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MINI-REVIEW ***Developments in reproductive biology and medicine***

The emerging role of extracellular vesicles in the testis

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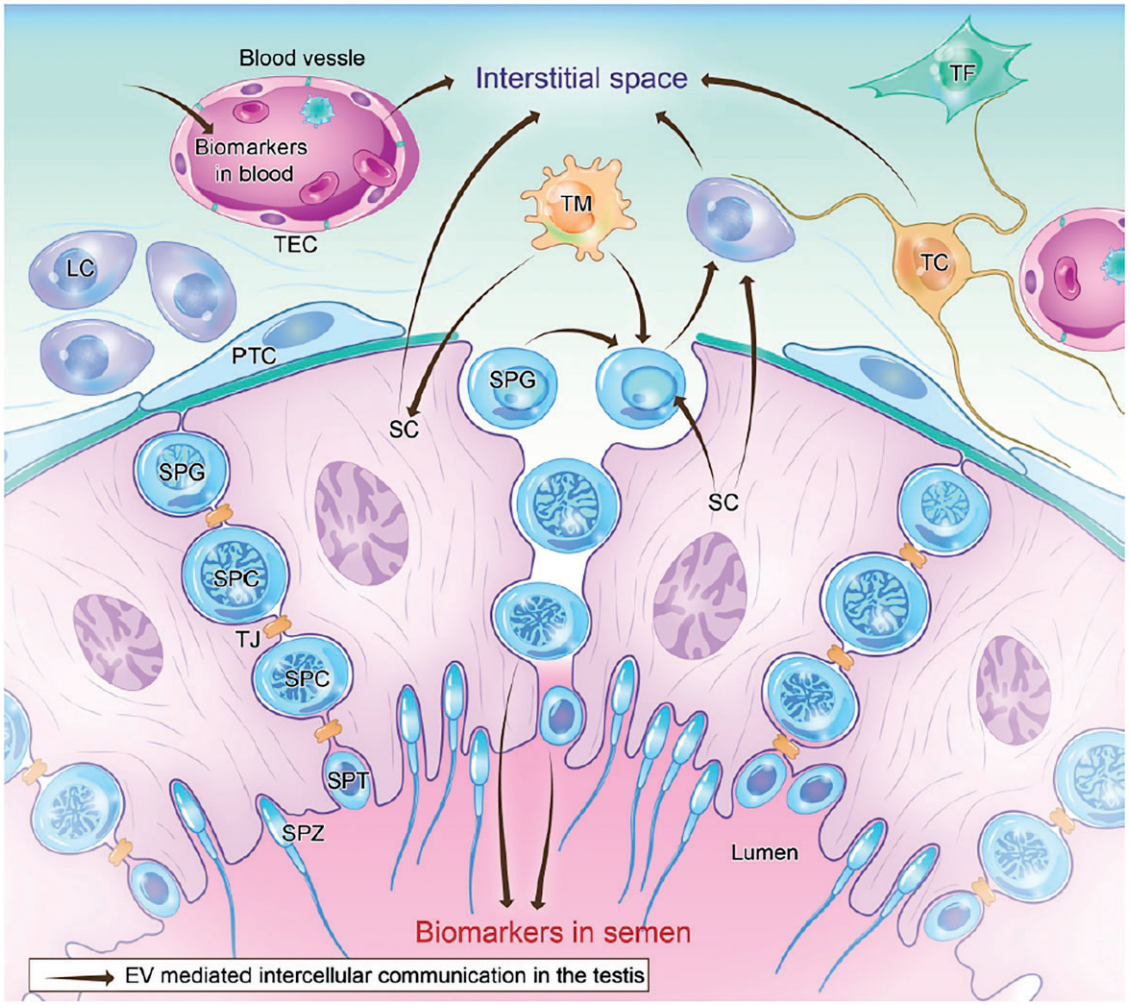
ABSTRACT: Extracellular vesicles (EVs) are nano-sized membrane-bounded particles, released by all cells and capable of transporting bioactive cargoes, proteins, lipids, and nucleic acids, to regulate a variety of biological functions. Seminal plasma is enriched in EVs, and extensive evidence has revealed the role of EVs (e.g. prostasomes and epididymosomes) in the male genital tract. Recently, EVs released from testicular cells have been isolated and identified, and some new insights have been generated on their role in maintaining normal spermatogenesis and steroidogenesis in the testis. In the seminiferous tubules, Sertoli cell-derived EVs can promote the differentiation of spermatogonial stem cells (SSCs), and EVs secreted from undifferentiated A spermatogonia can inhibit the proliferation of SSCs. In the testicular interstitium, EVs have been identified in endothelial cells, macrophages, telocytes, and Leydig cells, although their roles are still elusive. Testicular EVs can also pass through the blood–testis barrier and mediate inter-compartment communication between the seminiferous tubules and the interstitium. Immature Sertoli cell-derived EVs can promote survival and suppress the steroidogenesis of Leydig cells. Exosomes isolated from macrophages can protect spermatogonia from radiation-induced injury. In addition to their role in intercellular communication, testicular EVs may also participate in the removal of aberrant proteins and the delivery of antigens for immune tolerance. EVs released from testicular cells can be detected in seminal plasma, which makes them potential biomarkers reflecting testicular function and disease status. The testicular EVs in seminal plasma may also affect the female reproductive tract to facilitate conception and may even affect early embryogenesis through modulating sperm RNA. EVs represent a new type of intercellular messenger in the testis. A detailed understanding of the role of testicular EV may contribute to the discovery of new mechanisms causing male infertility and enable the development of new diagnostic and therapeutic strategies for the treatment of infertile men.

Key words: blood–testis barrier / biomarkers / endothelial cells / exosome / extracellular vesicles / germ cell / male infertility / Leydig cell / Sertoli cell / spermatogonia

GRAPHICAL ABSTRACT



Intercellular communication mediated by extracellular vesicles (EVs) in the testis. LC: Leydig cell; PTC: peritubular myoid cell; SC: Sertoli cell; SPC: spermatocyte; SPG: spermatogonia; SPT: spermatid; SPZ: spermatozoa; TC: telocyte; TEC: testicular endothelial cell; TF: testicular fibroblast; TJ: tight junction; TM: macrophage.

Introduction

All cells can release various types of membrane vesicles, known as extracellular vesicles (EVs) (van Niel *et al.*, 2018). Although EVs are highly heterogeneous in their origins, properties, and functions (Tkach and Thery, 2016), they are composed of a lipid bilayer membrane and have enclosed biologically active cargoes of nucleic acids, lipids, and proteins. Three main classes of EVs are defined. EVs can be released by budding from the cell plasma membrane; these EVs, ranging from 100 to 1000 nm in diameter, are known as microvesicles (Fig. 1) (van Niel *et al.*, 2018). On the other hand, apoptotic bodies are EVs produced by plasma membrane blebbing in cells undergoing programmed cell death. Apoptotic bodies are described to be 1–5 μm in diameter (van Niel *et al.*, 2018; Battistelli and Falcieri, 2020). The third class, exosomes, are generated from multivesicular bodies (MVBs) and secreted when MVBs fuse with the plasma membrane (Fig. 1). Exosomes are vesicles of 30–150 nm (Tkach and Thery, 2016) or even 200 nm (Pegtel and Gould, 2019) in diameter. Of the three EVs, exosomes have received the most attention.

We now know that EVs are important regulators of biological functions in health and disease (Tkach and Thery, 2016; Raposo and Stahl, 2019). EVs can act as signalling vehicles and participate in intercellular communication to maintain the body's homeostasis through the transfer of lipids, proteins, and nucleic acids (Mathieu *et al.*, 2019; Raposo and Stahl, 2019; Chen *et al.*, 2020). When EVs are attached to a target cell, they can be internalized by the recipient cell or fuse with the target cell's membrane to deliver their contents, thereby regulating diverse physiological or pathological processes in the recipient cell

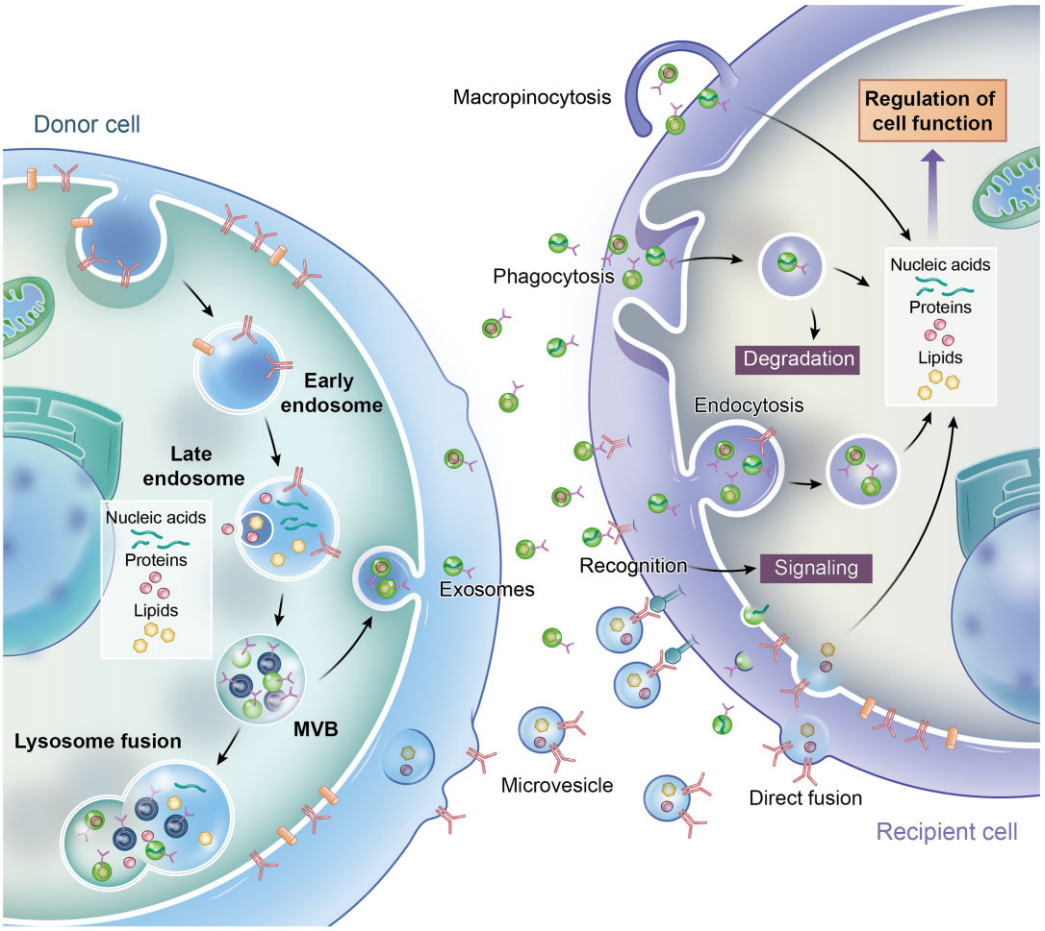


Figure 1. The biogenesis, release and internalization of extracellular vesicles (EVs). A simplified depiction of the biogenesis and release of exosomes and microvesicles (left). Internalization of EVs by target cells through multiple pathways (right), including macropinocytosis, phagocytosis, endocytosis, and direct fusion; vesicle contents can be released or degraded. EVs can also bind to the surface of the recipient cell and initiate signalling pathways. MVB: multivesicular bodies.

(Fig. 1) (Tkach and Thery, 2016; van Niel *et al.*, 2018). The majority of EV cargo is ubiquitous and has no biomarker potential, while other cargo may contain unique biomarkers that can reflect their cell origin and disease status, rendering them useful tools for disease diagnosis and prediction. In addition, because EVs have the functions of delivering biomolecules and crossing major biological barriers, they can be used as clinical drug delivery carriers (Zhang *et al.*, 2020), although in this regard, there are still many technical and regulatory barriers to be overcome (e.g. the immunogenicity of exogenous EVs and the isolation method for pure endogenous EVs).

EVs can be found in most biological fluids, such as plasma, breast milk, amniotic fluid, saliva, urine, cerebrospinal fluid, and semen (Keller *et al.*, 2011; Baskaran *et al.*, 2020; Ciferri *et al.*, 2021). Seminal plasma is particularly rich in EVs (Vojtech *et al.*, 2014; Hoog and Lotvall, 2015; Johnson *et al.*, 2015), and their diversity has been revealed by cryo-electron microscopy (Hoog and Lotvall, 2015). Seminal plasma consists of secretions from the testes, epididymis, prostate, seminal vesicles, and bulbouretheral and periurethral glands, which indicates that EVs in seminal plasma may also originate from these glands. Semen EVs from the epididymis and prostate are known as epididymosomes and prostasomes, respectively. These EVs can protect sperm and regulate sperm motility, morphology, capacitation, and acrosome reaction (Simon *et al.*, 2018), thus positively influencing these key processes associated with sperm functions. Epididymal epithelium-secreted epididymosomes can transfer to spermatozoa some molecules (CD52, GliPr1L1, MIF, P25b, P34h, ATP2B4, SPAM1, AKR1B1, SLC27A2, EDDM3B, KRT19, and WFDC8) that are involved in sperm maturation (Boue *et al.*, 1996; Frenette and Sullivan, 2001; Frenette *et al.*, 2003; Martin-DeLeon, 2006, 2015; Sullivan *et al.*, 2007; Caballero *et al.*, 2012; 2013; Baskaran *et al.*, 2020; Barrachina *et al.*, 2022). The cargo in epididymosomes can also protect sperm from oxidative stress (ELSPBP1, BLVRA, GPX5, and glutathione-S-transferase) (Hinton *et al.*, 1995; Taylor *et al.*, 2013; Sullivan, 2015; D'Amours *et al.*, 2016; Baskaran *et al.*, 2020) and regulate sperm morphology and motility

(ADAM7 and ATP2B4) (Oh *et al.*, 2009; Choi *et al.*, 2015; Martin-DeLeon, 2015). Epididymosome cargo (small RNA) also modulate gene expression in spermatozoa and, thus, affect paternal epigenetic inheritance (Peng *et al.*, 2012; Chen *et al.*, 2016; Sharma *et al.*, 2016; Trigg *et al.*, 2019; Conine and Rando, 2022). Prostasomes are released from the epithelial cells lining the prostate gland. Their cargo can enhance sperm motility (progesterone receptors, Ca2+ signalling cascade components, and aminopeptidase N) (Stegmayr and Ronquist, 1982; Arienti *et al.*, 1997; Subiran *et al.*, 2008; Park *et al.*, 2011; Andrews *et al.*, 2015) and protect spermatozoa from oxidative stress (NOSs and ATP2B4) (Andrews *et al.*, 2015), and from immunity, bacteria and hostile acidic environments in the female reproductive tract (PH, LGALS3, and CD48) (Skibinski *et al.*, 1992; Kitamura *et al.*, 1995; Arienti *et al.*, 1999; Jones *et al.*, 2010; Tarazona *et al.*, 2011). The cargo in prostasomes can also prevent premature capacitation and acrosome reaction (cholesterol) (Cross and Mahasreshti, 1997; Bechoua *et al.*, 2011; Pons-Rejraji *et al.*, 2011) and are involved in the subsequent induction of capacitation, sperm hypermotility, and acrosome reaction at the moment of fertilization (cAMP, progesterone receptors, hydrolases, and lipoxygenases) (Oliw *et al.*, 1993; Minelli *et al.*, 2002; Palmerini *et al.*, 2003; Siciliano *et al.*, 2008; Aalberts *et al.*, 2013).

Recently, some progress has also been made in clarifying the role of EVs in testes, providing some new insights into how testicular cells interact with one another through EVs to drive spermatogenesis. This article specifically reviews evidence on the release of different EVs by testicular cells, as well as their markers and cargo, and their capacity to communicate between cells through the transfer of their cargo contents. Prospective applications of testicular EVs as biomarkers and treatment methods for male infertility are also discussed in this review.

Search strategy and selection criteria

PubMed and Google Scholar were searched using keywords including ('extracellular vesicles' or 'exosomes' or 'microvesicles' or 'microparticle' or 'apoptotic bodies' or 'apoptotic blebs') and ('semen' or 'seminal plasma' or 'testis' or 'Sertoli cells' or 'Leydig cells' or 'sperm' or 'spermatocytes' or 'spermatogonia' or 'germ cells' or 'testicular endothelial cells' or 'testicular immune cells' or 'testicular telocyte' or 'peritubular myoid cells' or 'blood-testis barrier'). Only studies that included both EVs and reproduction and those reporting transmission electron microscopy (TEM), nanoparticle tracking analysis, or molecular markers of EVs (e.g. ALIX, CD9, CD63) were included. When testicular primary cells were isolated and identified, only studies reporting molecular markers (e.g. 3b-HSD, VIMENTIN, SYCP3) were included. Studies which did not adhere to the MISEV (minimal information for studies of EVs) 2018 naming convention (Thery *et al.*, 2018) were also included, provided that their reliability was not impaired. Only English-language publications and full-length articles on either animal or human studies were considered. The references of the included articles were also reviewed to obtain relevant articles. Studies without description of EV isolation or identification were excluded. Duplicate articles were eliminated. Studies found to be irrelevant to testicular EVs were excluded. There was no restriction on the publication date.

EVs as vectors for intercellular communication in the testis

The adult testis has two distinct components: the seminiferous tubules and the interstitial compartment (Fig. 2). After puberty, the seminiferous tubules produce spermatozoa, and the interstitial compartment is mainly responsible for steroidogenesis. The seminiferous tubules are composed of Sertoli cells (SCs) and germ cells (GCs). SCs rest on the tubular basement membrane and extend cytoplasmic extensions into the lumen of the tubules. SC is a key component of the germline stem cell niche and they provide structural and functional support for GC development. Beyond their physical support, SCs also play a vital role in regulating other cells through the secretion of various endocrine, paracrine, and autocrine factors (discussed in later sections). Direct cell-to-cell contacts, including gap junctions, ectoplasmic specializations, desmosomes, and tight junctions, can be found between SCs and GCs or between SCs and SCs (Mao *et al.*, 2020). Gap junctions are involved in intercellular communication through the exchange of ions and small molecules. Ectoplasmic specialization and desmosomes can maintain stable attachments between GCs and SCs to prevent the sloughing of immature GCs into the seminiferous epithelium. The tight junctions between SCs form intercellular barriers that divide the seminiferous tubule space into the basal (basement membrane) and abluminal (lumen) compartments. This anatomical structure forms the basis of the blood–testis barrier (BTB), protects GCs from antigens, antibodies, large molecules and immune cells, and maintains a GC microenvironment entirely distinct from that of plasma (Mruk and Cheng, 2015). The human seminiferous tubule is surrounded by peritubular myoid cells (PTCs), which are thought to have a contractile function. PTCs can provide physical support for spermatogenesis and play a role in the expulsion of spermatozoa out of the seminiferous tubules and into the epididymis. PTCs also profoundly affect the differentiation or synthetic functions of SCs, GCs and Leydig cells (LCs) by producing numerous paracrine factors (IGF1, LIF, TGFα, TGFß, GDNF, CSF1, and P-Mod-S) (Potter and DeFalco, 2017; Zhou *et al.*, 2019).

The testis interstitial compartment contains blood vessels, lymphatics, macrophages, mast cells, dendritic cells, T cells, telocytes, and LCs (Fig. 2) (Mruk and Cheng, 2015). LCs, blood vessels, and macrophages are important modulators of spermatogonial stem cell (SSC) niches (Potter and DeFalco, 2017; Heinrich and DeFalco, 2020). LCs are responsible for the production of testicular androgen. By binding androgen-binding protein, a high level of testosterone is maintained within the seminiferous tubules to regulate SC function and support spermatogenesis. Blood vessels influence SSCs through GDNF, CSF1, and VEGF, and by modulating oxygen levels (Potter and DeFalco, 2017). Testicular macrophages have a unique role in maintaining immune tolerance to GCs to establish an immune-privileged niche for spermatogenesis (Heinrich and DeFalco, 2020). Macrophages also interact with SSCs and induce the proliferation or differentiation of SSCs through CSF1 and RA (Potter and DeFalco, 2017). Mast cells, T cells, and dendritic cells are involved in maintaining the normal testicular immune and inflammatory environment.

A complex network of intercellular communication exists among testicular cells, which can be regulated by either direct cell-to-cell contacts (e.g. junctional intercellular complexes, cell-surface interactions)

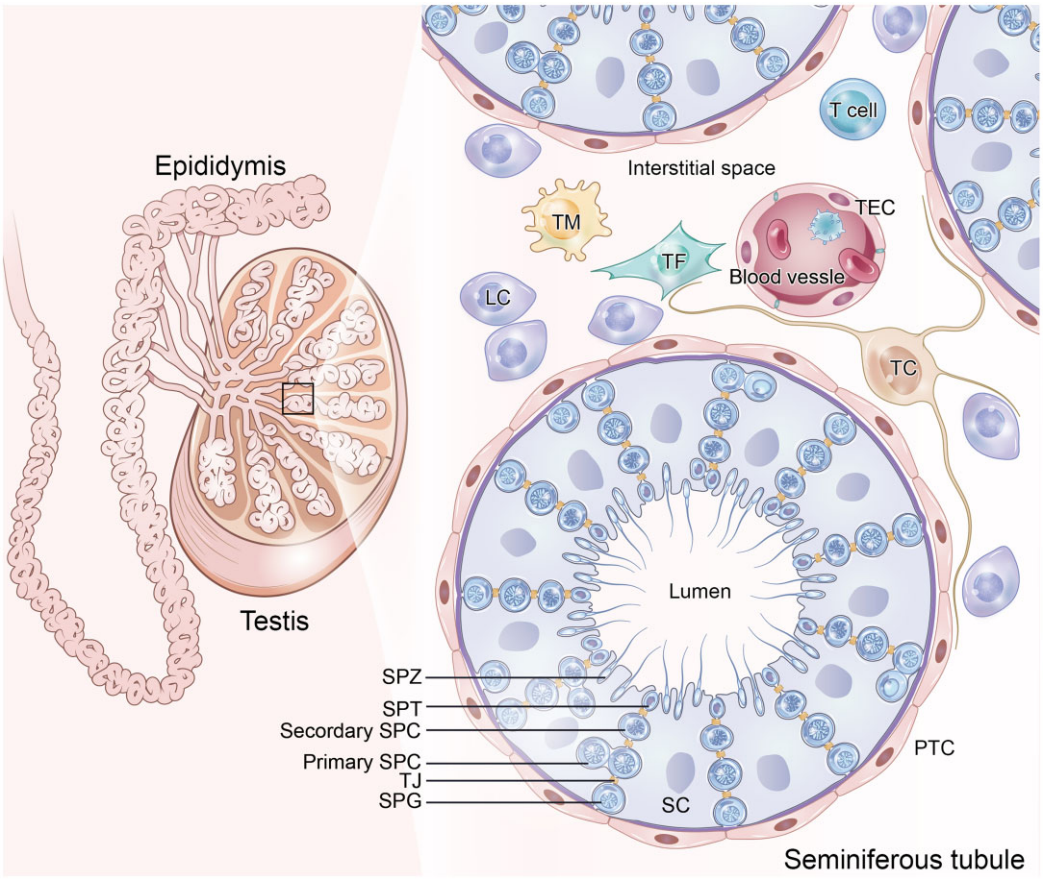


Figure 2. Organization of the testis. A diagrammatic cross-section of the testis showing the location of the seminiferous tubule and interstitium (left). In the seminiferous tubules (right), germ cells at different stages of maturation are embedded in Sertoli cells (SCs). Spermatozoa are shown in the lumen. The seminiferous tubule is surrounded by peritubular myoid cells (PTCs). In the interstitial space, blood vessels, Leydig cells (LCs), telocytes (TCs), testicular fibroblasts (TFs), testicular macrophages (TMs), and some other immune cells are present. SPC: spermatocyte; SPG: spermatogonia; SPT: spermatid; SPZ: spermatozoa; TEC: testicular endothelial cell; TJ: tight junction.

or cell-secreted factors (e.g. hormones and cytokines) (Martin, 2016). Recently, a third pathway has been discovered that involves the intercellular transfer of EVs. EVs are released by testicular cells, play important roles in the crosstalk between the various cells (Table I), and may be involved in testicular development and spermatogenesis. It has been reported that knockout of *Smpd3* in mice, a known regulator of EV biogenesis and release (Trajkovic *et al.*, 2008; Mathieu *et al.*, 2019), delays testis development and reduces fertility (Stoffel *et al.*, 2005).

Sertoli cell-released EVs

SCs are essential for normal testis development and are a major determinant of adult testis size (O'Donnell *et al.*, 2022). SCs can secrete many products, including extracellular matrix components, ceruloplasmin, transferrin, growth factors, and somatomedin, which are involved in SC–GC interactions (Mruk and Cheng, 2004). SCs control LC development and PTC fate by releasing IGF1, FGF2, TGFs, IL1, PDGF, inhibin, activin, oestrogen, DHH ligands, kit ligand, notch ligands, and desert hedgehog (Rebourcet *et al.*, 2014). SCs are also important for testicular vasculature. In SC-ablated testes, there is a reduction in total testicular vascular volume, vascular branch number, and microvessel number (O'Donnell *et al.*, 2022). SCs can also regulate interstitial immune cell function through some immunomodulatory factors (O'Donnell *et al.*, 2022).

EVs in SCs were first found in turtle testes (Ahmed *et al.*, 2016). EV-like structures and MVBs were detected in CD63-positive SC cytoplasmic processes around developing GCs during early and late spermatogenesis in turtle seminiferous tubules (Tarique *et al.*, 2020). The main types of EVs secreted by TM4 SCs under cryo-electron microscopy have been summarized, and exosome-like structures constitute ~50% of the EV population (Yefimova *et al.*, 2020). Some molecular markers have been identified as markers of EVs released by SCs, namely, CD9, CD63, CD81, TSG101, and HSP70 (Table I).

Human SC-secreted EVs contain diverse components (Tan *et al.*, 2022). The most abundantly expressed microRNAs (miRNAs) are

Table I Summary of studies demonstrating the role of EVs in the intercellular communication of testicular cells.

Source of EVs	Cell types	Species, age	EV types	Isolation method	Culture condition	Specific markers	Negative markers	Studied cargo	Targeted cells	Conclusion	References
SCs	Cell line (HSerCs)	Human	EVs	Ultracentrifugation[A]	Basal medium (EV-depleted FBS), 48 h.	CD9, CD63, TSG101	CANX	Proteins and miRNAs	/	EVs derived from HSerCs contain diverse components such as proteins and miRNAs.	Tan *et al.* (2022)
SCs	Primary cells	Mouse, 3 weeks	Exosomes	Ultracentrifugation[B]	Basal medium (FBS excluded), 72 h.	CD9, CD63, CD81	CANX	miR-145-5p	LCs	Immature SCs-derived exosomal miR-145-5p suppressed testosterone production in LCs by down-regulating SF-1.	Liang *et al.* (2021)
SCs	Primary cells	Mouse, 3 weeks	Exosomes	Ultracentrifugation[B]	DMEM (Exosome-depleted FBS), 48 h.	CD9, CD63, CD81	CANX	miR-486-5p	SSCs	Immature SCs-derived exosomal miR-486-5p down-regulated PTEN and promotes SSCs differentiation.	Li *et al.* (2021)
SCs	Primary cells	Mouse, 4 weeks	Exosomes	Isolation kit (Umibio, UR52121)	DMEM/F12 (FBS excluded), 24–48 h.	CD63, TSG101	/	miR-9-3p	LCs	PFOS increased SCs-derived exosomal miR-9-3p, which suppress testosterone secretion through targeting *Star* in LCs.	Huang *et al.* (2022)
SCs	Primary cells	Mouse, 3–6 days	Exosomes	Ultracentrifugation[C]	DMEM/F12 and BSA (FBS excluded), 48 h.	/	/	/	SSCs	Immature SCs-derived exosomes can ameliorate the effect electromagnetic fields on SSCs through suppressing ROS generation.	Salek *et al.* (2021)
SCs	Tissue and Cell line (TM4)	Mouse, 19–21 weeks	Myelinosomes	/	Transfection of mutant proteins, 48 h.	/	/	Abnormal proteins	/	Myelinosome mediated elimination of mutant proteins in SCs.	Yefimova *et al.* (2016)
SCs	Primary cells	Rat, 3 weeks	Exosomes	Isolation kit (SBI, EXOQ5A-1)	DMEM/F12 (FBS excluded), 72 h.	CD81, HSP70	CANX	*Ccl20* mRNA	LCs	Immature Sertoli cells-released exosomes may cross the BTB and promote the survival of Leydig cells.	Ma *et al.* (2022)
SCs	Primary cells	Pig, 15–20 days	EVs	Molecular weight cut-off filtration.	HAM's F-12 with retinoic acid and ITS. (FBS excluded) Testosterone and FSH treatment, 48 h.	/	/	Proteome	/	FSH and testosterone can alter protein expression in immature SC EVs.	Mancuso *et al.* (2018)
SCs	Primary cells	Pig, one week.	Exosomes	Ultracentrifugation[D]	α-MEM media (FBS excluded), 72 h.	/	/	/	/	Sertoli cell-released exosomes increase spermatogonial proliferation.	Thiageswaran *et al.* (2022)

(continued)

Table I Continued

Source of EVs	Cell types	Species, age	EV types	Isolation method	Culture condition	Specific markers	Negative markers	Studied cargo	Targeted cells	Conclusion	References
SCs	Tissue	Turtle, >3 years	Exosomes	/	/	CD63	/	/	/	MVBs and CD63 positive exosomes were found in turtle Sertoli cells and autophagy inhibition increased CD63-enriched exosomes.	Tarique *et al.* (2020)
SSCs	Primary cells	Mouse, 6 days	EVs	Ultracentrifugation[E]	Seminiferous tubules were digested and the supernatant was centrifuged.	CD9, CD81	/	/	SSCs	The EVs from thy1-positive spermatogonia suppress the proliferation of SSCs.	Lin *et al.* (2021)
SSCs	Cell line (C18-4)	Mouse	EVs	Ultracentrifugation[E]	Exosome-free medium, 72 h.	/	/	/	LCs	The EVs isolated from C18-4 cells stimulated TM3 LCs to secrete testosterone.	Yun *et al.* (2019)
TECs	Cell line (HTECs)	Human	Exosomes	Ultracentrifugation[A]	Medium with 5% exosome-depleted FBS, 48 h.	CD9, CD63, TSG101	CANX	proteins and miRNAs	/	Exosomes derived from testicular endothelial cells contain diverse components such as proteins and miRNAs.	Song *et al.* (2021)
Macrophages	Cell line (RAW264.7)	Mouse	Exosomes	Isolation kit (Umibio, UR52101)	DMEM (FBS excluded). Cells were treated with MPLA and CHX, 24 h.	/	/	G-CSF and MIP-2	Spermatogonia (GC1)	Macrophage-derived exosomal G-CSF and MIP-2 protect against radiation-induced testis damage.	Liu *et al.* (2020)
Mixed Testicular cells	Primary cells	Mouse, 8 weeks	EVs	Ultracentrifugation[E]	The tissue was digested and the supernatant was centrifuged.	CD9, CD81	/	/	LCs	Testicular cells-derived EVs can promote testosterone production in TM3 LCs and elevate serum testosterone and LH levels in mice.	Yun *et al.* (2019)
Mixed Testicular cells	Primary cells	Mouse, 8 weeks	EVs	Ultracentrifugation[F]	Seminiferous tubules were digested and the supernatant was centrifuged.	CD9, CD81, CD63, β-tubulin	CANX, GOLGA1	Proteins and small RNA	/	The study provides a resource on the repertoire of cargo carried by testicular EVs.	Choy *et al.* (2022)
Dissected testis	Tissue	Rabbit, 14 months	EVs	Isolation kit (101 Bio)	DMEM (FBS excluded), 24 h.	/	/	mRNA	Cumulus cells and oocytes	Testis-derived EVs can promote cumulus expansion and change transcript expression in cumulus cells and oocytes	Abumaghaid *et al.* (2022)

EVs: extracellular vesicles; FBS: foetal bovine serum; LCs: Leydig cells; miRNA: microRNA; SCs: Sertoli cells; SSCs: spermatogonial stem cells; TECs: testicular endothelial cells. A: Ultracentrifugation protocol 1. Remove cells and debris (2000 g 10 min, 10 000 g 30 min); precipitate EVs (110 000 g 75 min); samples were filtered (0.22-μm filter); precipitate EVs again (110 000 g 75 min). B: Ultracentrifugation protocol 2. Samples were filtered (0.22-μm filter); remove cells and debris (300 g 10 min, 10 000 g 30 min). Precipitate EVs (100 000 g 70 min); precipitate EVs again (160 000 g 60 min). C: Ultracentrifugation protocol 3. Remove cells and debris (300 g 15 min, 3000 g 30 min and 10 000 g 30 min). Samples were filtered (0.22-μm filter); precipitate EVs (100 000 g 70 min twice). D: Ultracentrifugation protocol 4. Remove cells and debris (300 g 15 min, 2000 g 30 min and 10 000 g 30 min). Precipitate EVs (100 000 g 70 min); samples were filtered (0.22-μm filter); precipitate EVs again (100 000 g 70 min). E: Ultracentrifugation protocol 5. Remove cells and debris (3000 g 30 min and 10 000 g 30 min). Samples were filtered (0.22-μm filter); precipitate EVs (100 000 g 90 min twice). F: Ultracentrifugation protocol 6. Remove cells and debris (10 000 g 30 min). Precipitate EVs (100 000 g 90 min).

miR-3960, miR-6087, miR-3665, miR-7704, and miR-4787-5p; however, these data are only from a single study (Tan *et al.*, 2022). Some miRNAs (e.g. miR-638, miR-149-3p, miR-1246, miR-455-3p, miR-101-5p, let-7b-5p, miR-572, and et-7c-5p) in these EVs have been reported in the pathological conditions of male reproduction (asthenozoospermia, oligozoospermia, teratozoospermia, and nonobstructive azoospermia), and some proteins (e.g. ADAM, EIF5A2, SEPTIN, and PRKAG1) have been found in the important process of spermatogenesis (Tan *et al.*, 2022), which indicates that EVs may regulate spermatogenesis. From molecular function analysis (gene ontology term), these proteins and miRNAs were most enriched in binding (e.g. enzyme binding, ATP binding and cell adhesion molecule binding). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis has shown that the shared enriched pathways are 'focal adhesion' and 'endocytosis' in the top 20 enriched signalling pathways of proteins and miRNAs (Tan *et al.*, 2022). The limitation of this study was that the cells were isolated from only one male infant donor. *In vitro* EV secretion by cells is inevitably affected by the external environment (culture conditions), so it is very important to make use of controls when functional effects are assessed.

Some factors can regulate the release or contents of EVs by SCs. Hypoxia significantly promotes EV release in rat primary SCs (Ma *et al.*, 2021). FSH or testosterone stimulation can regulate the mRNA (*Amh*, *Inhb*, *Abp*, and *Fshr*) and protein (INHA, INHB, PLKA, HPT, PHGDH, AT1A1, TPA, EGFL8, and EF1G) sorting of EVs released by prepubertal SCs in pigs (Mancuso *et al.*, 2018). Virus infection can alter miRNA expression in small EVs (<150 nm) released by sheep testicular cells (Fig. 3) (He *et al.*, 2019).

EVs secreted from SCs may positively regulate the differentiation of SSCs in mouse testes (Li *et al.*, 2021). SC-derived exosomes are enriched in miR-486-5p. When miR-486-5p is transferred from SCs to SSCs through exosomes, it directly targets *Pten* and down-regulates its expression, which up-regulates the expression of STRA8 and SYCP3 and, thus, promotes SSC differentiation (Li *et al.*, 2021). Inhibition of SC-released exosomes by GW4869 *in vitro* can decrease spermatogonial proliferation (Thiageswaran *et al.*, 2022). However, whether GW4869 is cytotoxic to spermatogonia remains unclear. Electromagnetic fields can reduce the viability and colonization of SSCs via oxidative stress in mice. The administration of SC-derived small EVs (<150 nm) ameliorates the effect on SSCs by suppressing reactive oxygen species generation (Fig. 3) (Salek *et al.*, 2021), although the molecular mechanism is unknown.

Immature primary SC-released EVs may promote the survival of LCs through *Ccl20* in rats (Ma *et al.*, 2022). *Ccl20* mRNA in the EVs released by immature SCs can be delivered to LCs, where it is translated into protein and activates AKT (Ma *et al.*, 2022). Exosomes released from immature SCs also participate in the regulation of LC steroidogenesis. miR-145-5p is highly expressed in mouse (3 weeks old) immature primary SCs compared with adult SCs and can be delivered to LCs by exosomes (Liang *et al.*, 2021). miR-145-5p in LCs directly targets steroidogenic factor-1 (Sf-1), which is involved in the transcription of steroidogenesis genes. The inhibition of Sf-1 by miR-145-5p leads to decreased expression of *Star*, *Hsd3b*, *Hsd17b3*, and *Cyp11a1*, and ultimately suppresses testosterone synthesis in LCs (Liang *et al.*, 2021). Perfluorooctane sulfonate (PFOS) is an organic pollutant associated with male reproductive disorders. PFOS significantly increases miR-9-3p levels in mouse SC-released exosomes, which can further suppress testosterone secretion by directly targeting the *Star* gene in the primary LCs of 4-week-old mice (Fig. 3) (Huang *et al.*, 2022).

SCs can secrete an EV with a diameter of 200–700 nm (Yefimova *et al.*, 2020). These vesicles are composed of two areas: an outer layer with osmiophilic membranes and an electron-transparent matrix (Yefimova *et al.*, 2020; Neyroud *et al.*, 2021). The EVs were reported to be myelinosomes, which are different from exosomes, microvesicles, and apoptotic bodies in their origin and morphology, and are devoid of the molecular marker, CD63 (Yefimova *et al.*, 2016). MVBs support the trafficking and excretion of myelinosomes. Myelinosomes were identified in the seminiferous tubules of the testis and are secreted by SCs. They appear in pathological situations (such as Huntington's disease or cystic fibrosis) and are thought to remove the misfolded/mutant proteins in SCs (Yefimova *et al.*, 2016). Myelinosomes can be released by SCs and are present in human seminal fluid. The authors (Yefimova *et al.*, 2020) described four types of EVs, including myelinosomes, in seminal plasma; however, no EV had a diameter greater than 700 nm, and their centrifugation protocol may be a reason. Myelinosomes may be used as a potential biomarker for testicular disorders (Fig. 3). However, it is important to note that other cells from the male reproductive tract may also release myelinosomes, and many other EVs are also CD63-negative; also, no specific marker of myelinosomes has been found to date.

SCs may also remove apoptotic GC- or residual body-derived proteins into the interstitial fluid through EVs (Tung *et al.*, 2017; O'Donnell *et al.*, 2021). These proteins have been detected in human and mouse testicular interstitial EVs and human blood plasma (O'Donnell *et al.*, 2021). It has been reported that proteins from sperm can emerge into the interstitial space from seminiferous tubules to promote peripheral immune tolerance (Tung *et al.*, 2017). MHCII+ peritubular macrophages accumulate around the seminiferous tubules and may participate in the uptake and presentation of the antigen (Fig. 3). However, the mechanism by which SCs release these EVs is still unknown. Interestingly, human seminal EVs can reduce the ability of antigen-presenting cells to activate T cells and prevent immune responses (Vojtech *et al.*, 2019); however, it is unknown whether SC-released EVs are involved.

Germ cell-released EVs

SSCs are undifferentiated spermatogonia that subsequently become differentiating spermatogonia, spermatocytes, spermatids, and mature spermatozoa. MVBs have been found in type A spermatogonia, and a large number of testicular EVs have been found close to the basement membrane of seminiferous tubules in mouse, rat, rabbit, and human testes (Lin *et al.*, 2021). EVs can be secreted from mouse undifferentiated A spermatogonia with the marker Thy1. These EVs were purified and found to have an inhibitory effect on the proliferation of SSCs (Fig. 3) (Lin *et al.*, 2021). In addition, the authors found that ~83% (40/48) of testicular EVs physically interact with spermatogonia, whereas only 17% (8/48) interact with SCs, according to their TEM analysis (Lin *et al.*, 2021). However, they only used the snapshot provided by TEM for a static observation; *in vitro* EV real-time live imaging may trace the EVs better. The EVs in this study were isolated from enzymatically digested testicular tissue. As a stress factor, enzymatic

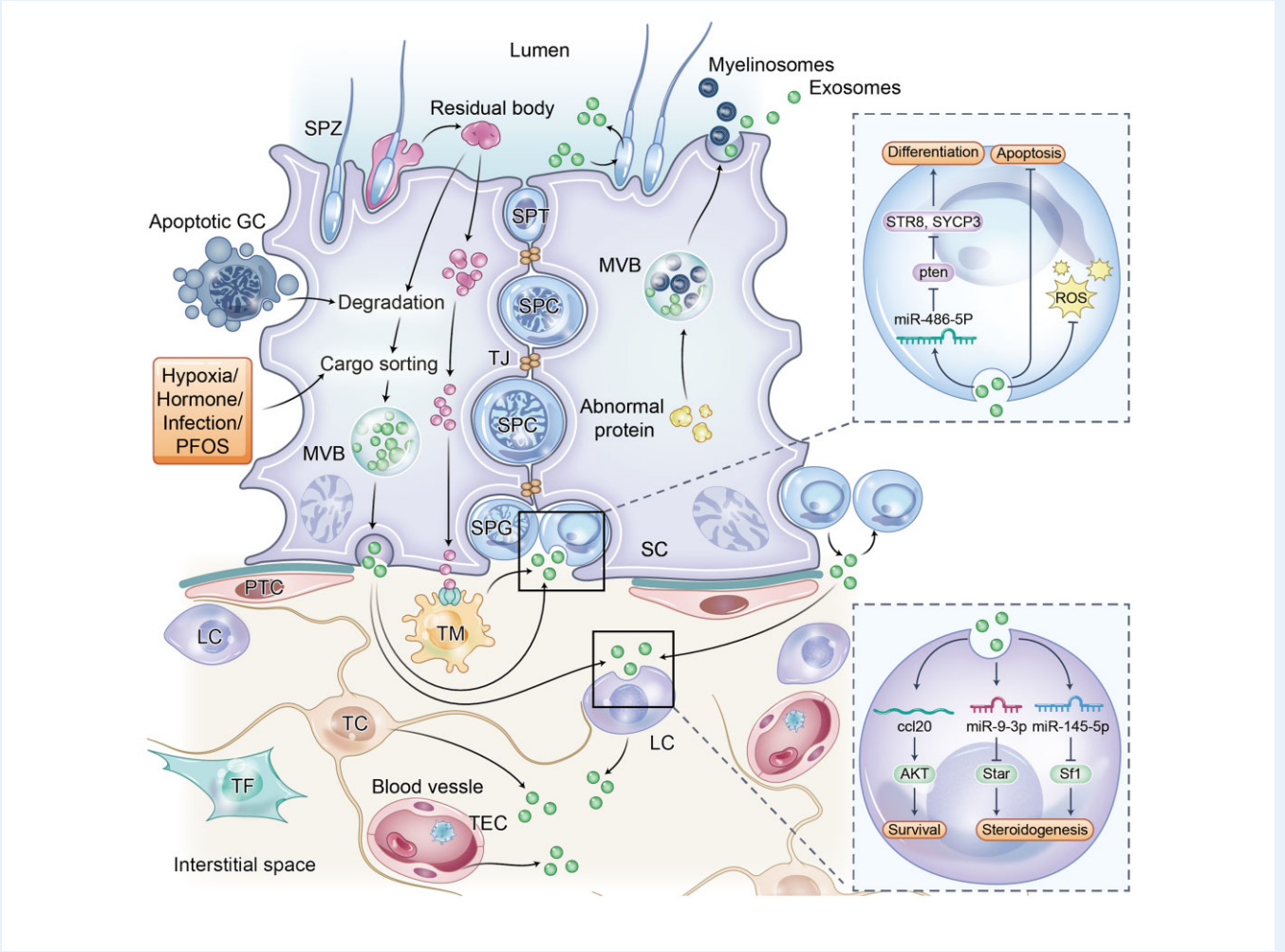


Figure 3. Extracellular vesicles (EVs) mediate intercellular communication in the testis. In the seminiferous tubules, the cargo sorting of exosomes in Sertoli cells (SCs) can be regulated by hypoxia, hormones, infection, and perfluorooctane sulfonate (PFOS), an organic pollutant. Apoptotic germ cells (GCs) and residual bodies can be phagocytosed and degraded by SCs, and some contents may be released into the interstitial space through EVs, which may participate in the delivery of antigens to testicular macrophages (TMs) for immune tolerance. Aberrant proteins can be removed by SC-released EVs (myelinosomes). SC-derived exosomes can promote the differentiation of spermatogonial stem cells (SSCs) through miR-486-5p (top insert). SC-derived exosomes can also reach Leydig cells (LCs) in the interstitium, stimulate cell survival through CCL20 and suppress steroidogenesis through miR-9-3p and miR-145-5p (bottom insert). Undifferentiated A spermatogonia (SPG) can release exosomes and inhibit the proliferation of SSCs. Spermatozoa (SPZ) may uptake or release EVs from or to the lumen. In the testicular interstitium, EVs can be secreted from testicular endothelial cells (TECs), TMs, telocytes (TCs), and LCs. Exosomes isolated from TMs can protect spermatogonia from radiation-induced injury. MVB: multivesicular bodies; PTC: peritubular myoid cell; SPC: spermatocyte; SPT: spermatid; TF: testicular fibroblast; TJ: tight junction.

digestion may affect the secretion of EVs by cells and even alter the contents of EVs, thereby reducing the reliability of the study. This is also an issue for the other studies using enzymatically digested testicular tissue in this review (Table I). In another study from the same research group, EVs were isolated from C18-4 SSCs and found to stimulate testosterone secretion by TM3 LCs (Yun *et al.*, 2019). It is important to note that the study used differential ultracentrifugation to isolate EVs, where precipitation of non-EV structures can confound the results. This is also an issue with the other studies in this review using this method (Table I).

EVs not only transport cargo to spermatozoa but also aid in the removal of sperm-binding proteins. Fusion or budding of vesicles to or from the sperm tail has been detected under cryo-electron microscopy in human semen (Hoog and Lotvall, 2015). In fact, many proteins found in sperm have also been detected in human seminal EVs (Yao *et al.*, 2021), which indicates that sperm may shed EVs into the fluid of seminiferous tubules or seminal plasma. Spermatocytes and spermatogonia are enriched in TEX101. TEX101 must be shed and must disappear from GCs to maintain normal sperm fertilization. TEX101 can be shed from the ram sperm membrane during epididymal transit (Leahy *et al.*, 2020). Other TEX101-associated proteins, including LY6K, DPEP3, and VAMP3, have also been shown to be shed from the sperm membrane and are present in seminal EVs (Leahy *et al.*, 2020). One study showed that 1034 identified proteins were removed from

mouse sperm during epididymal transit (Skerget *et al.*, 2015). In addition, numerous spermatogenesis-associated mRNA fragments specifically expressed in GCs (Spata4, Smcp, Spata20, Spata8, Gapdhs, and Odf1) can be detected in human seminal exosomes (GSE56076) (Vojtech *et al.*, 2014). However, it is also possible that some of these exosomes are released by SCs after their phagocytosis of apoptotic GCs or residual bodies. Some proteins (e.g. CMTM2/CKLFSF2) specifically expressed in GCs and residual bodies were recently found to be abundantly present in ram seminal EVs (Leahy *et al.*, 2020).

Testicular endothelial cell-released EVs

Testicular endothelial cells (TECs) are a vital population in the germline stem cell niche and can promote the proliferative and self-renewal capacity of SSCs. TECs have been reported to restore spermatogenesis in busulfan-induced depletion of SSCs in mice (Bhang *et al.*, 2018). Proteomic analysis has identified 945 proteins, and miRNA profiling has identified 2578 miRNAs in human TEC-derived small EVs (<150 nm) (Song *et al.*, 2021). Bioinformatics analysis revealed that 11 out of the 945 proteins (e.g. ADAM10, HMGB2, and SEPTIN2) are closely related to spermatogenesis, and 30 out of 2578 miRNAs (e.g. miR-6087, miR-5787, and miR-4459) may be associated with spermatogenic dysfunction or other male reproductive disorders. In addition, 11 out of the 30 miRNAs (e.g. miR-638, miR-320a, and miR-26a-5p) have been reported to be involved in spermatogenesis (Song *et al.*, 2021).

EVs released by other testicular cells

Testicular macrophages are critical cells involved in innate immunity and inflammation in the testis. In addition to promoting SSC proliferation and differentiation via CSF1, macrophages also interact with LC and influence LC proliferation and steroidogenesis through some biofactors (IL-1, TNF, and 25-hydroxycholesterol) (Heinrich and DeFalco, 2020). Macrophage-derived exosomes were recently reported to play a role in mouse testis radiation protection through exosomal G-CSF and MIP-2 stimulated by the low-toxicity TLR4 agonist monophosphoryl lipid A (Liu *et al.*, 2020).

Telocytes were identified as novel interstitial cells present in the turtle, rat, mouse, rabbit, and human testis (Yang *et al.*, 2015). Telocytes are characterized by a small cell body and 1–5 long and thin extended filamentous structures, which indicates their role in intercellular communication. TEM demonstrated that telocytes shed EVs that may communicate with neighbouring cells, including LCs, PTCs, and TECs, in turtles (Chen and Holt, 2021). However, the effect of this communication is unknown.

LCs synthesize testosterone and insulin-like 3 (INSL3), which are essential for normal male sexual development and testicular reproductive function (Martin, 2016). LCs also produce CSF-1, IGF1, vasopressin, prostaglandins, and oxytocin to regulate the function of multiple cell types within the testis (including LCs, SCs, PTCs, TECs, and SSCs) (Potter and DeFalco, 2017; Heinrich and DeFalco, 2020). One study reported that LCs were enriched with a set of proteins found in mouse testicular EVs (Choy *et al.*, 2022), which indicates that LCs can secrete or take up EVs. However, the role of LC-released EVs in the testis is still unclear.

EVs isolated from mouse mixed testicular cells (cell suspension after testis dissociation) were found to be internalized by SCs, GCs, and LCs *in vivo* (Choy *et al.*, 2022). The testicular EV proteome was then examined, and a total of 553 proteins were identified (Choy *et al.*, 2022). The authors matched the identified proteins with the corresponding gene expression from the single-cell sequencing dataset and found that EVs may be secreted by LCs, macrophages, GCs, SCs, and other somatic cells in the testis. In addition, 11 of the top 50 proteins in testicular EVs were related to male subfertility. The study also explored the RNA species in these EVs and found that testicular EVs mainly carried small RNAs, with PIWI-interacting RNAs (piRNAs) (17%) and miRNAs (4%) representing the dominant subtypes. miR1, miR34a, and let-7b were found to be the most abundant miRNAs in testicular EVs. These miRNAs were also found in spermatozoa, indicating the contribution of testicular EVs to sperm RNA. Finally, the authors found that repetitive injections into the interstitial space with GW4869 (a small molecule that inhibits EV release) significantly increased the number of apoptotic cells in seminiferous tubules, suggesting that testicular EVs may regulate spermatogenesis (Choy *et al.*, 2022). However, it is also possible that the reagent itself can lead to apoptosis. In another study using mouse mixed testicular cells (Yun *et al.*, 2019), injection via the tail vein of EVs isolated from these cells was found to promote mouse serum LH and testosterone levels.

Can EVs pass through the BTB?

Recent studies have shown the vital roles of exosomes in many biological barriers, including the blood–brain barrier, blood–air barrier, stromal barrier, blood–retinal barrier, and placental barrier (Elliott and He, 2021). The mammalian BTB is created mainly by SCs, tight junctions, gap junctions, desmosomes, and ectoplasmic specializations (Mruk and Cheng, 2015; Mao *et al.*, 2020). Although the BTB divides testes into two separate compartments, active communication exists between different testicular cells in these two compartments (Martin, 2016). EVs may also be involved in this process. It has been reported that *EGFP* (enhanced green fluorescent protein) RNA in EVs can be released from xenografted human cells into the bloodstream and eventually found in the epididymal spermatozoa of mice (Cossetti *et al.*, 2014), which indicates that EVs may cross the BTB or the blood–epididymis barrier and transfer information from the soma to the germline. After the injection of labelled SC-secreted small EVs into the rat efferent duct, the presence of EVs in both seminiferous tubules and interstitial space indicated that the EVs can cross the BTB from seminiferous tubules into the interstitium (Ma *et al.*, 2022). The results also showed that the injected EVs in the seminiferous tubules do not diffuse evenly, but they seem to enter the interstitium along with the intercellular space (Fig. 3B of Ma *et al.*, 2022)). Another recent study involved the injection labelled testicular EVs into the interstitial space in the mouse testis, revealing that the EVs can be seen in both interstitial cells and seminiferous tubules, which indicates that EVs can pass through the BTB from the interstitium to seminiferous tubules (Choy *et al.*, 2022). Although the current evidence demonstrates that EVs can cross the

BTB, better-designed experiments are still needed to explore the detailed mechanism and verify the ability of EVs to cross the BTB *in vivo*.

Testicular EVs as potential clinical biomarkers

Human semen contains abundant EVs (Vojtech *et al.*, 2014), some of which originate in the testis (Ma *et al.*, 2021). Pathological conditions (e.g. heat, hypoxia and infection) in the testis may alter the cargo in EVs; therefore, EVs in semen can reflect testicular function in disease progression (Table II).

Non-obstructive azoospermia (NOA) is characterized as the absence of sperm in the ejaculate and the failure of spermatogenesis and is believed to be the most severe form of male infertility. Microdissection testicular sperm extraction (mTESE) has been considered the gold standard method for surgical sperm retrieval in patients with NOA. It has been reported that exosomal miRNAs in seminal plasma can predict the presence of sperm in testes according to a multivariate analysis (Barcelo *et al.*, 2018). HIST1H2BA, a protein specifically expressed in GC, can be found in seminal EVs and predicts the presence of GCs in the testes of NOA patients (Yao *et al.*, 2021). piRNA is enriched in testicular EVs and can be present in seminal plasma (Choy *et al.*, 2022). One study identified eight piRNAs in seminal EVs that were associated with spermatogenic ability and the authors developed a model based on the expression of pir-61927, which can predict mTESE outcomes in NOA patients (Chen *et al.*, 2021). Long noncoding RNAs (lncRNAs) can also be found in testicular EVs (Choy *et al.*, 2022). Xie *et al.* (2020) established a panel consisting of nine testis-specific lncRNAs, including LOC100505685, SPATA42, CCDC37-DT, GABRG3-AS1, LOC440934, LOC101929088, LOC101929088, LINC00343, and LINC00301, which was thought to be a sensitive and specific method for predicting sperm retrieval in NOA patients. Transfer RNA-derived fragments (tRFs) are small fragments derived from mature tRNAs or pre-tRNAs; plasma circulating exosomal tRF-Gly-GCC-002 and tRF-Glu-CTC-005 were found to be useful biomarkers for predicting successful mTESE in patients with NOA (Zhang *et al.*, 2022) (Table II). Sperm prediction in most current studies is not based on the cause of NOA. Therefore, we do not know whether the prediction model is still effective when the backgrounds of NOA patients change.

Varicocele is one of the most common causes of male infertility and is characterized by abnormal dilatation and tortuosity of the pampiniform plexus veins in the spermatic cord. Ma *et al.* (2021) found that the hypoxic microenvironment in varicocele up-regulates miR-210-3p expression in rat SC-released small EVs. The retrospective analysis revealed that in patients with varicocele, seminal exosomal miR-210-3p was significantly increased in those with grade II and III varicocele, and miR-210-3p was negatively correlated with sperm count and seminal inhibin B expression. The results indicate that seminal exosomal miR-210-3p may be a novel biomarker of SC damage in varicocele (Ma *et al.*, 2021).

EVs may be biomarkers reflecting testicular injury. Testicular injury can be induced by drugs, toxins, infection and external force. In a rat model of testicular injury induced by ethylene glycol monomethyl ether, Kawata *et al.* (2020) identified serum exosomal miR-423-5p and miR-128-3p as non-invasive biomarkers for testicular injury. Scrotal heat, as a stress factor, impairs spermatogenesis. A recent study demonstrated that scrotal heat stress down-regulated three EV-miRNAs (miR-23b-5p, miR-489, and miR-1248) in bull seminal plasma (Alves *et al.*, 2021), suggesting that these miRNAs may be used as indicators of scrotal heat stress. EVs have also been proposed as biomarkers of the outcomes of assisted reproduction. Lal *et al.* (2022) analysed EVs in seminal plasma obtained from IUI treatments and found that a panel of exosomal mRNAs and lincRNAs were associated with positive pregnancy outcomes. Some of the mRNAs are specifically expressed in the testis (e.g. DMBX1, RUNDC3A, GPC2) (https://www.proteinatlas.org/). In summary, testicular EVs have demonstrated their potential as biomarkers, but high-quality research is still needed prior to the clinical use of these EVs.

Testicular EVs as potential treatments for male infertility

In the current decade, EVs as delivery carriers of therapeutics have received much attention. Compared with other vectors, exosomes have the advantages of higher stability, lower immunogenicity, lower toxicity, better biocompatibility, and biological barrier permeability (Zeng *et al.*, 2022). Nanoparticles have been used to control sperm movement (Chang *et al.*, 2019), deliver protein (BMP4) for GC differentiation (Esfandiari *et al.*, 2017), and introduce transgenes into the embryo via spermatozoa (Wang *et al.*, 2017). EVs, as biological endogenous carriers, may be superior to nanoparticles. EVs released from extratesticular cells (HEK293T) can interact with sperm without affecting their basic function (Vilanova-Perez *et al.*, 2020), which provides possibilities for EV-based drug delivery in the treatment of male infertility. However, it has been reported that spermatogonia (C18-4 and GC1-spg) and SCs (TM4) take up many more EVs isolated from testicular cells than those from other cells (HEK293FT) (Choy *et al.*, 2022). The authors found that the testicular cell lines were highly efficient in taking up testicular EVs, and labelled EVs could be detected in 95% of targeted testicular cells. A similar efficient uptake of testicular EVs was also found in spermatids and spermatozoa (Choy *et al.*, 2022). The results indicate that testicular EVs may be more advantageous than EVs originating from other cells for drug delivery, probably due to the specific targeting mechanisms of testicular EVs.

Nevertheless, some challenges severely hinder the use of EVs in clinical applications. The current methods for exosome isolation cannot achieve a pure and abundant isolate (Wang *et al.*, 2021). Other barriers include EV storage, drug loading efficiency, uptake efficiency of EVs by recipient cells, non-specific biodistribution of EVs in the body, rapid elimination from blood circulation, and possible side effects (Wang *et al.*, 2021).

Testicular EVs regulate the female reproductive system

Seminal plasma in the female reproductive tract not only protects sperm against attack by coating the sperm surface but also interacts with female reproductive tissues to facilitate conception. Human and

Table II Summary of EVs as clinical biomarkers in male infertility.

Disease/operation	Species	EV types	EV sources	Isolation method	Specific markers	Studied cargo	Conclusion	References
NOA	Human	Exosomes	Seminal plasma	Ultracentrifugation[A]	/	miRNAs	The expression of miR-539-5p and miR-941 can predict the presence of residual spermatogenesis.	Barcelo *et al.* (2018)
NOA	Human	EVs	Seminal plasma	Ultracentrifugation[B]	ALIX, TSG101, CD81	HIST1H2BA	HIST1H2BA can predict the presence of germ cells in testis.	Yao *et al.* (2021)
NOA	Human	EVs	Seminal plasma	Ultracentrifugation[A]	ALIX, TSG101, CD9, CD63	piRNAs	pir-61927 can predict the microTESE outcome in NOA patients.	Chen *et al.* (2021)
NOA	Human	EVs	Seminal plasma	Ultracentrifugation[A]	ALIX, TSG101, CD9, CD63, CD81	lncRNAs	A panel consisting of nine testis-specific lncRNAs can predict sperm retrieval in NOA patients.	Xie *et al.* (2020)
NOA	Human	Exosomes	Blood plasma	Isolation kits (Umibio, UR52136)	/	tRFs	tRF-Gly-GCC-002 and tRF-Glu-CTC-005 were useful for predicting successful microTESE.	Zhang *et al.* (2022)
Varicocele	Rat and human	Exosomes	Rat Sertoli cells and human seminal plasma	Isolation kit (SBI, EXOQ5A-1)	CD63, CD81, HSP70	miRNAs	Seminal exosomal miR-210-3p may reflect Sertoli cell damage in varicocele.	Ma *et al.* (2021)
Testicular injury	Rat	Exosomes	Blood serum	Ultracentrifugation[C]	CD63	miRNAs	miR-423-5p and miR-128-3p are biomarkers for testicular injury.	Kawata *et al.* (2020)
Scrotal heat	Bull	EVs	Seminal plasma	Ultracentrifugation[D]	ALIX, CD9, HSP70	miRNAs	Scrotal heat stress down-regulated three EVs-miRNAs (miR-23b-5p, miR-489 and miR-1248)	Alves *et al.* (2021)
IUI	Human	Exosomes	Seminal plasma	Liquid chromatography	/	mRNAs and lincRNAs	A panel of mRNAs and lincRNAs were associated with positive pregnancy outcomes.	Lal *et al.* (2022)

EVs: extracellular vesicles; miRNA: microRNA; NOA: non-obstructive azoospermia; piRNAs: PIWI-interacting RNAs; tRFs: transfer RNA-derived fragments. A: Ultracentrifugation protocol 1. Remove cells and debris (4000 g 10–15 min, 12 000 g 30 min); samples were filtered (0.22-μm filter); Precipitate EVs (100 000 g 70 min twice). B: Ultracentrifugation protocol 2. Remove cells, debris and microvesicles (4000 g 30 min, 20 000 g 60 min); precipitate EVs (199 700 g 70 min twice). C: Ultracentrifugation protocol 3. Precipitate EVs (208 000 g 70 min); samples were filtered (0.22-μm filter); precipitate EVs again (208 000 g 70 min). D: Ultracentrifugation protocol 4. Remove cells and debris (700 g 10 min, 4000 g 20 min, 16 500 g 30 min); precipitate EVs (120 000 g 70 min twice).

porcine seminal EVs have been reported to regulate the immunoinflammatory responses of the endometrium (Bai et al., 2018; Paktinat et al., 2019). Human seminal EVs can induce interleukin-6 and interleukin-8 secretion by endometrial stromal cells *in vitro* (Paktinat et al., 2019), and the EVs can also enhance the decidualization of endometrial stromal cells and increase their secretion of prolactin, which is an essential hormone in implantation (Rodriguez-Caro et al., 2019). In pigs, when endometrial epithelial cells were treated with seminal EVs, transcripts related to the 'immune response' and 'inflammatory response' were up-regulated (Bai et al., 2018). CCL20, a cytokine that recruits lymphocytes, is increased in endometrial cells and is suggested to regulate the uterine environment and facilitate sperm survival and activation (Bai et al., 2018). Interestingly, *Ccl20* mRNA has been found in SC-released EVs and can be translated into protein in other cells (Ma et al., 2022). EVs secreted by SCs can be present in seminal plasma (Yefimova et al., 2020; Ma et al., 2021) and, thus, may participate in regulating the female reproductive tract. In rabbits, testis-derived EVs can promote cumulus expansion and change the transcript expression of cumulus cells and oocytes *in vitro*, which indicates that the EVs may regulate female ovulation and oocyte maturation (Abumaghaid et al., 2022). Sperm RNAs derived from the epididymis can regulate embryonic development in mice (Trigg et al., 2021); therefore, it is postulated that testicular EVs can also affect embryonic development by altering sperm RNA. Testicular EVs may also be the cause of improper gene expression in spermatozoa, which is involved in recurrent pregnancy loss (Jena et al., 2021). However, the role of testicular EVs in the female reproductive system is largely unknown.

Future perspectives and outstanding questions

The roles of testicular EVs in the development of the male reproductive system and the regulation of spermatogenesis and steroidogenesis require further exploration. The intra- or inter-compartmental regulation by EVs between different cell types in the testis should be clarified in detail. Until now, there has been no study on EVs released by LCs, PTCs, and some immune cells, which may participate in many biological processes in the testis. Choy et al. (2022) found that 10% of rRNA and 60% of small noncoding RNA of testicular EV RNA were classified as bacterial rRNA and transfer-messenger RNA. The bacterial microbiome has been reported as a possible extracellular microenvironment component in the testis (Alfano et al., 2018). Bacteria can release membrane vesicles that carry RNA cargo and are involved in the modulation of host cells. Microbial RNA can be detected in human sperm (Swanson et al., 2020), which indicates that sperm may internalize bacterial EVs. In addition, obesity-induced changes in testicular microbiota can lead to decreased sperm motility in obese zebrafish (Su et al., 2021). However, the origin of bacteria-released EVs and their roles in the regulation of spermatogenesis, the spermatogenic microenvironment and paternal epigenetic inheritance are still elusive.

The present research on EVs is still limited to the interaction in the testis; it is unknown whether there is any EV communication between the testis and non-reproductive tissue (soma-to-germline communication). Cossetti et al. (2014) found that grafting GFP-positive melanoma cells into recipient mice resulted in detectable GFP mRNA levels in epididymal sperm, and EGFP-specific RNA could be amplified from the exosomal fraction of mouse plasma. In another study, the authors found that virus-mediated expression of human pre-miR-941 in the mouse brain led to detectable miR-941 in mature sperm and embryos (O'Brien et al., 2020). However, whether EVs play a role in this process is elusive. Paternal environmental exposure can alter the EV cargo entering testicular cells and regulating cell function or survival. If blood-derived EVs can pass through the BTB, GCs may also be affected, thereby probably affecting spermatogenesis or changing the sperm epigenome and early embryogenesis through sperm RNA. It has been reported that paternal stressors alter the EV cargo that enters sperm and produce offspring with changed neurodevelopment and adult stress reactivity (Chan et al., 2020). Recent studies have revealed that dysbiosis of gut microbiota causes male infertility (Ding et al., 2020) while improved gut microbiota restores spermatogenesis (Zhao et al., 2020). EVs derived from gut bacteria EVs are capable of crossing intestinal and cerebral barriers and have emerged as possible carriers in gut–brain communication (Cuesta et al., 2021). Can these EVs affect testicular function? EV-mediated soma-to-germline RNA or protein communication is an area of research that warrants further study.

Although significant progress has been made in the field of ART, there are still certain problems, such as abnormal embryonic development, recurrent implantation failure, and genetic abnormalities. It is unknown whether testicular EVs are involved. EVs in seminal plasma may participate in the development of embryos and the regulation of the female reproductive tract; thus, the outcomes of IVF/ICSI cycles can probably be improved by adding testicular/seminal EVs to the medium of gametes or embryos before fertilization or transplantation. EVs may also be used as a protective 'umbrella' that prevents short-lived additives such as proteins or siRNA from degradation in the culture medium or female reproductive tracts.

Conclusions

Existing studies have demonstrated some vital functional roles of testicular EVs in the proliferation and differentiation of SSCs, survival and steroidogenesis of LCs, removal of aberrant proteins, delivery of antigens, and protection of testicular cells. However, it is important to note that conclusive experimental evidence has not yet been provided for many of the functions of EVs in testes discussed in the present review. Further studies are still needed to explore their emerging roles in the complex regulation of spermatogenesis through intercellular communication. We found that EVs have been shown to have overlapping functions with many other testicular signalling factors (e.g. hormones and cytokines) in most recent studies, potentially indicating a fail-safe mechanism to maintain fertility. However, since EVs represent a new type of intercellular messenger in the testis, there may be some new EV-mediated regulatory targets and modes.

We also provide the current knowledge on the present use of testicular EVs as clinical biomarkers. EVs can be released into semen and blood, so they have the potential to be designated as non-invasive biomarkers for monitoring disease progression in varicocele, NOA and testicular injury. Although we still face many challenges in the clinical application of EVs, great interest has been paid to testicular EVs for their untapped biological roles. We anticipate that the development of

testicular EV-based diagnosis, therapeutics, and prognosis of male infertility may provide exciting opportunities in the future.

Data availability

The data underlying this article are available in the article.

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Authors' roles

Y.M. had the idea for the article. Y.M. and Q.-W.M. performed the systematic literature search. Y.M., Q.-W.M., Y.S., and X.-F.C. participated in the writing and revision of the article.

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Conflict of interest

None to declare.

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