVs derived from the epididymis

Epididymosomes are produced by epididymal epithelial cells through apocrine secretion. They are a kind of exosomes with a diameter of 50–250 nm and characterized by high cholesterol/phospholipid ratio. Epididymosomes were first found in the intraluminal compartment of the epididymis of Chinese hamsters, and then in mice, rats, cattle, sheep, and humans. Epididymosomes can carry and transport numerous proteins with biological functions (including enzymes, adhesion molecules, transport, and signal transduction proteins) and non-coding RNA (such as miRNA). These proteins and non-coding RNA play an important biological role in the process of sperm maturation and fertilization, participating in the acquisition of motility, the acquisition of fertilization ability, and protection against oxidative stress [50, 53–55].

The proteins of epididymosomes including aldo-keto reductase family 1 member B (AKR1B1), phosphatidylethanolamine binding protein 1 (PEBP1), macrophage migration inhibitory factor (MIF), polyol pathway enzymes, glutathione peroxidase 5 (GPX5), plasma membrane Ca^{2+} -ATPase 4a (PMCA4), ubiquitin, and SPAM1/PH-20. AKR1B1 and PEBP1 can jointly regulate the sperm state, keeping sperm at a quiescent state during transportation until ejaculation [56]. MIF and polyol pathway enzymes are involved in sperm maturation and fertilization. GPX5, together with ubiquitin, is transferred to the sperm acrosome region during sperm epididymal transport, which protects sperm from oxidative stress, maintains the integrity of DNA, and prevents premature acrosome reaction. As a calcium efflux pump, PMCA4 carried by epididymosomes can regulate calcium concentration, affect the activation of calcium signaling pathway and maintain the homeostasis of Ca^{2+} in spermatozoa. Sperm delete PMCA4 leads to loss of hyperactivation and capacitation. GPI anchoring proteins include P34h and SPAM1/PH-20. As mentioned earlier, they are sperm binding proteins related to epididymosomes, which locate on the sperm surface during sperm epididymal transport, mediating the docking between epididymosomes and sperm and providing a "highway" for epididymosomes to transport their cargos to participate in the process of fertilization. Similar to their function are the P25b/P26 that facilitate sperm binding to the zona pellucida [33, 57–61].

Epididymosomes are also mediator of cell communication. New surface antigens are obtained on the sperm surface during epididymal transport, which are related to the acquisition of fertilization ability. Epididymosomes transmit Notch signals between epididymal epithelial cells and between epididymis and SPZ to influence sperm motility [62].

Epididymosomes are also rich in non-coding RNA. Epididymosomes contain many kinds of miRNA, such as miR-888, miR-182, miR-24, and miR-15b. MiR-888 of epididymosomes maintains sperm flagella peristalsis and mature sperm structure by regulating SPAG6 and SPAG1. Target gene prediction shows that miR-15b can specifically regulate the expression of IDH3A and control energy metabolism in TCA cycle, while miR-182 and miR-24 can specifically regulate the expression of glycogen synthase kinase 3α (GSK3A), and phosphorylation of GSK3A can affect sperm motility. Functional enrichment analysis shows that these miRNAs play important roles in embryonic development. There are also inflammation-related miRNAs in epididymosomes, such as miR-181a and miR-1224. Studies have shown that miR-181a participates in inflammatory response by regulating B cell differentiation and T cell receptor signals. MiR-146 can inhibit inflammation and innate immune response by down-regulating various pro-inflammatory cytokines, but its specific mechanism

needs to be further studied. When inflammation exists, miR-1224 activates the immune response by down-regulating the expression of TNF- α. Epididymosomes also contain miR-29a, which enhances the expression of nuclear autoantigen sperm protein (NASP) and inhibits the proliferation of epididymal epithelial cells [63–68].

In addition, epididymosomes contain a small molecule: tRNA-derived small RNAs (tsRNA). Studies have shown that tsRNAs have the functions of regulating gene transcription, cell proliferation and apoptosis, and stress response [69, 70].

4. Implications of EVs in reproductive pathology

4.1 Polycystic ovarian syndrome

Polycystic ovary syndrome (PCOS) is one of the most common reproductive endocrine diseases in women of childbearing age (global prevalence rate: 4–21%). The clinical features of PCOS are: hyperandrogenemia, polycystic ovary (PCO) morphology, and oligo-ovulation/anovulation [71]. PCOS as a disease of follicular abnormal, there is a close relationship between EVs in follicles and the pathogenesis of PCOS [72].

The amount of EVs (mainly exosomes) in plasma of patients with PCOS are significantly increased and positively correlated with the number of follicles [73]. The expression of non-coding RNAs altered in exosomes of FF in PCOS, including miRNAs, piRNAs, and tRNAs [74]. Further study found that there is differential expression of miRNAs in plasma exosomes of women with PCOS, which was related to the menstrual cycle, antral follicle count (AFC, means the number of antral follicle) and hormone levels [75]. LncRNAs and circRNAs are also differentially expressed in follicular exocrine bodies of patients with PCOS. They may regulate ovarian steroid production, aldosterone synthesis, and secretion, and involved in Jak–STAT signal pathway, hippo signal pathway, and MAPK signal pathway. A high-throughput lncRNAs sequencing study found that there are 1253 upregulated and 613 down-regulated lncRNAs in FF exosomes of patients with PCOS infertility compared with patients with non-PCOS infertility, and nine lncRNAs with significant changes may play an important role in the pathogenesis of PCOS (lncRNA-LINC00173, lncRNA-H19, lncRNA-HDAC6, lncRNA-POP4, lncRNA-PTEN, lncRNAAKT3, lncRNA-DICER1, lncRNA-NF1, and lncRNA-MUM1). High-throughput sequencing of circRNAs also found the expression of 167 circRNAs in FF exosomes of PCOS patients are significantly upregulated and 245 circRNAs are significantly down-regulated compared with the control group, suggesting that these abnormally expressed circRNAs may play some roles in the study of pathophysiological mechanism of PCOS [76–78]. Similarly, proteins in exosomes of FF also changed. A proteomic study of exosomes from FF in patients with PCOS and healthy controls found that exosomes rich in S100-A9 can activate NF-κB signaling pathway in GCs and may play key roles in the progression of PCOS [79].

4.2 Endometriosis

Endometriosis (EMs) is an estrogen-dependent inflammatory disease, characterized by the deposition and growth of endometrial stromal cells (ESCs) outside the uterine cavity, resulting in the appearance of endometrial tissue with growth activity

outside the uterine body. Pelvic peritoneum and ovary are the most common sites of ectopic endometrial stromal cells (EuESCs) growth. The main clinical manifestations are pelvic pain, pelvic adhesion, infertility and so on. EMs affects millions of women around the world, and the cause remains to be further determined [80].

The number of EVs in cervical and vaginal samples of rhesus monkeys with EMs decreased, indicating that the synthesis pathway of EVs in EMs has changed [81]. EMs are closely related to the formation of blood vessels. The biologically functional exosomes released by ESCs are transported to other parts through blood countercurrent in the endometrial and peritoneal microenvironment, and mediate intracellular signal transduction to ESCs itself or neighboring cells through the intercellular space in an autocrine or paracrine manner, thus regulating angiogenesis. *In vitro*, exosomes mediate the promotion of angiogenesis by EuESCs in the development of EMs [82]. Previous studies have shown that miRNAs can be extracted from the exosomes of ESCs, and these miRNAs potentially regulate the angiogenesis of ESCs. The expression of miRNA-21 related to angiogenesis in the exosomes of women with EMs is significantly higher than that of women without Ems [83]. Our research group identified the differential expression patterns of exosomal miRNAs in patients with EMs and found that 49 miRNAs expressed differentially in EuESC exosomes compared with normal endometrial stromal cells (NESCs) exosomes. Many exosomal miRNAs may be involved in regulating endometrial receptivity in women with EMs-related infertility through their predicted target genes: homeobox A10 (HOXA10) and leukemia inhibitory factor (LIF) which are essential for normal implantation. Our finding provides a new sight on how EVs participate in the occurrence and development of EMs [84].

4.3 Pregnancy complications

Common pregnancy complications include gestational hypertension, diabetes, and preeclampsia. Serious pregnancy complications may endanger the lives of fetuses and mothers [85]. The concentration and biological activity of EVs changed in a variety of pregnancy complications, such as preeclampsia (PE), gestational diabetes mellitus (GDM), preterm delivery (PTB), intrauterine growth restriction (IUGR), recurrent abortion and unexplained abortion, suggesting that EVs are closely related to pregnancy complications [86, 87].

Maternal obesity is a risk factor for GDM and several other pregnancy complications. Adipose tissue hypertrophy or metabolic stress can change the cargos in EVs (mainly miRNAs), leading to systemic inflammation and insulin resistance (IR) in obese patients with gestational diabetes. These altered EVs may also change the physiological function of placenta and remove the regulation of placental nutrition signal pathway, resulting in obesity-related pregnancy complications [88–90].

There is a class of syncytial nuclear aggregates (SNAs) in placental-derived EVs the level of which increases with the progression of pregnancy and is associated with pregnancy complications such as PE [91]. Numerous placental EVs were detected in the serum of pregnant women with PE. These EVs have pro-inflammatory, anti-angiogenic, and procoagulant activities, which may lead to activation of the blood coagulation system, systemic inflammation, and vascular endothelial dysfunction [92]. In addition, the abundance of syncytin-2 in serum-derived EVs attenuates in women with PE. This may lead to immunosuppressive reduction and pathological inflammation in pregnancy complications [93].

4.4 Male infertility

Infertility has becoming a global health problem, the incidence rate is as high as 15%, of which about 50% of infertility cases are caused by male reproductive disorders [94]. Just as oocytes need normal FF microenvironment to provide nutrition and support, sperm also need SP to provide a safe environment in order to survive and transport in the female reproductive tract. As mentioned earlier, there are a train of semen proteins in SP, most of which are transported by EVs (prostasomes and epididymosomes). Since only EVs proteins from normal sperm can regulate the movement of SPZ and trigger SPZ capacitation, the changes of proteomic characteristics in semen EVs may indicate male reproductive tract dysfunction; at the same time, they can be used as a biomarker of male infertility. In male infertility, the proteins transported by reproduction-related EVs are differentially expressed in azoospermia, asthenospermia, oligozoospermia, teratospermia, or other male infertility compared with normal sperm, and the prostatic proteins related to sperm energy production and sperm activity are under expressed in abnormal sperm, indicating that SP proteome map may be a potential indicator of sperm dysfunction [36].

Azoospermia may be one of the causes of male infertility. There are two types of azoospermia: non-obstructive (NOA) and obstructive azoospermia (OA) caused by seminal tract obstruction [95]. A study found that deficiency of EVs contributes to lower ejection volume, and changes of various nutritional components in semen [96].

At present, a sea of EV cargos has been identified to be differentially expressed in male infertility patients, which can be used as diagnostic markers and treatment of infertility as detailed in the next section.

4.5 Ovarian and cervical cancer

Gynecological malignant tumors such as ovarian cancer (OC) and cervical cancer (CC) are two of the three major tumors of the female reproductive system, the former with the highest mortality rate and the latter with the second incidence and the third fatality rate [97, 98]. The difficulty of early diagnosis, high metastasis rate, and strong drug resistance are still the main obstacles in gynecological cancer diagnosis and treatment. As a member of tumor microenvironment, exosomes not only play important roles in tumor occurrence and development, drug resistance and immunosuppression, but also can be used as a new tumor marker and clinical target molecule in clinical work.

The latest study has proved that there is a difference in the expression of exosomes in the blood of patients with OC and normal people, and the expression in the body fluid of patients with OC is related to the stage of the tumor [99]. A total of 1017 co-expressed proteins were screened from the exosomes secreted by two kinds of OC cells, among which tubulin beta 3 class III (TUBB3), epithelial cell adhesion molecule (EpCAM), claudin 3 (CLDN3), proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), and fatty acid synthase (FASN) were highly enriched in tumor-related signal pathways. Claudin-4 positive exosomes can be used in the diagnosis of OC, with a specificity of 98% and a sensitivity of 51%, while CA125 has a specificity of 98% and a sensitivity of 71%. Although claudin-4 is not a better diagnostic marker than CA125, this study confirms that exosome-related proteins can be used in the diagnosis of OC. These results suggested that the related proteins in exosomes may become markers for the diagnosis of OC [100, 101]. In addition, the level of exosomal proteins extracted from the serum of patients with OC were

higher than that of patients with benign ovarian disease and healthy women, and that of patients with advanced OC was higher than that of patients with early stage. The expression of tumor-specific antigen MAGE3/6 and transforming growth factor β1 (TGF- β1) in patients with OC was significantly higher than that in patients with benign ovarian disease and healthy women [102]. CD24 is a marker of poor prognosis in OC and other types of cancer. CD24 positive exosomes were screened from ascites of patients with OC, which is a good marker for early diagnosis of OC [103].

There are a large number of exosomes in cervicovaginal lavage specimens of women with CC, which carry miRNAs playing important roles in CC. It was found that many differentially expressed miRNAs, such as miR-483-5p, miR-1246, miR-1275, microRNA-21, microRNA-146a and miR-222-3p, were up-regulated in vaginal lavage and cell culture of CC, while some miRNAs, such as let-7d-5p, miR-92a-3p, miR-20a-5p, miR-378a-3p, miR-423-3p, miR-7-5p, miR-99-5p, miR-100 5p and miR-320a, were down-regulated. It is suggested that the contents of miRNAs in exosomes may be related to the occurrence of CC and is expected to become a new diagnostic marker of CC [104]. There was a significant difference in the expression of exosome-mediated let-7d-3p and miR-30d-5p between cervical tumors and adjacent normal tissues, suggesting that these two plasma exosomes let-7d-3p and miR-30d-5p can be used as valuable biomarkers for non-invasive screening of CC [105]. The exosomes of cervical squamous cell carcinoma (CSCC) transfers miR-221-3p from cancer cells to vascular endothelial cells and promote angiogenesis by down-regulating thrombospondin-2 (THBS2) [106]. In addition, exosomal miR-221-3p secreted by CC cells promotes the invasion, migration, and angiogenesis of CC microvascular endothelial cells (MVECs) by down-regulating the expression of MAPK10 [107]. The expression of activating transcription factor 1 (ATF1) and RAS genes were significantly upregulated in primary and recurrent CC mouse models, and ATF1 and RAS could also be detected in blood exosomes of mouse models. These results suggest that exosome-mediated ATF1 and RAS may become potential diagnostic markers for CC, which provides a new idea for individual detection and treatment of CC [108].

5. Clinical and therapeutic applications of EVs in reproductive disorders

5.1 EVs as biomarkers

The optimal age to have a baby is a medical social problem that needs to be faced by the whole society. Based on the risk of fetal disease, male and female fertility, and many other factors, it is generally believed that the best childbearing age for men and women is between 25 and 35 years old. With the increase of age, the semen quality of men decreases, the sperm concentration and motility decrease, and the deformity rate increases; the quality of oocytes decreases in women, which limits the success of pregnancy [109]. Therefore, looking for reproductive-related markers that change with age has become a meaningful research direction. It is found that the miRNAs characteristics of follicular EVs vary with the age of women, suggesting that miRNAs of EVs are possible markers and predictors of age-related oocyte quality decline [9]. A study compared the EVs of FF in young women with that in older women and found four significantly different miRNAs: miR-99b, miR-134, and miR-190b were upregulated, and miR-21-5p was down-regulated in older women. These miRNAs regulate genes related to cell apoptosis, p53 signaling, and cytokine-cytokine-receptor interaction, so their changes may affect follicular development and oocyte maturation [110].

There are numerous EVs in SP which can easily detach and collection. A study identified that prostasomal proteins relating to sperm activity and energy production pathways have changed in non-normozoospermic men. Among them, HIST1H2B, KLK2, MIF, MPO, and MSMB are related to liquefaction of semen (in order to break through SP) and sperm-oocyte binding. LDHC, HK1, PNP, APRT, and SLC2A14 are involved in sperm energy production [111]. Others include ELSPBP1/BLVRA, GPX5, SPAM1, P34H, Aldose reductase and sorbitol dehydrogenase, PAP, PSA, TMPRSS2, pTGase, PSCA, KIF5B, ANXA2, and so on [50]. Due to these variations may have a relevant correlation with male infertility, the cargos of EVs can potentially serve as useful biomarkers of male infertility.

In ART, FF can be taken out together with the follicles, so it is an attractive biomarker to detect the quality of oocytes. The expression of miRNAs in EVs is related to fertilization status and embryo quality [112]. It has been found that the overexpression of miR-92a and miR-130b can lead to adverse results of *in vitro* fertilization (IVF), while the differential expression of miR-214, miR-454, and miR-888 is related to high quality embryos [113].

Peripheral blood is the most widely used biological sample in clinical diagnosis. The cargos in EVs in peripheral blood are good indexes for the detection of many diseases [114]. Prostasomes produced by prostate tumor cells may be involved in the spread of prostate cancer. Prostate corpuscles in peripheral plasma and their specific proteins, such as PAP, PSA, TMPRSS2, and PSCA, may be valuable biomarkers of prostate cancer [115]. Since EVs and their content in women's peripheral blood can be detected from early pregnancy, they can be used as biomarkers for the prediction or diagnosis of pregnancy complications, fetal developmental disorders, and preterm birth (PTB). About 11% of infants around the world are born prematurely every year. PTB, defined as delivery before 37 weeks of pregnancy, is the leading cause of neonatal morbidity and mortality. The exosomes in plasma can be used to predict whether pregnant women will develop PTB, so as to prepare for prenatal intervention. Several miRNAs such as hsa-miR-381, hsa-miR-154, hsa-miR-377, and hsa-miR-150-5p in circulating EVs have been predicted to be potential biomarkers of PTB. A longitudinal study analyzed the miRNAs in plasma EVs collected from multiple pregnant women throughout pregnancy and found that the number and expression profile of miRNAs of EVs in maternal plasma of PTB changed significantly compared with the term birth, especially these miRNAs associated with TGF-β, p53, and glucocorticoid receptor signaling. It is worth pointing out that these miRNAs are known to be associated with fetal membrane apoptosis [116–119]. Fetal cell-free DNA (cfDNA) carried by maternal circulating plasma and serum exocrine has been used in non-invasive prenatal diagnosis as a biomarker of pregnancy complications, such as PE and GDM [120].

MVs from vaginal microbes (e.g., Group B Streptococcus) can affect fetal placental tissue and lead to pregnancy complications [121]. Therefore, it may be possible to detect the contents of vaginal MVs to find significantly differential cargos, so as to detect pregnancy complications as soon as possible.

With the deepening of research, studies in mounting numbers have shown that the miRNAs in EVs can be used to diagnose pregnancy-related diseases, including miR-136, miR-155, miR-210, miR-486, miR-494, miR-495, miR-517-5p, miR-520a-5p, miR-525-5p, and miR-548c-5p [122].

Wrong or late diagnosis is one of the main problems of EMs. Therefore, it is of great significance to find biomarkers for early diagnosis. Extracellular nucleases are key enzymes in the process of inflammation, which participate in the occurrence and development of EMs by regulating the levels of extracellular ATP and adenosine.

A study has found that the extracellular nuclease activity in the aspiration fluid of endometrial tumors is significantly higher than that of simple cysts and comes from the exosomes of these fluids, and therefore the exosomes of these lesions may become biomarkers of EMs. The proteins and miRNAs transported by endEVs may be very valuable biomarkers of human endometrial diseases [123].

The expression of DENN domain containing 1A (DENND1A) in urine-derived exosomes of PCOS patients was significantly higher than that of normal controls, suggesting that it can be used as a potential PCOS marker [124].

5.2 Clinical and therapeutic applications

As biological substances are produced by normal human bodies, the in-depth study of EVs in different physiological and disease states is helpful to identify specific proteins related to EVs functional defects, thus promoting the development of new diagnoses and treatment strategies for reproductive dysfunction. EVs have attracted much attention in the field of translational medicine because compared with other commonly used synthetic drug delivery carriers (such as liposomes), bioengineered EVs have not only have inherent targeting ability but also are low immunogenicity, easy to obtain, selective assembly, high modification flexibility, and biological barrier permeability. Considering EVs can deliver functional cargos to target cells, it is hopeful for us to use them as a drug delivery tool. For example, EVs may be able to pass through the tissue barrier (such as endothelial barrier, blood–brain barrier, endothelial barrier, and blood–brain barrier) through endocytosis. At present, the application of exosome therapy has been explored in a variety of reproductive diseases, such as the treatment of female infertility, POI, EMs, IUA, and so on. For instance, exosomes can carry specific miRNAs into receptor cells to target genes. There are also studies to target the treatment of related diseases by putting modified small interference RNA (siRNAs) into exosomes [125, 126].

EuESCs can secrete a large number of exosomes, which contain a variety of miRNAs may not only be closely related to the occurrence, development and complications of EMS, but also have different expression levels from normal endometrial cells. Therefore, exosomal miRNAs have certain application value in the early diagnosis, judgment of disease progression, and prognosis of EMs. In EMs mouse models, the delivery of miR-214 rich exosomes isolated from EuESCs can inhibit fibrosis and regulate the development of EMs [127].

Exosomes derived from endometrial epithelial cells can enhance the adhesion, implantation, and growth ability of embryo *in vivo* [128]. EVs obtained by uterine lavage carries proteins that regulate and predict embryo implantation, and its protein composition, metastatic and invasive properties, and antioxidant function are dynamically regulated throughout the menstrual cycle, so it has the potential to be used as a biomarker of embryonic development, implantation, and successful pregnancy [129].

PCOS and GDM are closely related to obesity. Adipose tissue macrophages (ATM) of obese mice secrete exosomes containing miRNAs, which can lead to impaired glucose tolerance and IR when given to thin mice. In contrast, ATM exosomes obtained from lean mice can improve glucose tolerance and insulin sensitivity when given to obese mice. These miRNAs can be transferred to insulin target cells through paracrine or endocrine regulation, and have a strong effect on cellular insulin action, insulin sensitivity, and global glucose homeostasis [88]. Therefore, the differentially expressed miRNAs of ATM in obese and thin people may be used as biomarkers of PCOS and GDM and used in their treatment.

Premature ovarian failure (POF) is defined as ovarian function lost in women before 40 years of age. The incidence of POF in women is about 1%. The occurrence of POF is closely related to the depletion of ovarian follicles, but the specific mechanism is still not clear [130]. The combination of stem cell therapy and EVs provides a glimmer of hope for the treatment of POF. In one study, exosomes collected from Bone mesenchymal stem cells (BMSCs) were injected into POF mice, and then found that follicles, FF, and corpus luteum increased in mice ovaries, while apoptosis-related genes p53 and caspase3 were down-regulated, indicating that BMSC-derived exosomes can improve the phenotype of POF. Further in *vitro* experiments showed that the expression of miR-664-5p was increased in BMSC-derived exosomes and could reverse the injury of POF granulosa cells, which was regulated by p53 [131]. Another study found that miR-144-5p in BMSC-derived exosomes inhibit POF GCs apoptosis by targeting PTEN and activating the PI3K/AKT pathway in *vitro* [132]. Furthermore, exosomes derived from human amniotic epithelial cells (hAECs), amniotic fluid stem cells (AFSCs), and placenta-derived mesenchymal stem cells (PD-MSCs) can regulate the apoptosis pathway to reduce the apoptosis of ovarian follicles through their different cargos, such as miR-10a, miR-146a, miR-1246, and antioxidant enzymes [133]. Taken together, these studies demonstrated that exosomes can mediate ovarian function, thus pointing out a new direction for the treatment of POF.

6. Conclusions

Gametogenesis, fertilization, implantation and early embryonic development are complex processes that are highly dependent on communication between cells and organs. The transmission of EVs between cells as a newly discovered way of cell communication, is increasingly found to play crucial and multiple roles in the field of reproduction. In the male reproductive system, prostasomes and epididymosomes derived from prostate/epididymis are found in semen, which contribute to maturation, motility, activation, capacitation of SPZ and acrosome reaction (**Figure 2**). In the female reproductive system, the two-way communication between oocytes and

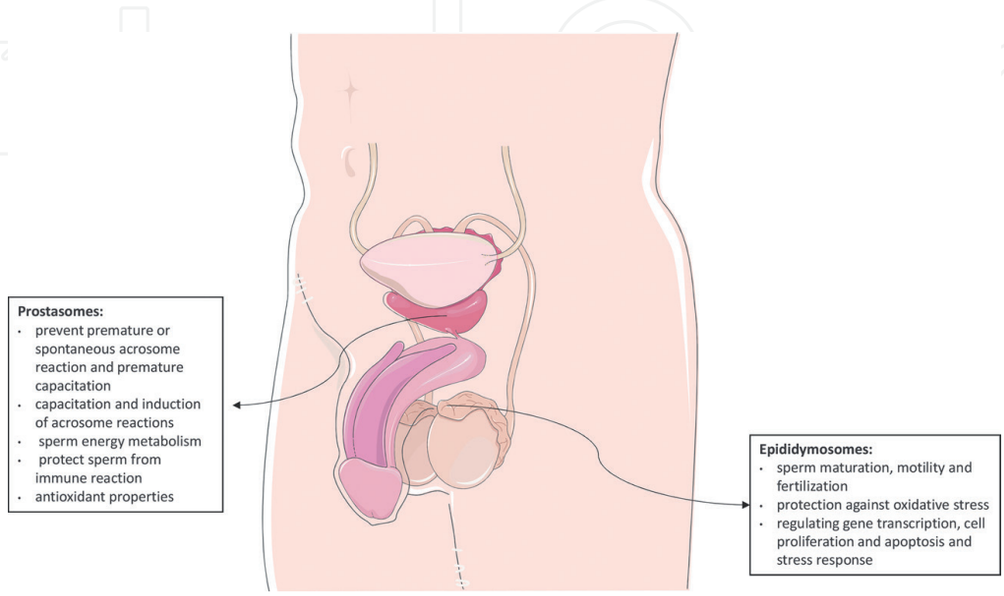

Figure 2.
The functions of EVs in male reproductive system.

somatic cells around them (companion somatic cells) is important for the development of oocytes fertilization and embryogenesis. EVs in ovary, oviduct, endometrium and placenta are the carriers of information during gametogenesis, fertilization and embryo-maternal dialog, helping follicle and oocyte development and maturation. While in fertilization, they can regulate maternal immunity, promote early embryo development, assist implantation, and maintain pregnancy, which are all vital for successful pregnancy. In pathologies, EVs play important roles in female reproductive system disorders, such as PCOS, EMs, pregnancy complications (**Figure 3**).

In the process of EVs synthesis, different RNAs, proteins and other contents are selectively packaged into EVs, therefore the data of genomics, proteomics and

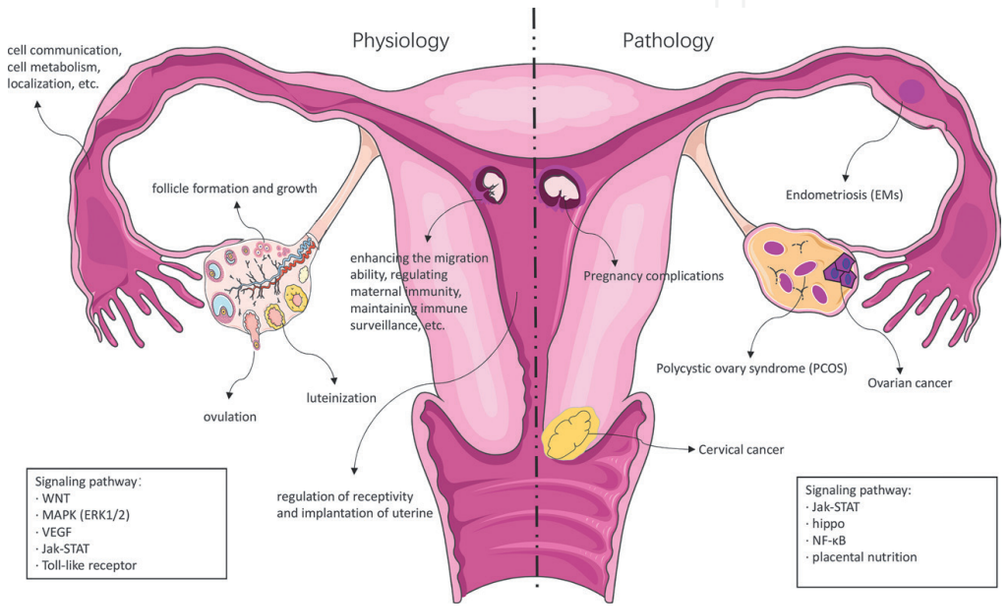

Figure 3.
The functions of EVs in female reproductive physiology and pathology conditions.

Sex	Diseases	EV cargos	References
Female	Polycystic ovary syndrome	LncRNAs: LINC00173, H19, HDAC6, POP4, PTEN, AKT3, DICER1, NF1 and MUM1 Protein: S100-A9	[76–78]
	Endometriosis	MiRNAs: miR-21, miR-615-3p, miR-6873-3p, miR-3195, miR-196a-5p, miR-4483, miR-1273 h-3p, miR-4262, miR-1269a, miR-6859-5p	[83, 84]
	Ovarian and cervical cancer	Proteins: TUBB3, EpCAM, CLDN3, PCNA, EGFR, FASN, MAGE3/6, TGF- β1, ATF1, RAS MiRNAs: miR-483-5p, miR-1246, miR-1275, microRNA-21, microRNA-146a and miR-222-3p, let-7d-5p, miR-92a-3p, miR-20a-5p, miR-378a-3p, miR-423-3p, miR-7-5p, miR-99-5p, miR-100 5p and miR-320a, let-7d-3p, miR-30d-5p, miR-221-3p	[100–102, 104–108]
Male	Male infertility	Proteins: HIST1H2B, KLK2, MIF, MPO, MSMB, LDHC, HK1, PNP, APRT, SLC2A14	[111]

Table 1.
Potential EVs biomarkers in different reproductive diseases.

metabolomics in EVs may be different between normal physiological state and conditions, and the number and composition of EVs may reflect the functional state of its cell origin. In view of the importance of this mode of intercellular communication in the reproductive system, EVs can be used as biomarkers or therapeutic targets for reproductive diseases in the field of reproductive system diseases and assisted reproductive technology (ART) (**Table 1**).

Future research can not only continue to focus on the role of EVs in the normal reproductive physiology, but also focus on the changes of their cargos in a variety of reproductive diseases, so as to find biomarkers for prediction and diagnosis of diseases, and how to transform them into targeted drug therapy and other clinical applications.

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Conflict of interest

The authors declare no conflict of interest.

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