CHAPTER FOUR

Exosomes of male reproduction

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Contents

Abstract

Exosomes are nanosized membrane vesicles secreted by wide variety of cells and found in abundance in biological fluids including semen. They contain cargo of lipids, proteins, microRNAs and mRNAs, and are known to play a major role in intracellular communication. Seminal exosomes mainly include epididymosomes and prostasomes. Most of the proteins associated with the epididymosomes are transferred to the sperm subcellular or membranous domains during their epididymal transit and are involved in the acquisition of fertilizing ability, modulation of motility and protection against oxidative stress. Proteins associated with prostasomes stimulate sperm motility and regulate the timing of capacitation to avoid premature induction of acrosome reaction. Furthermore, prostasomes protect the sperm from immune responses within the female reproductive tract. Overall, exosome-associated proteins play an indispensable role in maturation of spermatozoa and therefore, serve as an excellent biomarker in early diagnosis of male infertility.

1. Background

The study of proteins secreted by a cell or organism under specific conditions is referred to as secretome. These secreted proteins are encoded by 10% of the human genome and found in biological fluids [1]. They are

categorized into three main groups: (1) soluble proteins released via classical secretion, (2) ectodomain shedding of transmembrane proteins and (3) the proteome released via microvesicles from the endosomal compartment as exosomes [2,3].

In this review, we refer exosomes as membranous, nanometer-sized vesicles (50–500 nm) that are released via fusion of multivesicular bodies with plasma membrane (exocytosis) into the extracellular space and body fluids. They contain cargo of lipids, proteins, microRNAs and mRNAs, while devoid of DNA [4,5]. Their content is derived from the endosomal compartment of the target cells. A wide variety of cells secrete exosome including epithelial cells, T-cells, B-cells, platelets, dendritic cells and they are found in biological fluids such as plasma, breast milk, amniotic fluid, saliva and semen under physiological and pathophysiological conditions [6–14].

Exosomes orchestrate a plethora of biological functions based on their cellular origin including immunomodulation, cell migration, cell differentiation and cell–cell communication [15,16]. These vesicles exhibit an exoplasmic outward orientation similar to the lipid bilayer of the donor cells that facilitate interaction with the recipient cells via receptor–ligand interaction. These bound vesicles fuse with target cells resulting in the integration of proteins, RNAs and lipids into the target cell thereby influencing its function [17,18]. Therefore, the quantity and composition of exosomes is reflective of the functional status of their cellular origin and serve as novel biomarker for prognosis and diagnosis of various pathological conditions including cancer [17].

The role of exosomes in male reproduction has recently gained more attention and extensive research has been focused on delineating the implications of exosomes in germ cell development, sperm function and epididymal maturation, as well as their role as a modulator of fertility [19–21]. The current chapter discusses the composition, secretion and role of major seminal exosomes, epididymosomes and prostasome, in male reproduction. Furthermore, the chapter sheds light on the potential role of exosome biomarkers for assessing male infertility.
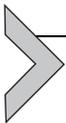
2. Exosome profile of semen

Semen contains cellular (spermatozoa) and non-cellular (seminal plasma) components. The secretions from testes (2–5%), epididymis and prostate (20–30%), seminal vesicles (65–75%) and bulbouretheral and peri-urethral gland (1%) constitute the seminal plasma [22]. The secretion is

enriched with lipids, sugar, growth factors, transcriptional factors and proteins that provides an ideal milieu for the nourishment and protection of spermatozoa during its journey through the male as well as female reproductive tract. Seminal plasma plays a vital role in sperm maturation, capacitation, acrosome reaction and fertilization. The heterogenous population of exosomes present in the seminal plasma is known to positively influence these key processes associated with sperm functions [21].

Seminal exosomes contribute 3% of total seminal plasma protein and mainly includes epididymosomes and prostasomes [22] (Fig. 1). Recently, Yang et al. conducted a comprehensive proteomic analysis of exosomes derived from human seminal plasma and identified 1474 proteins [23]. Furthermore, through Gene Ontology analysis, these exosomes-associated proteins were demonstrated to be mostly linked to "exosomes," "cytoplasm," and "cytosol." Bioinformatic analysis revealed the involvement of these proteins in biological processes such as cell growth and maintenance, metabolism, transport, energy pathways and protein metabolism [23]. The study conducted by Vojtech et al. reported that the human seminal exosomes contains distinct repertoire of small non-coding RNAs that modulate female reproductive tract to support embryo development [24].
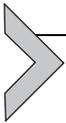
3. Exosomes in epididymis-epididymosomes

Upon leaving the testis, the differentiated spermatozoa undergo a long journey through the epididymis and reach the primary starting site of ejaculation, the vas deferens. The role of epididymis is not just limited to sperm storage (in the distal cauda) and transportation, but indeed plays an indispensable role in sperm maturation, and in imparting sperm heterogeneity. In humans, the epididymis is divided into three segments, namely, the proximal caput (head), the mid elongated corpus (body) and the distal cauda (tail). The epithelium of this highly differentiated organ is characterized by a unique set of tight junctions that contribute to the formation of distinct intraluminal microenvironment [25]. This blood-epididymal barrier regulates the movement of molecules in and out of lumen, resulting in epididymal intraluminal composition of electrolytes and macromolecules different from that of circulating body fluids. In fact, the intraluminal fluid composition of proteins varies from one segment of epididymis to another [26]. Though the spermatozoa entering the epididymis is immotile and lacks fertilizing ability, their passage through the continually changing, yet optimal, microenvironment of epididymal duct facilitates their maturation into
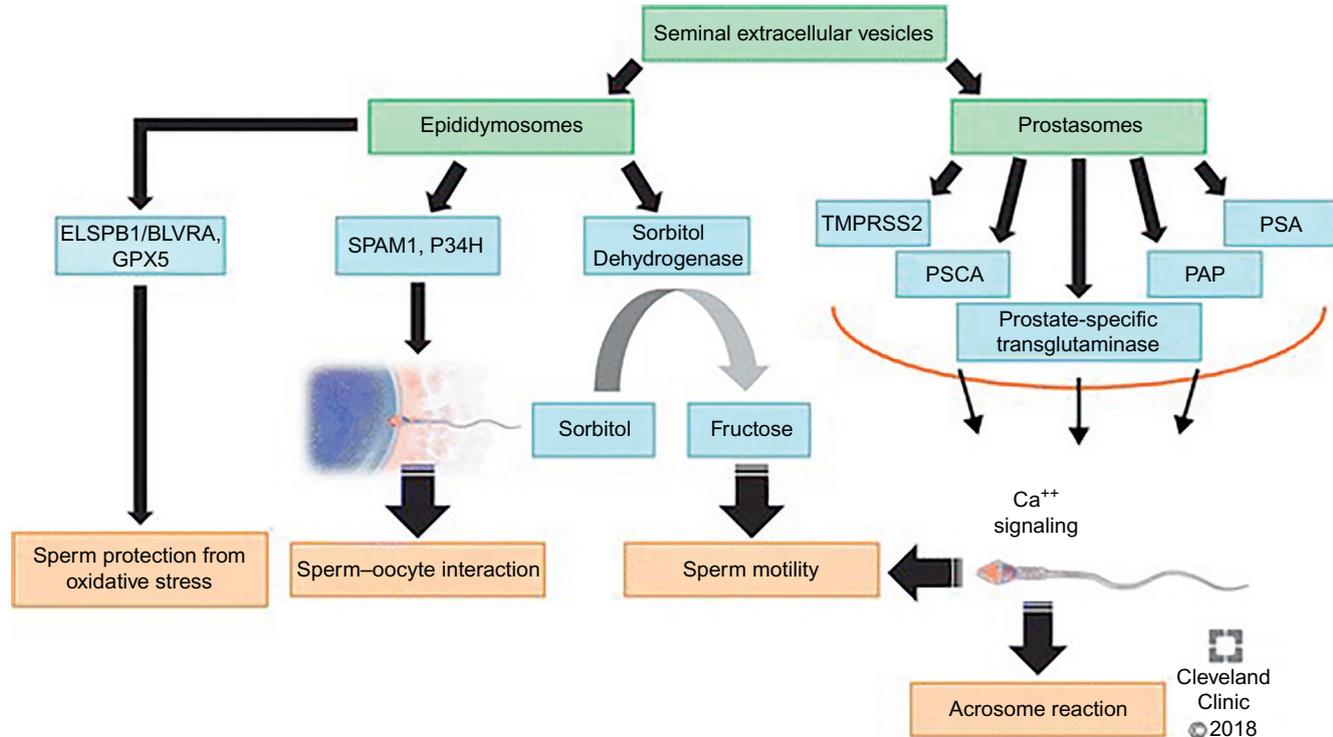
**Fig. 1** Role of proteins associated with seminal exosomes, epididymosomes and prostasomes, in sperm functions. *This figure has been adapted from L. Samanta, et al., The enigmatic seminal plasma: a proteomics insight from ejaculation to fertilization, Reprod. Biol. Endocrinol. 16 (1) (2018) 41. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved.*

fully functional sperm [25]. This event of sperm maturation is orchestrated by the sequential interaction of proteins secreted into the intraluminal fluid of epididymis with the spermatozoa resulting in morphological and bio-chemical alterations of the male gamete [27]. Some of the acquired proteins behave as a coating protein and modify the sperm surface by electrostatic interactions, while some behave as an integral protein and others cross the plasma membrane and get incorporated themselves into the intracellular structures of spermatozoa [27–29].
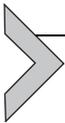
4. Secretion and composition of epididymosomes

The classical mechanism of protein secretion by an epithelium is via the merocrine pathway. The coding sequences of some secreted proteins in the intraluminal compartment of epididymis lack N-terminal signal peptide and associate with the cellular membrane via a glycosylphosphatidylinositol (GPI) anchor [27,30]. These indicated that some intraluminal proteins are not processed through the endoplasmic reticulum-Golgi complex, which led to the postulation of an alternative pathway of secretion in the epididymis. Apocrine secretion is an important secretory pathway of epididymis [31]. This pathway involves formation of cytoplasmic blebs at the apical pole of the principal secretory cells that detach into the intraluminal compartment. Subsequently, these apical blebs disintegrate and release their content, includ-ing epididymosomes [31–33]. These small membranous vesicles are about 50–500 nm in diameter with cholesterol and sphingomyelin concentrated lipid rafts, also termed as detergent resistant membrane (DRM). Yanagimachi et al. were the first to describe these vesicles at electron microscopic level in the intraluminal epididymal fluid and their association with sperm surface in Chinese hamsters [34]. They hypothesized that these vesicles could be involved in the transfer of cholesterol to sperm plasma membrane in order to stabilize it. Since then, the presence of small membranous vesicles or epididymosomes has been described in many mammalian species including mouse [35], rat [36], bull [37] and humans [38]. Epididymosomes are char-acterized by a high cholesterol/phospholipid ratio and contains adhesion molecules, such as tetraspanins, integrins and milk fat globule-epidermal growth factor protein (MFGE8) [38,39]. Proteins associated with the epididymosomes are transferred to the sperm's subcellular or membranous domains and are involved in the acquisition of fertilizing ability, modulation of motility and protection against oxidative stress [34,40–43].
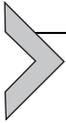
5. Proteins associated with epididymosomes and their functions

Two distinct populations of epididymosomes have been characterized (via differential ultracentrifugation protocol) in bovine epididymal fluid with discrete functions within the epididymis [19,44]. One sub-population is enriched with CD9 and other tetraspanin proteins (CD26 and CD224) that interact with live spermatozoa, while another sub-population is characterized by epididymal sperm binding protein 1 (ELSPBP1) that interacts with only dying/dead spermatozoa [19,44]. CD9-positive epididymosomes bind to or fuse with live spermatozoa and transfer proteins involved in the acquisition of sperm functions. Proteins p25b and GliPriL1 involved in sperm–egg interaction, and proteins MIF and AKR1B1 involved in sperm motility are enriched in CD9-positive epididymosomes [45]. Sperm motility and egg-recognition are key properties acquired by the spermatozoa via CD9-positive epididymosomes during its phase of epididymal maturation. In addition, CD9-positive microvesicles are also known to play a role in transfer of lipids to sperm membrane and hence, involved in plasma membrane remodeling during epididymal sperm maturation [45].

The epididymosomes enriched in ELSPBP1interacts with dead or dying spermatozoa in a Zn^{2+}-dependent manner [44]. ELSPBP1, described as HE12 in human, shows structural similarity with BSP (Binder of Sperm Proteins, the major constituent of seminal vesicle secretion) and has affinity for phospholipid choline group of sperm plasma membrane [46]. ELSPBP1 in association with biliverdin reductase (BLVRA) reduces biliverdin to bilirubin using NADPH as a proton donor [19]. Subsequently, bilirubin uses reactive oxygen species (ROS) to regenerate biliverdin in presence of Zn^{2+}. This enzymatic loop serves as a scavenger of ROS generated by the dying spermatozoa and protects the surviving spermatozoa from oxidative stress. Therefore, acquisition of ELSPBP1/BLVRA complex by the maturating spermatozoa via interaction with epididymosomes enable tagging of sperms that must be eventually eliminated while protecting the live spermatozoa from the detrimental effects of molecules generated by the dying sperms [19].

Ubiquitin is another protein associated with epididymosomes and transferred to spermatozoa during their transit. Since, ubiquitin is involved in enzymatic degradation of proteins via the proteasome, it is also thought to play a role in elimination of defective spermatozoa [47,48]. Glutathione peroxidase type 5 (GPX5) is another enzyme secreted in association to

epididymosomes and protects the sperm from oxidative stress and preserves DNA integrity during epididymal transit [35,49]. GPX5 is seleno-independent glutathione peroxidase and has a weak enzymatic activity toward hydrogen peroxide. It is transferred to the acrosomic region of spermatozoa during their epididymal transit and are thought to be involved in protection of sperm against premature acrosome reaction [50,51]. Similarly, Glutathione-S-transferase secreted by the principal cells into the intraluminal compartment in association with epididymosomes are involved in protecting the spermatozoa from free radical damage [52].

Human epididymis protein 5 (HE5) is expressed in the human epididymis and by lymphocytes [53,54]. It is highly glycosylated and GIP-anchored to cellular membranes [55]. HE5/CD52 is secreted in association with epididymosomes and transferred via these vesicles to sperm plasma membrane during epididymal maturation. This protein is thought to be associated with human immunological infertility [55,56]. P34h is another sperm binding protein secreted in association with epididymosomes and GIP-anchors, and targeted to the sperm surface during epididymal transit. It is involved in the binding of sperm to zona pellucida, an indispensable step for fertilization [57–59]. Sperm adhesion molecule 1 (SPAM1, also known as PH–20) is another GIP-anchored protein transferred to sperm plasma membrane via epididymosomes and is involved in sperm-zona pellucida adhesion [60,61]. It is a hyaluronidase that increases penetration of sperm through cumulus layer around the oocyte. Glioma Pathogenesis-related protein 1 (GliPriL1), which belongs to cysteine-rich secretory protein (CAP) family, is also GIP-anchored to sperm plasma membrane and plays a crucial role in fertilization [62].

Macrophage migration inhibitory factor (MIF) is a cytokine secreted into the intraluminal compartment of the epididymis in association with exosomes [28,63]. MIF has three cysteines present as free thiols and possess thiol-protein oxido-reductase activity. The MIF containing vesicles interact with the spermatozoa during their epididymal passage resulting in the transfer of MIF as a new component to the sperm flagellar outer dense fibers [29]. Based on the thiol-protein oxido-reductase activity of MIF, it has been postulated that free thiol groups could be involved in the chelation of zinc associated with outer dense fibers resulting in disulfide bond formation between the structural proteins of the sperm flagellum [29]. Thus, MIF modulates sperm motility during epididymal maturation [42].

In addition to MIF, enzymes of polyol pathway associated with epididymosomes also modulate sperm motility during epididymal transit [41,43].

The first step of this pathway involves reduction of glucose to sorbitol by aldose reductase using NADPH as electron donor. Subsequently, sorbitol is oxidized by sorbitol dehydrogenase using NAD^+ as electron acceptor to fructose, the primary energy source of spermatozoa. In bovine, it is interesting to note that the enzymatic activity of aldose reductase is high all long the epididymis except at distal cauda where the activity of sorbitol dehydrogenase was reported to be higher, favoring oxidation of sorbitol to fructose [64]. Based on this observation, it was hypothesized that most of the epididymal milieu is enriched with sorbitol, which is poorly permeable through sperm plasma membrane. Therefore, aldose reductase associated with epididymosomes deprives the sperm of energy source and maintains the maturing spermatozoa in a quiescent state. However, higher sorbitol dehydrogenase activity at the distal cauda and vas deferens oxidizes the sorbitol to fructose providing spermatozoa the energy required for their journey through female genital tract.

6. Exosomes in prostate-prostasomes

Prostasomes were first visualized 35 years ago in the human prostatic fluid and seminal plasma using electron microscope [65,66]. Size of the prostasomes varies from 30 to 500 nm. They are secretory microvesicles released into the prostrate ducts from the epithelial cells lining the prostate gland [67], similar to process observed during the secretion of exosomes form other origin. Prostasomes are localized inside endosomes in cells, and in the extracellular space (prostatic duct and seminal plasma) [65]. The plasma membrane of prostasomes is multilamellar having cholesterol and phospholipids in the ratio of 2:1 [68]. Nearly 50% of the phospholipid is sphingomyelin with very rigid membrane [69]. This unique composition of prostasome membrane allows prostasomes to fuse with other cells and transfer its contents to the same [70]. Ejaculated semen contains prostasomes mixed with the epididymal sperm and seminal vesicle fluid.

Protein composition of prostasomes was identified using global proteomic approaches [13,71]. Important prostasome specific proteins such as PAP, PSA, TMPRSS2 and PSCA serve as biomarkers of prostasomes and prostate cancer [72]. Two types of prostasomes were identified using transmission electron microscope based on their size and molecular composition. Smaller vesicles are of 56 ± 13 nm in size, whereas larger vesicles are 105 ± 25 nm in size [73]. Proteins GLIPR2 and ANXA1 were enriched in smaller prostasomes and larger prostasomes, respectively. Expression of these two

proteins on prostasomes are proposed as biomarkers for prostate dysfunction and male infertility [73]. Prostasomes have a direct influence on sperm function. Utleg et al. profiled 139 proteins in prostasomes using a microcapillary HPLC-tandem mass spectrometry technique [71]. The majority of those proteins (33.8%) are enzymes and 19% of them are transport and structural proteins [71]. Prostasomes are enriched with annexins I, II, IV, V, VII and XI and are associated with membrane trafficking and fusion [71,74]. Sperm motility is affected by the intracellular pH and calcium (Ca^{2+}) concentration. The annexins present on the prostasomes regulate the calcium channels to increase the intracellular Ca^{2+} levels of the sperm and thereby, exerts positive effect on motility [70,71]. Prostasomes also play a major role in the capacitation and induction of acrosome reaction that are essential for sperm–oocyte interaction. Capacitation and acrosome reaction is activated by the removal of prostasomal cholesterol transferred to sperm plasma membrane during prostasome fusion [75–77]. Ecto-diadenosine polyphosphate hydrolase is transferred from prostasomes to the sperm plasma membrane during sperm-prostasome fusion and it modulates the acrosome reaction [78,79]. Sperm deposited in the female reproductive tract are protected by the prostasome transferred proteins such as galectin 3 and CD48 [80,81]. These proteins modulate the immune response pathways such as complement pathway [82], lymphocyte proliferation [83], and phagocytosis [84] in the female reproductive tract.
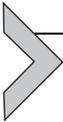
7. Exosome proteins as biomarkers for male infertility

Exosomal proteins can potentially serve as biomarkers of male infertility. They are composed of several proteins and play a vital role in sperm motility, capacitation, acrosome reaction, and fertilization process [70]. Advancement in the global proteomic tools and purification of exosomes has made it possible to identify many exosomal proteins. Yang et al. identified a total of 1474 exosomal proteins in normozoospermic men [23]. The exosomal proteins are primarily associated with protein metabolism, cell growth and maintenance [23]. Similarly, García-Rodríguez et al. identified a total of 1282 proteins in prostasomes and reported that the prostasomal proteome of normozoospermic men differs from non-normozoospermic men [85]. Prostasomal proteins associated with spermatozoa's energy production pathways and sperm activity were underexpressed in non-normozoospermic men [85]. Recent study on proteomic profiling of seminal plasma in unilateral varicocele patients showed the dysregulation

Table 1 Seminal exosomal proteins and their functions.

| Protein	Function	Reference
ELSPBP1/BLVRA	Scavenger of reactive oxygen species	[44]
GPX5	Epididymal sperm maturation and protects sperm from oxidative stress	[22]
SPAM1, P34H	Binding with zona-pellucida and sperm-oocyte interaction	[19]
Aldose reductase and sorbitol dehydrogenase	Modulates sperm motility	[22]
PAP, PSA, TMPRSS2, pTGase, PSCA	Sperm motility, capacitation, acrosome reaction	[13,87,88]
KIF5B	Release and proper functioning of the exosomes	[86]
ANXA2	Membrane trafficking and fusion	[86]
LDHC, HK1, PNP, APRT, SLC2A14	Spermatozoa's energy production	[85]
HIST1H2B, MSMB, MPO, MIF, KLK2	Sperm DNA organization, semen liquefaction, sperm-oocyte binding	[85]

of annexin II protein and proposed it as potential biomarker of infertility [86]. Table 1 summarizes the role of important exosomal proteins in male reproduction. Aberrant expression of these exosomal proteins could affect sperm functions and impact fertilization.
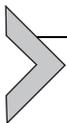
8. Future perspectives

Development in the omics techniques may allow for the identification of a biomarker related to exosomal dysfunction, associated with male infertility. Recent proteomic studies have reported altered expression of exosomal proteins in unilateral varicocele and non-normozoospermic subjects [85,86]. These two proteomic studies serve as a foundation to explore the role of exosomal proteins in specific male infertility associated conditions. Currently available molecular markers are able to identify the cause of male infertility; however, the identification of exosomal markers will further increase the understanding of defects in spermatozoa as a result of accessory sex organ/gland dysfunction. Proteomics relies on bioinformatics analysis and tools such as IPA, Metacore, Cytoscape, and Reactome that make

the interpretation of results more versatile and feasible. One of the major limitations with exosomal proteomic studies is the isolation and inadequate yield of the exosome for profiling of proteins. Overcoming these limitations and further validation of exosomal marker in large cohort can be useful for implementation of these biomarkers in clinical diagnosis of male infertility associated with defective exosome functions.
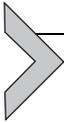
9. Conclusion

Exosome-associated proteins play a crucial role in male reproduction. Expression levels of these proteins can provide first-hand information related to the root cause of male infertility. In fact, the exosomal proteins have the potential to be designated as biomarkers for spermatozoa maturation failure. In-depth omics studies on seminal exosomes could help in identification of specific proteins related to defective exosome function that could facilitate development of new diagnostic and therapeutic strategies for treating exosomal dysfunction in infertile men.

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