

Prostasomes: extracellular vesicles from the prostate

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Abstract

The term 'prostasomes' is generally used to classify the extracellular vesicles (EVs) released into prostatic fluid by prostate epithelial cells. However, other epithelia within the male reproductive tract also release EVs that mix with 'true' prostasomes during semen emission or ejaculation. Prostasomes have been proposed to regulate the timing of sperm cell capacitation and induction of the acrosome reaction, as well as to stimulate sperm motility where all three are prerequisite processes for spermatozoa to attain fertilising capacity. Other proposed functions of prostasomes include interfering with the destruction of spermatozoa by immune cells within the female reproductive tract. On the other hand, it is unclear whether the distinct presumed functions are performed collectively by a single type of prostasome or by separate distinct sub-populations of EVs. Moreover, the exact molecular mechanisms through which prostasomes exert their functions have not been fully resolved. Besides their physiological functions, prostasomes produced by prostate tumour cells have been suggested to support prostate cancer spread development, and prostasomes in peripheral blood plasma may prove to be valuable biomarkers for prostate cancer.

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Introduction

All mammalian cell types examined to date have been shown to release extracellular vesicles (EVs) *in vitro*, and EVs have been detected in all body fluids. In recent years, it has also become clear that EVs can be important means of intercellular communication, supplementing the more conventional signalling mechanisms provided through direct cell-to-cell contact or by freely soluble signalling molecules (Raposo & Stoorvogel 2013). The idea that EVs from healthy cells could serve important biological functions was initially treated with scepticism by many scientists, based on the long-standing awareness that in addition to apocrine secretion by some specialised cell types, such as fat globules secreted by mammary epithelial cells, EVs are also abundantly released by apoptotic and necrotic cells. This scepticism ceased, however, in response to the numerous recent publications on molecular mechanistic aspects of EV formation and selective incorporation of 'cargo' including (glyco)proteins, lipids and RNA, and with the discovery of molecular pathways that can explain some of the demonstrated functions of EVs in body fluids. EVs include so-called exosomes, which are most often referred to as intraluminal vesicles of multivesicular bodies (MVBs) and that are released when MVBs fuse with the plasma membrane. Other types of EVs may be produced through shedding from the plasma membrane, and we here refer to these as microvesicles. In the literature, it is often

unclear whether reported EVs are exosomes or microvesicles, and, therefore, it is often more adequate to use the more comprehensive term 'EVs'. Cell-derived EVs have been demonstrated to regulate a variety of physiological processes, including adaptive immune responses, as well as to impact on pathological processes, like tumour development. Moreover, pre-existing EV pathways are thought to be exploited for the secretion of certain enveloped viruses, prions and amyloid peptides (Raposo & Stoorvogel 2013).

EVs in seminal plasma and prostatic fluid were first reported in the 1970s (Ronquist & Hedstrom 1977, Ronquist *et al.* 1978a, 1978b) and have been the topic of excellent recent reviews (Ronquist 2012, Sullivan & Saez 2013). EVs from the prostate epithelium, now known as prostasomes, correspond in size to vesicles found inside the so-called 'storage vesicles' within prostate epithelial cells (Brody *et al.* 1983, Ronquist & Brody 1985). That storage vesicles are the source of prostasomes was suggested by electron microscopic images from both benign and malignant prostate epithelial cells (Sahlen *et al.* 2002). Because these storage vesicles resemble MVBs found in other cell types, at least one population of prostasomes may be considered to be exosomes. Consistent with an exosomal nature, prostasomes from the prostate cancer cell line PC-3 have been shown to express proteins that are generally enriched on MVBs, such as MAL, caveolin-1, Tsg101 and Hrs

(Llorente *et al.* 2004, 2007). It should be noted, however, that it has never been directly demonstrated that storage vesicles are the exclusive or even the primary source of EVs in seminal plasma. Thus, populations of EVs in semen may originate from the plasma membrane of prostate epithelial cells or even from other sources within the male genital tract, including other accessory sex glands. To appreciate the potential contributors of EVs to seminal plasma, it is important to first evaluate the male reproductive tract and the potential role of EVs therein. The composition of seminal fluid and the biological roles of its components vary widely between mammalian species (Aumüller & Seitz 1990). Of note, this may include variation in tissue sources and biological functions of EVs, and conclusions drawn for one species cannot be automatically transferred to other species without experimental confirmation.

EVs in the male reproductive tract

Haploid spermatids mature into sperm cells within the seminiferous tubules. Sperm cells that leave the testis to enter the epididymis have limited biosynthetic capacity and are dependent on transfer of secreted products from epididymal epithelial cells for further maturation (Cooper 1998). Many of these molecules are transferred by epididymosomes, membrane vesicles that are thought to be shed from the epididymal plasma membrane in an apocrine fashion and can thus be characterised as microvesicles (Cornwall 2009). Epididymosomes have heterogeneous diameters ranging from ~ 0.1 to $1 \mu\text{m}$ and are produced throughout the epididymal duct, with their precise protein composition depending on the segment of epididymis in which they originate (Girouard *et al.* 2011). Epididymosomes transfer specific subsets of epididymal membrane proteins to the sperm cell surface (Frenette *et al.* 2002; Fig. 1). Among these is the GPI-anchored protein P34H. P34H (Legare *et al.* 1999), and its counterparts in other species, such as P26h in the hamster (Gaudreault *et al.* 1999), P31m in the monkey (Lamontagne *et al.* 2001) and P25b in the bull (Frenette & Sullivan 2001), are proteins that enable sperm cells to properly bind to the zona pellucida of an oocyte. Similarly, the transmembrane protein 'a disintegrin and metalloproteinase 7' (ADAM7 or GP-83) is also transferred by epididymosomes to sperm cells, where it has been proposed to play a role in sperm–oocyte interaction (Sun *et al.* 2000, Oh *et al.* 2009). Besides membrane proteins, epididymosomes may also transfer cytosolic proteins, including aldose reductase and sorbitol dehydrogenase, both of which are enzymes of the polyol pathway; the former reduces glucose to sorbitol, while the latter oxidises sorbitol to fructose. As fructose is a major energy source for spermatozoa, these enzymes may play an important role in sperm cell motility (Frenette *et al.* 2006). In a recent study, *in vitro* fusion of isolated epididymosomes with sperm was

reported (Schwarz *et al.* 2013), presumably explaining how epididymal epithelial cell cytosolic and membrane constituents can be integrated into sperm cells. Sperm capture of epididymosomes is apparently quite efficient, or epididymosomes are subsequently recaptured by epididymal epithelial cells, as the contribution of non-sperm associated free epididymosomes to the total population of EVs in the ejaculate appears to be low.

Depending on the species, spermatozoa may be stored for between a few days and several weeks in the cauda epididymis (Cornwall 2009). From the epididymis, sperm cells are transported through the ductus deferens to the ampulla, the dilated terminal segment of the distal ductus deferens that serves as an additional short-term sperm reservoir in many mammals but not in humans (Nistal *et al.* 1992). Secretions of the ductus deferens include vas deferens protein, a protein that lacks a signal sequence and is therefore thought to be released in association with EVs rather than via a conventional secretory pathway (Manin *et al.* 1995). The ampulla discharges its contents into the prostatic urethra, the location at which both the vesicular glands (confusingly also called the 'seminal vesicles') and the prostate secrete their contents. Although there are between-species differences, in humans the secretions from the vesicular glands account for 50–80% of seminal volume and contain prostaglandins, fructose and semenogelin I, one of the components responsible for semen coagulation after ejaculation (Aumüller & Riva 1992, Duncan & Thompson 2007). The vesicular glands also secrete

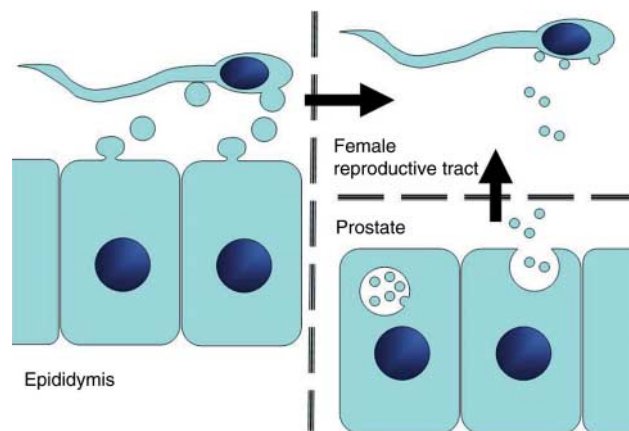


Figure 1 EVs in the reproductive tract. The epididymal epithelium produces EVs that are thought to be shed from the plasma membrane in an apocrine fashion. These vesicles then fuse with the sperm cell plasma membrane enabling the transfer of specific subsets of membrane and cytosolic proteins from epididymal cells to the sperm cells. In the female reproductive tract, sperm cells encounter another subset of EVs: prostasomes. We hypothesise that these vesicles first bind to capacitated sperm cells at a neutral or slightly alkaline pH and finally fuse with sperm cells only when they encounter a lower pH possibly in cumulus cell matrix in close proximity to the oocyte. The epididymis, prostate and female reproductive tract are separate compartments as indicated by the dashed lines.

EVs, which have a protein composition distinct from both epididymosomes and true prostasomes, and unknown function(s) (Sahlen *et al.* 2010). A prostate is apparent in all mammalian species, and although prostatic secretions may not be absolutely required, they are highly beneficial for fertility (Aumüller & Seitz 1990). Prostatic fluid accounts for 20–30% of seminal volume and contains high concentrations of citric acid, zinc and specific enzymes. Two major ones are prostate acid phosphatase (PAP) and prostate-specific antigen (PSA) (Veveris-Lowe *et al.* 2007, Graddis *et al.* 2011). The major function of the protease PSA is to liquefy the semen coagulum, although PSA has become better known as a biomarker for prostate cancer (Tosoian & Loeb 2010). The prostate also produces prostasomes, the primary topic of this review. Finally, the bulbourethral glands, also known as Cowper's glands, discharge into the urethra just before the sciatic arch at the base of the penis. This secretion is the primary component of pre-ejaculatory fluid and accounts for 2–5% of the total human ejaculate. It contains glycoproteins that lubricate the urethra for spermatozoal passage and neutralises the pH of any residual urine in the urethra and of the acidic vaginal secretions that the sperm are expected to encounter (Chughtai *et al.* 2005). We are not aware of any study reporting on the nature of EVs in secretions from the bulbourethral glands.

In short, although the name prostasome is generally applied to all EVs that can be recovered from semen, and the prostate is probably the major contributor of these EVs, it should be taken into account that heterogeneity in the composition or functions of seminal EVs may reflect EVs from distinct sources within the male reproductive tract. Moreover, the presence and functions of the accessory sex glands vary between mammalian species and this may well affect the nature and relative proportions of the contained EVs. For example, dogs do not have bulbourethral or vesicular glands, whereas the vesicular glands in the bull produce significant quantities of membrane vesicles (Agrawal & Vanha-Perttula 1987). It cannot, therefore, be concluded for all species that the prostate is the major contributor of EVs in the ejaculate.

The molecular composition of prostasomes

Prostasomes isolated from human semen have been characterised by comprehensive proteomic approaches (Utleg *et al.* 2003, Poliakov *et al.* 2009). Interesting components included prostate-specific proteins such as PAP, PSA, type 2 transmembrane serine protease (TMPRSS2), prostate-specific transglutaminase and prostate stem cell antigen (PSCA). These proteins have already been examined as (candidate) markers for prostate cancer (Cho *et al.* 2010, Bjartell *et al.* 2011, Graddis *et al.* 2011, Zhao *et al.* 2011), among which PSA is the most well known (Tosoian & Loeb 2010). Given their mutual presence on prostate epithelial cells and

prostasomes, these proteins may also serve as markers for 'true' prostasomes. In a recent study, we characterised EVs from seminal fluid obtained from vasectomised men so as to ensure exclusion of contributions from the testes or epididymides (Aalberts *et al.* 2012). Using this material, two distinct populations of EVs were purified and their further characterisation indicated distinct protein compositions. Both types of EVs resembled exosomes in terms of buoyant density, size and the presence of CD9, an ubiquitous exosome marker. The prostatic origin of both was indicated by the presence of PSCA. One of the populations had a diameter of 56 ± 13 nm and was selectively enriched in GLIPR2/GAPR-1, a protein that regulates autophagy by interacting with beclin 1 (Shoji-Kawata *et al.* 2013), possibly indicating an exosomal origin of this population of prostasomes. The second prostasome population constituted larger vesicles (105 ± 25 nm) with annexin A1 as an exclusive marker. The most extensively studied proteins in EVs isolated from semen are, however, enzymes that had already been described before this association was revealed. For example, dipeptidyl peptidase IV (DPP4; CD26) was shown to be associated with prostasomes (Bellezza *et al.* 2005, Carlsson *et al.* 2006), after having previously been proposed to be transferred to sperm cells by prostasomes (Arienti *et al.* 1997b). DPP4 is in fact expressed by many cell types and involved in many processes (Boonacker & Van Noorden 2003), although its role on EVs/sperm cells is unclear. Aminopeptidase N has also been reported to be transferred to sperm cells by prostasomes (Arienti *et al.* 1997a, Carlsson *et al.* 2003, 2006, Siciliano *et al.* 2008). Like DPP4, it is found on a wide variety of cell types (Mina-Osorio 2008), but in seminal fluid, aminopeptidase N has been proposed to regulate sperm cell motility through modulation of enkephalin levels (Subiran *et al.* 2007). In a recent study on EVs isolated from stallion seminal plasma, we found that both DPP4 and aminopeptidase N are largely confined to vesicles that are much larger than the CD9 carrying prostasomes (Aalberts *et al.* 2013). It is possible that these large EVs also originate from the prostate, e.g. by shedding from the plasma membrane; however, alternative sources such as the epididymis cannot be excluded (Dubois *et al.* 2009). Other enzymes that have been reported on prostasomes include 5' nucleotidase (Fabiani & Ronquist 1993, 1995), alkaline phosphatase, alkaline phosphodiesterase I (Fabiani & Ronquist 1995), neutral endopeptidase (Renneberg *et al.* 2001), TMPRSS2 (Chen *et al.* 2010), ecto-diadenosine polyphosphates hydrolase (Minelli *et al.* 2002), protein kinases A and C (PKA/PKC), casein kinase II and membrane-bound ATPase (Babiker *et al.* 2006).

Prostasomes have a peculiar lipid composition. The main phospholipid in prostasomes is sphingomyelin (SM), whereas mammalian cells contain more abundant quantities of phosphatidylcholine (PC) and

phosphatidylethanolamine (PE) (Arienti *et al.* 1998). The prostasomal membrane also has an unusually high cholesterol content. Early studies indicated that the cholesterol:phospholipid ratios in isolates of human and equine prostasomal membranes were 2.0 and 1.7 respectively, whereas the ratio was only 0.67 in the human sperm cell plasma membrane (Carlini *et al.* 1997, Arienti *et al.* 1998). The fatty acids in the prostasomal membrane are mostly saturated (stearic and palmitic acid) or monounsaturated (oleic acid) (Arienti *et al.* 1998). Together with a high concentration of cholesterol, this probably accounts for the very high stability of the prostasomal membrane (Arvidson *et al.* 1989). In a recent study, we used a lipidomic approach to analyse the lipid composition of two distinct populations of prostasomes that had been purified from the semen of vasectomised men (Brouwers *et al.* 2013) and confirmed the relatively high enrichments in cholesterol (55% of total lipid) and SM. In addition, we detected relatively high amounts of monohexosylceramides. Exosome formation is dependent on hydrolysis of SM by neutral sphingomyelinase in the endosomes (Trajkovic *et al.* 2008). The resulting product, ceramide, may serve as a precursor for the synthesis of hexosyl ceramides or of ceramide 1-phosphate, which we also detected in prostasomes (Brouwers *et al.* 2013). We speculate that these ceramide derivatives may play a role in the formation of prostasomes in endosomes within prostate epithelial cells. The prostasome glycerophospholipids consisted mainly of monounsaturated species while the sphingosine-based lipids, SM and the monohexosylceramides were characterised by the near absence of unsaturated species (Brouwers *et al.* 2013).

EVs from many other sources have been reported to contain RNA, including mRNA, miRNA and other small non-coding RNA biotypes with potential regulatory functions (Raposo & Stoorvogel 2013). Similarly, prostasomes have also been found to contain RNA. For example, in EVs from a prostate tumour cell line, RNA transcripts of the prostate cancer-specific fusion gene TMPRSS2:ERG were detected (Jansen *et al.* 2009). These transcripts, along with mRNA for GOLPH2, SPINK1 and prostate cancer antigen 3, were also found in EVs isolated from the urine of prostate cancer patients (Laxman *et al.* 2008, Nilsson *et al.* 2009, Leyten *et al.* 2012). In semen, cell-free mRNA and miRNA were found both in association with EVs and membrane-free protein complexes (Li *et al.* 2012). We have recently analysed the RNA content of prostasomes isolated from the ejaculate of vasectomized men by deep sequencing and found that the majority is neither mRNA nor miRNA (non-published data).

Prostasomes have also been reported to contain chromosomal DNA, representing an array of small DNA fragments randomly selected from the entire human genome (Ronquist *et al.* 2009, 2011). In our opinion, however, it cannot be excluded that small

apoptotic vesicles, which are known to contain fragmented DNA, were co-isolated with prostasomes.

Sperm cell capacitation and the role of prostasomes

Sperm cells that are deposited in the female reproductive tract are mature but not yet completely primed for fertilisation. In order to acquire fertilising potential, they first need to undergo a complex series of modifications in a process collectively referred to as capacitation (Bailey 2010, Fraser 2010). During this process, sperm cells attain the capacity to bind to the zona pellucida of the oocyte, are primed to undergo the acrosome reaction and acquire hyperactive motility and the potential to fuse with the oocyte plasma membrane. *In vitro*, sperm cell capacitation can be initiated by the presence of extracellular bicarbonate, Ca^{2+} and a cholesterol acceptor such as albumin. Bicarbonate rapidly (within minutes) activates adenylate cyclases, which produce cAMP (Harrison & Gadella 2005). Sperm cells contain several membrane-associated adenylyl cyclases, as well as a soluble adenylyl cyclase (Esposito *et al.* 2004). The latter is mainly expressed in the testis and is both Ca^{2+} and bicarbonate dependent (Buck *et al.* 1999, Baxendale & Fraser 2003, Kamenetsky *et al.* 2006). In a recent study, it was proposed that prostasomes help to increase intracellular cAMP at capacitating conditions (Pons-Rejraji *et al.* 2011). cAMP stimulates PKA, which is the central activator of several capacitation-related processes in the sperm cell, including a reorganisation of plasma membrane lipid and protein distribution (Harrison & Miller 2000, Fraser 2010). PKA indirectly stimulates tyrosine phosphorylation of specific sperm proteins, which is considered as a hallmark of capacitation (Visconti *et al.* 2002, Bailey 2010). Major tyrosine-phosphorylated proteins in capacitated human sperm include the PKA anchoring protein AKAP and its precursor, pro-AKAP (Carrera *et al.* 1996), and their phosphorylation is considered as a terminal stage of capacitation. Others and we have found that prostasomes interfere with end-stage sperm capacitation *in vitro*, as measured by a decrease in protein tyrosine phosphorylation (Pons-Rejraji *et al.* 2011, Aalberts *et al.* 2013). One possible explanation is that cholesterol could be transferred from prostasomes to sperm cells. Another PKA-driven process is plasma membrane lipid scrambling. In non-capacitated sperm cells, lipid asymmetry is maintained by phospholipid transferases: asymmetry is characterized by location of the aminophospholipids PE and phosphatidylserine (PS) predominantly in the inner leaflet of the plasma membrane bilayer, whereas PC and SM are mainly present in the outer leaflet (Harrison & Gadella 2005). During capacitation, a phospholipid scramblase is activated, resulting in the bi-directional movement of phospholipids across the lipid bilayer. As a result, an increased proportion of PE and PS become exposed on the outer

leaflet of the sperm cell plasma membrane. This breakdown in membrane asymmetry renders the membrane less stable and enables cholesterol acceptors, such as serum albumin, but probably also lipoproteins, to extract cholesterol from the plasma membrane (Therien *et al.* 1998, Flesch *et al.* 2001). Cholesterol extraction leads to a further increase in membrane fluidity, which is associated with a reorganisation of the constituent lipids and proteins and the formation of so-called 'lipid rafts'. Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingolipids and specific membrane proteins, including SNAREs (Chamberlain *et al.* 2001, Lingwood & Simons 2010). The function of SNARE proteins is influenced by their position in the membrane rafts. In response to cholesterol removal, the SNARE proteins syntaxin 1 and 2, that reside in the sperm cell plasma membrane, along with the vesicle-associated membrane protein (VAMP), which is located in the outer acrosomal membrane, relocate from their original random distribution over the entire sperm head and become concentrated in the apical ridge area, which is the region of the sperm cell involved in oocyte binding (Tsai *et al.* 2007).

Sperm cell motility is regulated by intracellular pH and Ca^{2+} signalling (Darszon *et al.* 2011, Alasmari *et al.* 2013). In most species, sperm cells acquire motility shortly before arrival in the cauda epididymis, immediately after their release from the epididymis or soon after ejaculation. In aqueous solutions, sperm cells with activated motility swim with near linear trajectories as a result of their symmetrical flagellar beats. Within the female reproductive tract, sperm cells presumably migrate through more viscous fluids and interact with cells and cellular projections; most notably, they attach to the mucosal epithelium of the oviduct and, in several mammalian species, oviduct epithelium-bound sperm cells form a reservoir for later fertilisation (Suarez & Pacey 2006). During sperm hyperactivation, which is physiologically induced during capacitation of sperm within the oviduct, the flagellar movements change in character to large-amplitude, high-frequency and asymmetrical beats. Hyperactivated motility is required for sperm cells to detach from the oviductal epithelium, to swim through the oviductal mucus, pass the layer of follicular cells that surround the oocyte (the cumulus complex) and penetrate the zona pellucida (Quill *et al.* 2003, Ho *et al.* 2009). During sperm cell capacitation, the plasma membrane potential becomes hyperpolarised as a consequence of the opening of inwardly rectifying K^+ channels (Acevedo *et al.* 2006) and closure of epithelial Na^+ channels (Hernandez-Gonzalez *et al.* 2007), which results in the opening of voltage-dependent Ca^{2+} channels (Publicover *et al.* 2007). Sperm cell hyperactivation is triggered by a sustained Ca^{2+} influx (Quill *et al.* 2003). When sperm cells reach the zona pellucida of the oocyte, contact with the zona pellucida, and in particular zona pellucida

glycoprotein 3, induces a depolarisation of the sperm plasma membrane (Florman *et al.* 1998). This depolarisation in turn induces the proton channel, Hv-1, in the sperm cell flagellum to open, triggering an outward transport of protons to the extracellular milieu, thereby causing intracellular alkalisation (Lishko *et al.* 2010). As a result, sperm-specific pH-dependent CatSper Ca^{2+} channels are opened (Quill *et al.* 2003, Kirichok *et al.* 2006, Neri-Vidaurre Pdel *et al.* 2006). The initial influx of Ca^{2+} is thus followed by a sustained Ca^{2+} entry that requires the presence of the distinct components of the CatSper channel complex (Qi *et al.* 2007). Opening of the CatSper channels is also stimulated by direct interaction with progesterone, which is released by the cumulus granulosa cells surrounding the oocyte, as well as by prostaglandin E_1 and other ligands (Lishko *et al.* 2011, Strunker *et al.* 2011, Brenker *et al.* 2012). In this way, progesterone attracts spermatozoa towards the oocyte and helps them to penetrate the layer of granulosa cells and the zona pellucida. Ca^{2+} can also be recruited from intracellular Ca^{2+} storage organelles. Ryanodine receptors are Ca^{2+} channels that release Ca^{2+} from such intracellular stores in response to cyclic adenosine dinucleotide phosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (Park *et al.* 2011). Increase in intracellular Ca^{2+} via CatSper channel opening and release of stored Ca^{2+} at the sperm neck provides two separate mechanisms for regulating motility (Alasmari *et al.* 2013). Interestingly, long-lasting Ca^{2+} responses require the presence of prostasomes, and it has therefore been hypothesised that some Ca^{2+} signalling tools are not initially carried by the sperm cells but have to be delivered to them by prostasomes (Park *et al.* 2011). Both the ryanodine receptor and CD38, which helps generate cADPR and NAADP, are carried by prostasomes. Similarly, a pump for refilling Ca^{2+} in intracellular stores, SPCA1, and the plasma membrane-associated progesterone receptor, but not the CatSper elements, are transferred to sperm by prostasomes. Thus, some of the tools required for sustained Ca^{2+} signalling appear to be transferred to sperm by prostasomes.

The acrosome reaction and prostasomes

As well as influencing motility, Ca^{2+} influx also triggers the acrosome reaction in sperm cells. The acrosome is a sperm cell-specific organelle that originates from the Golgi system and is formed during spermiogenesis (Berruti & Paiardi 2011). The acrosome extends over the anterior aspect of the sperm cell nucleus in the head region and is in close proximity to the sperm plasma membrane. In response to capacitation, *trans*-SNARE complexes are formed from VAMP3 and syntaxin 1B, in the plasma membrane, and synaptosomal-associated protein 23 (SNAP23) in the outer acrosomal membrane (Tsai *et al.* 2010, Gadella & Evans 2011); these complexes hold the two membranes in close proximity.

During the acrosome reaction, completion of the SNARE interactions results in the outer acrosomal membrane fusing with the overlying plasma membrane. These membrane fusions occur simultaneously at multiple sites, as a consequence of which mixed membrane vesicles are formed, the integrity of the membranes is breached and the acrosomal contents are released. The liberated acrosomal enzymes assist the sperm cell with its penetration through the zona pellucida of the oocyte (Buffone *et al.* 2008). ZP3 molecules within the zona pellucida are thought to assist in triggering the acrosome reaction (Litscher *et al.* 2009), probably by activating the Rap guanine nucleotide exchange factors 3 and/or 4, which may induce depolarisation of the sperm cell plasma membrane, thereby stimulating Ca^{2+} influx (McPartlin *et al.* 2011). However, while the acrosome reaction can take place at the zona pellucida surface, it is probably initiated even earlier under the influence of cumulus cells, the follicle wall derived epithelial cells that surround the recently ovulated oocyte in the oviduct (Jin *et al.* 2011). Progesterone secreted by these cells (Therien & Manjunath 2003), coupled with the previously described transfer of progesterone receptors and other Ca^{2+} signalling tools by prostasomes (Park *et al.* 2011), may be important factors in this process.

Prostasomes regulate sperm cell function by first binding and then fusing

Prostasomes are brought into contact with sperm cells during ejaculation (Fig. 1). As illustrated above, prostasomes have both inhibitory and stimulatory effects on sperm cell function, and their role(s) is therefore complex (Table 1). We recently demonstrated that *in vitro* recruitment of highly purified prostasomes by live equine sperm cells required the presence of bicarbonate and an environment with $\text{pH} \geq 7.5$, circumstances that also favoured protein tyrosine phosphorylation in sperm cells, a hallmark of capacitation (Aalberts *et al.* 2013; Fig. 2). By contrast, dead sperm cells 'recruited' prostasomes independently of bicarbonate or pH. Binding specificity was further indicated by the observation that live cells recruited prostasomes primarily at their head region, whereas dead sperm cells

recruited prostasomes over both their head and tail regions. A common characteristic of capacitated, and indeed apoptotic or dying, sperm cells is a loss of plasma membrane lipid bilayer asymmetry (Harrison & Gadella 2005), which might therefore be a requirement for prostasome binding. Notably, however, while prostasomes required capacitating conditions to allow them to bind to live sperm cells, they then reduced the level of tyrosine phosphorylation (Aalberts *et al.* 2013), consistent with a negative regulatory effect on the progression of capacitation (Pons-Rejraji *et al.* 2011, Aalberts *et al.* 2013). How prostasomes inhibit or prevent the progression of capacitation is not known but transfer of cholesterol to the sperm cell's plasma membrane, independent of membrane fusion, is a potential candidate (Cross & Mahasreshti 1997, Pons-Rejraji *et al.* 2011). Cholesterol transfer would counteract the cholesterol efflux that normally occurs during *in vitro* capacitation of washed sperm cells (Cross 1996, Cross & Mahasreshti 1997). Recent evidence indicates that sterol extraction from sperm cells, a prerequisite for capacitation, depends on the formation of oxysterols in response to low doses of reactive oxygen species (ROS), which in their turn are formed in a bicarbonate-dependent process (Boerke *et al.* 2013). In another study, prostasomes were shown to interfere with ROS production by polymorphonuclear neutrophils from blood or semen (Saez *et al.* 2000). Although entirely speculative at this point in time, it is therefore possible that prostasomes put a brake on the depletion of cholesterol from the sperm plasma membrane by interfering with sperm cell ROS production. By binding to sperm cells early during capacitation, prostasomes would be in a position to inhibit progression to late capacitation events and therefore help avoid premature induction of the acrosome reaction (Cross & Mahasreshti 1997, Bechoua *et al.* 2011, Pons-Rejraji *et al.* 2011).

The physiological sites for prostasome binding and fusion with sperm cells have not been identified (Fig. 1). In humans, sperm are deposited in the anterior vagina during coitus after which sperm motility helps their passage through the cervical mucus. The acidic environment within the vagina would not be expected to support sperm cell capacitation nor, therefore, the recruitment of

Table 1 Reported effects of prostasomes on sperm cell functions.

Effects of prostasomes	References
Stimulation of early capacitation events	Stegmayr & Ronquist (1982) and Pons-Rejraji <i>et al.</i> (2011)
Inhibition of late capacitation events	Cross & Mahasreshti (1997), Bechoua <i>et al.</i> (2011), Pons-Rejraji <i>et al.</i> (2011) and Aalberts <i>et al.</i> (2013)
Inhibition of spontaneous acrosome reaction	Pons-Rejraji <i>et al.</i> (2011)
Inhibition/stimulation of acrosome reaction dependent on environmental conditions	Cross & Mahasreshti (1997), Palmerini <i>et al.</i> (2003), Siciliano <i>et al.</i> (2008) and Park <i>et al.</i> (2011)
Inhibition/stimulation of motility dependent on environmental conditions	Stegmayr & Ronquist (1982), Fabiani <i>et al.</i> (1994a, 1994b), Arienti <i>et al.</i> (1999) Bechoua <i>et al.</i> (2011) and Park <i>et al.</i> (2011)
Stimulation of fertility	Aumüller <i>et al.</i> (1990)

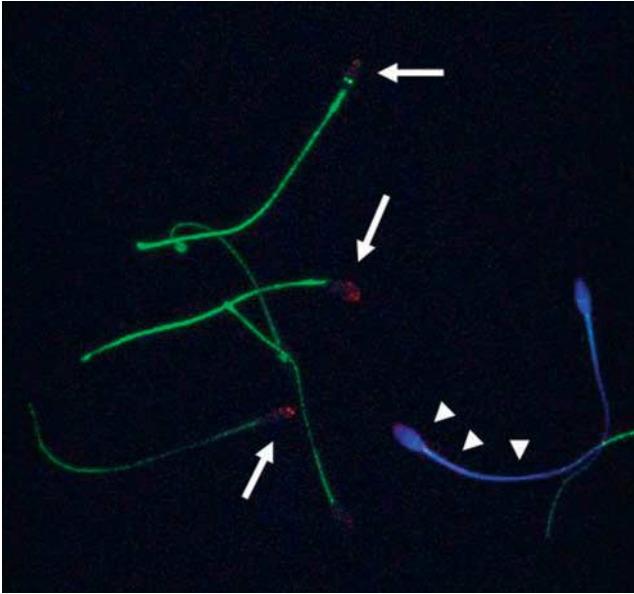


Figure 2 Prostasome binding to capacitated sperm cells. Prostasomes were isolated from stallion semen and labelled with biotin. Washed equine sperm cells were incubated at capacitating conditions (in the presence of Ca^{2+} , HCO_3^- and albumin at pH 7.5) in the presence of biotinylated prostasomes. After selective labelling of dead cells in violet, the cells were fixed, permeabilised and labelled in red with NeutrAvidin for associated biotinylated prostasomes and in green with anti-phosphotyrosine as a marker for capacitation. Cells were imaged by confocal laser scanning microscopy. Prostasomes associated primarily to the sperm head of live capacitated cells, assuming a punctate pattern (arrows). Binding to dead cells was diffuse and not confined to a specific region (arrowheads). For details, see our study (Aalberts *et al.* 2013).

prostasomes. By contrast, the uterus would be a relatively capacitation-friendly environment, due in part to cystic fibrosis transmembrane conductor regulator (CFTR)-regulated secretion of bicarbonate by the endometrium (Wang *et al.* 2003). That the uterus should be a physiological site for prostasome recruitment to sperm seems even more obvious for the mammalian species in which semen is deposited intracervically (e.g. the pig) or intrauterine (e.g. the horse). Fusion of previously bound prostasomes to the sperm cells has been reported to occur only in acidic buffers (Arienti *et al.* 1997c, Palmerini *et al.* 1999, Park *et al.* 2011). The neutral pH of the uterus and the local presence of bicarbonate would therefore represent a favourable location for prostasome binding but not fusion (Aalberts *et al.* 2013). We therefore hypothesise that, after binding in the uterus, prostasomes may 'piggy-back' onto the sperm cell's surface to enter the oviduct and accompany the sperm on its final approach to the oocyte-cumulus complex. Here, the role of prostasomes may change as a consequence of fusion with the sperm cells, allowing them to integrate contained cytosolic and membrane constituents into the sperm cell, although definitive proof of fusion at this time is lacking. Stimulatory effects

of prostasomes on the acrosome reaction (Palmerini *et al.* 2003, Siciliano *et al.* 2008) and sperm motility (Fabiani *et al.* 1994a, 1994b, 1995, Arienti *et al.* 1999, Park *et al.* 2011) have been reported, and as described above, these stimulatory functions can be explained by transfer of Ca^{2+} signalling tools as a result of the fusion of prostasomes to sperm cells (Park *et al.* 2011). Whether fusion at this location really occurs and whether it is indeed pH dependent, along with the nature of a postulated low pH compartment, are questions that remain to be solved. One possible location is the interstitial space between cumulus cells. The extracellular matrix between cumulus cells is a viscoelastic gel made up of hyaluronic acid containing glycosaminoglycan molecules. Sperm cells traverse this layer with the aid of multiple hyaluronidases (Cherr *et al.* 2001, Martin-Deleon 2011). In this context, it may be significant that some of these hyaluronidases have a low optimum pH and are important for sperm penetration of the cumulus. To our knowledge, the pH of the cumulus interstitial space has never been determined directly, but the requirement for acidic hyaluronidase activity to enable sperm penetration suggests that this could represent a compartment where low pH-induced prostasome fusion may occur. Although entirely hypothetical, such a timely delivery by integration of prostasome borne Ca^{2+} signalling molecules might explain their apparent facilitatory role in the acrosome reaction. Many *in vitro* studies have been performed with epididymal sperm cells, which by definition have never been in contact with prostasomes. This may explain some of the controversies in the literature concerning the mechanisms of Ca^{2+} signalling in sperm cells (Ren 2011). Successful artificial insemination with epididymal sperm has been demonstrated for many species, suggesting that prostatic secretions, and hence the presence of prostasomes, may not be an absolute requirement for successful fertilisation. Studies in the dog, however, showed that addition of prostate fluid to washed ejaculated sperm (Nöthling & Volkmann 1993) or epididymal sperm (Hori *et al.* 2005) significantly improved pregnancy and litter size, consistent with a facilitating role of prostasomes.

Prostasomes interact with immune cells

The female reproductive tract is equipped with a well-balanced immune system. On the one hand, it has to protect the carrier and a potential embryo or fetus against invading pathogens, but on the other hand, it must tolerate potentially highly immunogenic paternal allo-antigens in order to be able to establish and maintain pregnancy (reviewed in Wira *et al.* (2005) and Hansen (2011)). Macrophages and neutrophil granulocytes form a first line of defence against pathogens but also phagocytose sperm cells (Matthijs *et al.* 2003, Wira *et al.* 2005). Natural killer (NK) cells can also attack

pathogens, while apparently playing an important role during implantation of the embryo and the maintenance of pregnancy (Marlin *et al.* 2012). Finally, dendritic cells form a bridge between the innate immune system and the adaptive immune system of the female reproductive tract, which also involves T cells (Wira *et al.* 2005).

In vitro studies have demonstrated that prostasomes have a variety of immunomodulatory capacities. These may involve direct interactions with leukocytes, interactions through the complement system or direct antibacterial or antiviral mechanisms. For example, human prostasomes have been shown to adhere to lymphocytes, inhibit lymphocyte proliferation and to inhibit endocytosis by monocytes and neutrophil granulocytes (Kelly *et al.* 1991, Skibinski *et al.* 1992). In the female reproductive tract, inhibition of phagocytotic activity by prostasomes may protect sperm cells from attack by neutrophil granulocytes. Another immunomodulatory property of prostasomes involves inhibition of the generation of superoxide anions by NADPH oxidase in neutrophil granulocytes (Skibinski *et al.* 1992, Saez *et al.* 2000). Superoxide anions and other ROS also play a role in the defence by phagocytotic cells against bacteria and fungi. However, the sperm cell is also very sensitive to lipid peroxidation, due to the high content of polyunsaturated fatty acids in its plasma membrane and its limited ROS scavenging capacity as a result of a small cytoplasmic volume. After protein denaturation by heat treatment of the prostasomes, the protective effect against ROS was retained, suggesting that lipids rather than proteins may be involved (Saez *et al.* 1998). Prostasomes probably do not act as direct scavengers of ROS, unlike other antioxidants in seminal plasma (Tremellen 2008). Instead, prostasomes may modify the plasma membrane of neutrophil granulocytes by transfer of cholesterol and SM to the cells, making them more rigid and resulting in a decrease in NADPH oxidase activity (Lazarevic *et al.* 1995, Saez *et al.* 2000). Membrane vesicles from bovine seminal fluid have been demonstrated to have similar effects on proliferation, phagocytotic activity and superoxide production by leukocytes (Lazarevic *et al.* 1995). Additionally, human prostasomes have been shown to contain CD48, which is the ligand for activating NK cell receptor 2B4 (CD244) (Tarazona *et al.* 2011). NK cell activity decreases after incubation in the presence of prostasomes, suggesting that prostasomes may also play a role in controlling NK cell activity in the female reproductive tract. In addition, prostasomes have been described to interfere with the complement system, through their surface molecules CD59 and CD46. CD59 is an inhibitor of the membrane attack complex of the complement system. Transfer of CD59 from prostasomes to cells (Rooney *et al.* 1993, 1996) has been shown to inhibit complement-mediated cell lysis (Babiker *et al.* 2005). Membrane cofactor protein (CD46) is a transmembrane glycoprotein that possesses cofactor activity for proteolytic inactivation of C3b and

C4b of the complement cascade (Kitamura *et al.* 1995). CD46 is also known as a receptor for measles virus, and prostasomes have been shown to inhibit infectivity of this virus, probably by capture of the virus by CD46, as a kind of 'mock cell' (Kitamura *et al.* 1995). Another immunomodulatory prostasomal surface protein is galectin-3 (Jones *et al.* 2010), a β -galactoside-binding protein that plays a role in immunomodulation, pathogen–host interactions, cell–cell and cell–matrix interactions and cancer progression (Dumic *et al.* 2006). Galectin-3 lacks a signal sequence for transport into the endoplasmic reticulum and is therefore secreted via a non-classical pathway. The galectin-3 ligand, Mac-2 binding protein (M2BP), has also been found to be associated with prostasomes (Block *et al.* 2010) and has also been proposed to have an immunomodulatory function on macrophages and monocytes (Block *et al.* 2010).

Taken together, the interaction of prostasomes with the local female immune system may prevent sperm cells from being phagocytosed, damaged or killed, and thereby prolongs their lifespan in an otherwise hostile environment. Finally, prostasomes have antibacterial properties (Carlsson *et al.* 2000), probably through the antimicrobial protein human cationic antimicrobial protein-18 (hCAP-18) present in the prostasomal membrane. hCAP-18 belongs to the cathelicidin family and releases the antimicrobial peptide LL37 (Andersson *et al.* 2002).

Prostasomes and prostate cancer

Prostate cancer is the most common type of cancer suffered by men in the Western world (Center *et al.* 2012). As hyperplastic and malignant prostate cells, and even metastatic tumour cells, secrete prostasomes that share many characteristics with prostasomes from normal cells (Carlsson *et al.* 2003, Sahlen *et al.* 2004), information on prostasomes may provide valuable information for the early diagnosis of prostate cancer or incipient malignant transformation. While prostasomes are normally secreted from the apical side of the columnar prostate epithelial cells into the glandular lumen and are then excreted in the semen or urine, malignant prostatic epithelial cells become cuboidal and lose polarity. When these cells invade through the basement membrane, prostasomes can be secreted towards the interstitial compartment of the prostate tissue, or even directly into the blood circulation (Sahlen *et al.* 2002, Ronquist & Nilsson 2004). Tissues that are normally devoid of prostasomes are now challenged by the immunomodulatory properties of these vesicles, a process that has been proposed to assist tumour survival and metastasis. Moreover, the composition of prostasomes from prostate tumour cells may be altered in several respects, when compared with vesicles from normal prostate cells.

Characteristics of prostasomes that enable them to influence prostate cancer progression include their involvement in the complement pathway, their expression of enzymes on the prostasomal surface and the promotion of angiogenesis (Ronquist & Nilsson 2004). As described above, prostasomes contain the complement regulatory proteins CD46 and CD59 and are able to transfer CD59 to neighbouring cells (Kitamura *et al.* 1995, Babiker *et al.* 2005). Indeed, prostasomes from malignant cells have a higher ability to transfer CD59 to cells than prostasomes from normal cells (Babiker *et al.* 2005). Another difference between prostasomes from cancer cells and normal prostate epithelial cells is their enzymatic activity. Prostasomes from cancer cells show a higher kinase activity than prostasomes from normal seminal plasma and thereby enhance ability to phosphorylate complement protein C3 and fibrinogen (Babiker *et al.* 2006). This makes C3 inaccessible to physiological activation (Forsberg *et al.* 1990), while it may also alter the functional properties of fibrinogen, a change that has been implicated in cancer cell migration (Simpson-Haidaris & Rybarczyk 2001). Other enzymes that show a higher activity on prostasomes from tumour cells than normal cells include peptidoglycan hydrolyzing enzymes, matrix metalloproteinases and plasminogen activator, all of which play a role in extracellular matrix degradation and cancer cell invasion (Bellezza *et al.* 2005). Conversely, the expression of CD26 is reduced on prostate cancer cell-derived prostasomes, which may affect its role as a tumour suppressor (Bellezza *et al.* 2005). TMPRSS2 is a serine protease that is also expressed on prostasomes (Chen *et al.* 2010). But while the expression of TMPRSS2 is often dysregulated during prostate cancer due to gene fusion, its function remains unclear.

Membrane vesicles from the prostate cancer cell line DU-145 have been shown to promote the development, invasion and tube formation of endothelial cells (Kim *et al.* 2002). This is remarkable primarily because prostasomes derived from the seminal fluid of healthy men have been described to conversely inhibit angiogenesis (Delves *et al.* 2005, 2006). This may indicate that malignant transformation could alter prostasome function with regard to the stimulation of neovascularisation.

To conclude, prostasomes from tumour cells are highly equipped to promote survival of malignantly transformed prostate epithelial cells and tumour progression by inhibiting complement-mediated attack but stimulating invasion into extracellular matrices and neovascularisation. Besides an apparent role in the development and survival of prostate cancer cells, prostasomes from malignant prostate epithelial cells may also serve as a biomarker for this disease (Duijvesz *et al.* 2011). New biomarkers for prostate cancer would be welcomed because blood serum levels of the currently used and best known prostate cancer marker, PSA, do not always correlate well with the aggressiveness of the malignancy and therefore the prognosis of the

condition (Shariat *et al.* 2011). Qualitative and quantitative analysis of the protein, lipid and/or nucleic acid content of prostasomes may be indicative of prostatic abnormalities. These prostasomes may be isolated from urine or from blood. As the blood of healthy men is normally devoid of prostasomes, secretion of prostasomes into the blood is in itself indicative of pathology. Although EVs from other cell types are present in abundance in the blood, prostasomes could be distinguished using prostate-specific biomarkers. Such markers may be constitutively present in prostasomes from healthy tissue or specific to malignantly transformed prostate epithelial cells. Potential constitutive prostasome-based biomarkers include proteins and RNA. A recent development is a proximity ligation assay, which is based on the simultaneous recognition of four different epitopes by antibodies (Tavoosidana *et al.* 2011). The antibodies are conjugated to cDNA oligonucleotides, which can hybridise only when the antibodies are in close proximity. Hybridised oligonucleotides can be amplified by a qPCR, thereby allowing specific detection of small quantities of prostasomes (≥ 1 ng/ml) in patient blood.

Conclusions and future perspectives

Prostasomes have several special features that characterise them as multifunctional EVs. Prostasomes probably perform these functions within the female reproductive tract by local modulation of the immune system and by associating with sperm cells that find themselves in conditions capable of inducing capacitation, but long before they have encountered the oocyte. The precise physiological roles of distinct classes of prostasomes needs to be resolved and differentiated further, particularly with respect to sperm cell and immune cell modulating functions. In addition, prostasomes are extremely promising candidates as biomarkers (or biomarker carriers) for the diagnosis and/or in prognostication of prostate cancer; this potential needs to be explored further.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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