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## Roles of the reproductive tract in modifications of the sperm membrane surface

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**Abstract.** Successful fertilization requires viable and functional spermatozoa to recognize and fuse with the oocyte. In most mammalian species, mature spermatozoa are not capable of fertilizing the oocytes immediately after ejaculation. However, unlike somatic cells, spermatozoa, after leaving the testis, are transcriptionally and translationally silent; therefore, upon completion of spermiogenesis, spermatozoa carry only a minimal amount of essential proteins on their membranes as well as within their restricted volume of cytoplasm. To develop into a fully functional and competent sperm that is capable of successful fertilization, modifications of the sperm membrane surface during its transit in the reproductive tracts is critical. These post-spermatogenesis modifications advance the maturation of epididymal spermatozoa. In addition, components secreted into the lumen of the reproductive tracts that are later added onto the sperm membrane surface also regulate (inhibit or activate) the functions of the spermatozoa. This acquisition of additional proteins from the reproductive tracts may compensate for the inactivity of morphologically mature spermatozoa. In this review, we discuss the contributions of the male and female genital tracts to modifications of the sperm membrane surface at different stages of fertilization.

**Key words:** Fertilization, Membrane modification, Reproductive tract, Sperm

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**M**ammalian fertilization is a complex process consisting of sequential events including interactions between spermatozoa and the epithelium of the genital tracts, the activation of sperm forward motility, and most importantly, the direct contact of spermatozoa with the cumulus oocyte complexes (COCs) [1, 2]. Successful completion of all the above-mentioned events allows the sperm to fuse with the oocyte and the ultimate conversion of two individual gametes into a single embryo capable of full-term development. The general consensus is that freshly ejaculated spermatozoa cannot fertilize the oocyte, and only become functionally mature and fertilize the oocyte after maturation modifications are accomplished especially at the sperm head membrane surface [3]. As soon as the spermatozoa are released from the seminiferous tubules, the sperm membrane surface is subjected to continuous remodeling upon their transit in the epididymis, the uterus, and the oviduct [4]. During this transit,

spermatozoa undergo morphological (lose cytoplasmic droplets in most species) [5], physical (acquire progressive motility), and biochemical (alterations in protein and lipid profiles) changes. In addition to these changes, the activation of downstream signaling cascades is essential for successful fertilization [6]. Membrane surface reorganization continues until the spermatozoa reach the ampulla of the oviduct, where they meet the COCs. Prior to the fusion of the sperm with the oolemma (the oocyte's plasma membrane), selective processes favor the functionally mature and intact spermatozoa to optimally interact with the COCs [3]. Specifically, these selective interactions between the sperm and oviduct epithelium, sperm and cumulus cells, sperm and zona pellucida (ZP), and even spermatozoa and the fluids from the female reproductive tracts contribute to sperm surface remodeling and facilitate the fertilization of the oocyte by the first penetrating sperm cell [1, 2, 7]. In this review, we discuss protein dynamics at the membrane surface of the sperm head and point out crucial events that occur upon the transits of the sperms in both the male and the female genital tracts.

### Modifications of the Sperm Membrane Surface by the Male Reproductive Tract

The epididymis is located in between the testis, which is the

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sperm-producing organ, and the ductus deferens, the sperm ejaculatory tract, and is the primary site for the maturation and storage of the spermatozoa. Fully differentiated testicular sperm, once released into the convoluted epididymal tubule, constantly interact with the epididymal epithelium and its secretions. Based on histology, the epididymis can be divided into three major segments, the caput (head), corpus (body), and cauda (tail), and each compartmentalized segment carries out distinct functions during epididymal maturation of the sperm cells [8]. Inter-cellular communications between different types of epididymal epithelial cells or interactions between the sperm and the epididymal epithelium can alter the luminal microenvironment by modulating the activities of specific ion channels (e.g. via V-ATPase or Ca<sup>2+</sup>-ATPase) [9, 10]. Moreover, different gene expression profiles in the adjacent epididymal epithelial cells also account for the region-specific luminal environment and segmental-specific protein and secretome profiles of the epididymis [8, 11, 12]. These systemic differences, along with hormone oscillations, further adjust the luminal pH, ions, or small molecule concentrations and affect the uptake of luminal components by the spermatozoa. Secretions from the caput region of the epididymis contribute to 70–80% of the total epididymal proteins, and this large pool of proteins and molecules likely acts as an additional fertilization-relevant component and is designed to be delivered onto the sperm membrane surface by small vesicle-like structures called epididymosomes. Secretions from the caput region of the epididymis contribute 70–80% of the total epididymal proteins, and this large pool of proteins and molecules likely act as additional fertilization-relevant components and are designed to be delivered onto sperm membrane surface by small vesicle-like structure named epididymosomes. The concept of epididymosomes was revealed by Yanagimachi *et al.* in the late 1990s [13]; however, the exact mechanism underlying the transfer of proteins onto the surface of spermatozoa by the epididymosomes is not fully understood, and the functional significance of epididymosomes in fertilization is thus yet to be confirmed.

However, previously, researchers have proposed a hypothetical mechanism called “apical blebbing”, wherein these blebbing vesicles (mostly ranging from 90–300 nm) from the epididymal epithelium contain proteins (epididymal sperm binding protein 1, interleukins, matrix metalloprotease domain 7) and mRNAs originating from the male genital tract, which later attach to the spermatozoa via the fusion of these vesicles with the sperm membrane surface [14, 15]. Although this membrane shedding process has been observed in many organs (kidney, intestine, and uterus) including the epididymis [14, 15], the regulatory mechanism and the players involved in this process remain unclear. The other hypothesis follows a common secretory pathway in which the epididymal epithelial cells produce multivesicular bodies and release their contents into the lumen; however, how these luminal proteins are transported to the spermatozoa is not clear. Based on the current available information, material exchange between the spermatozoa and the epididymal epithelium is most likely carried out by apocrine secretion considering that membrane-bound vesicles have been observed at the stereocilia and the apical membrane surface of the epididymal principal cells [16]. Further supporting this notion is evidence indicating that secretory proteins originating from the testes or epididymis, such as CRISPs (sperm-associated cysteine-rich secretory proteins), that

are involved in sperm-ZP adhesion, sperm-oolemma binding, and fertilization can be found on the surface of sperm cells [17–21]. Since spermatozoa lose almost the entire cytoplasm during spermiogenesis [22], the subtle exchange of materials between the epididymosome and the sperm or the fusion of the epididymosome with the sperm membrane surface possible greatly compensates for the synthetically inactive property of a fully differentiated spermatozoa and provides relevant proteins that are required for fertilization. Recently, using high-resolution helium ion microscopy, Păunescu *et al.* revealed an association between an epididymosome-like structure (~100 nm) and the spermatozoa embedded within epididymal epithelium in mice [23]. The heterogeneous population of these membrane vesicles is likely due to the difference in their protein and lipid compositions; these vesicles aim to transport various essential proteins and lipids to the sperm membrane surface and modify its compositions in a step-wise and segment-specific manner.

After the spermatozoa are liberated from the ductus deferens, spatial and temporal modifications of the sperm head membrane surface continue with re- and de-coating events induced by the male accessory fluid called the seminal plasma. Seminal plasma is a mixture of various components originating from male accessory glands (70% from the seminal vesicle and 30% from the testis, epididymis, prostate, and ductus deferens). It contains proteins, lipids, amino acids, fructose, other carbohydrates, etc. and is known to provide nutrients and metabolites essential for sperm motility. Proteins from the seminal plasma modify the sperm membrane surface, likely acting as “decapacitation” (or “inactivation”) factors that inhibit the spermatozoa from undergoing immature or spontaneous acrosome reaction (AR), and thus prevent unwanted stickiness or aggregation between the sperm cells or the fusion of the spermatozoa with other components of the reproductive tracts [24, 25]. Moreover, the seminal plasma can serve to stabilize semen pH and osmolarity, which are essential for sperm motility [26, 27]. Removal of the seminal vesicle markedly decreases the fertility rate in mice [28, 29]. This is largely due to the lack of seminal vesicle proteins (heat-labile phospholipid-binding protein, SVS VII, and carcinoembryonic antigen protein, CEACAM10), which results in a decrease in sperm motility [30, 31] or premature capacitation before the spermatozoa reach the oocyte [32–35]. In humans, the protein compositions of the seminal plasma in azoospermic patients is very different from that in healthy males [36]. It is thus clear that the protein profile of the seminal plasma is highly correlated to fertility in humans; as an example, a seminal plasma protein called secretory actin-binding protein (SABP) is highly expressed on the surface of the sperm from subfertile males and has been used as a marker protein for the evaluation of male fertility [37]. Several seminal plasma-specific proteins have been identified, and their importance in sperm physiology has also been addressed [26]. For example, seminal plasma protein profiles have been correlated with sperm and semen quality in adult boars [38], and post-thawing application or pre-freezing exposure of spermatozoa to the seminal plasma improves sperm quality, motility, and cryo-survival, eventually improving the fertilization ability of the sperm cells [39]. It is therefore clear at this point, that epididymal maturation is essential for successful fertilization, and that components from the male genital tract can serve as indicators for the evaluation of male fertility and can modify the protein profiles of the sperm membrane

surface, which provides an alternative opportunity to regulate the function and fertilizing ability of the sperm.

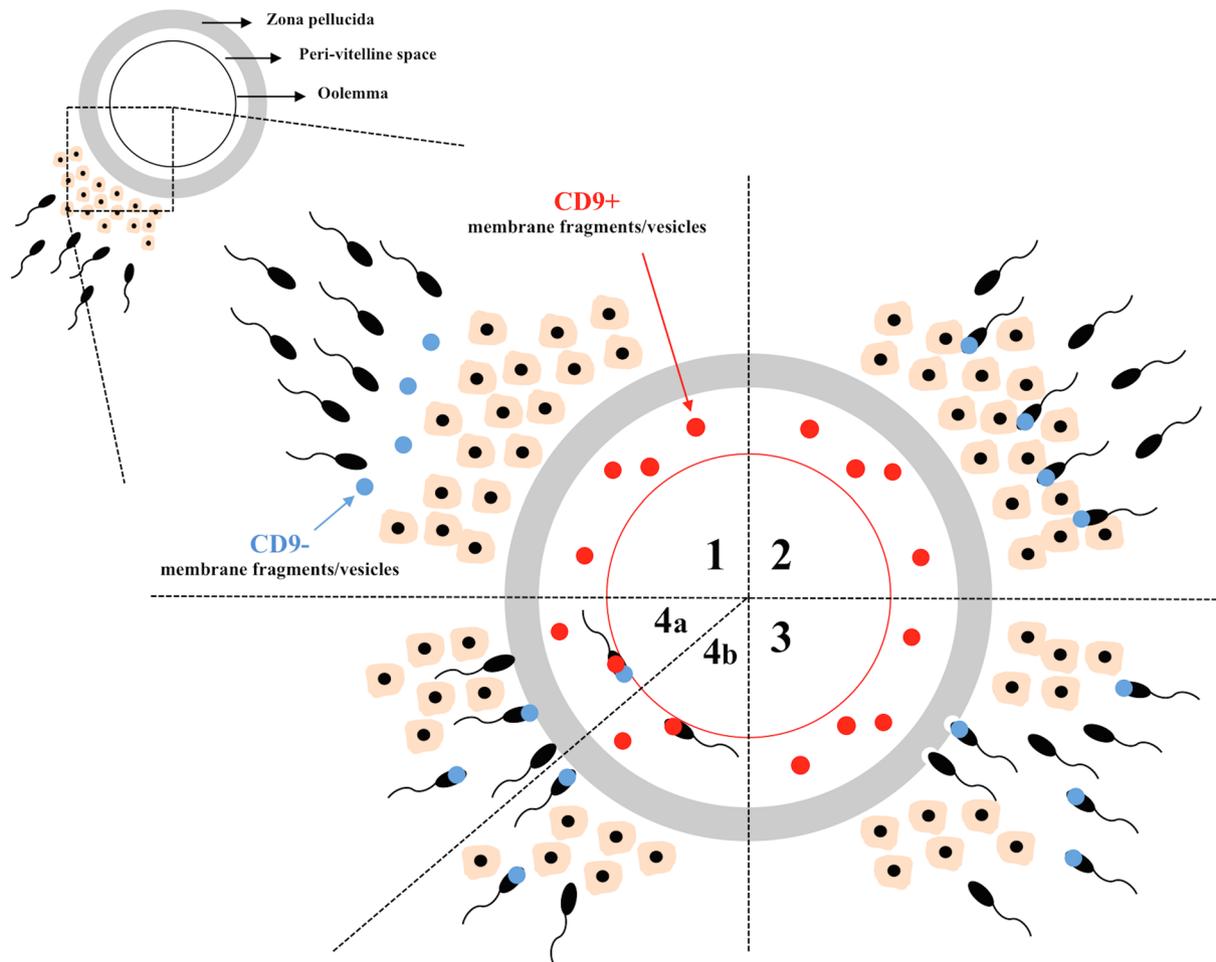
### Modifications of the Sperm Membrane Surface by the Female Reproductive Tract

After the spermatozoa enter the female reproductive tract, a direct contact with the oviduct epithelium, oviduct mucus, and the follicular fluid that surrounds the ZP further extends the surface modification process on the sperm membrane [4, 40–42]. However, compared to sperm surface modification by male factors, sperm modification by the female genital tracts is far less studied. Spermatozoa reside for hours to days in the cervix, uterus, and eventually the isthmic segment of the oviduct and await ovulation [43, 44]. The oviduct serves as a sperm reservoir and provides a proper oviductal environment to support sperm viability [42, 45]. When the spermatozoa reach the oviduct, interactions between the sperm cells and the epithelial cells of the oviduct regulate sperm capacitation status [46]. Yeste *et al.* and Moein-Vaziri *et al.* showed that when epithelial cells in the oviduct are co-cultured with viable spermatozoa, the gene expression profile of these epithelial cells is altered. These changes coincide with the restoration of sperm membrane integrity and fluidity that are essential for sperm capacitation [47, 48]. Based on morphology, the oviduct can be subdivided into four segments, the uterotubulo junction, isthmus, ampulla, and infundibulum (from the uterus toward the ovary); each segment contains similar sorts of cells, but in different proportions. The columnar non-ciliated secretory cells represent the population that actively synthesize and secrete proteins as they show stage-wise (according to estrus cycle) and segment-dependent variations in the amount and the size of secretory granules on the membrane surface [49, 50]. Recently, Leemans *et al.* demonstrated that oviduct-bound stallion spermatozoa showed a time-dependent protein tyrosine phosphorylation response with a gradual increase in intracellular pH, which was not observed in oviduct-unbound sperm cells [51]. The appearance of large alkaline vesicles in the oviduct epithelium towards which the sperm progresses suggests that the interaction between the sperm and the oviduct epithelium may facilitate sperm capacitation [51].

The oviduct fluid is equally important to modulate the functions of spermatozoa and gamete interactions. The oviduct secretions contain various proteins that can be added onto the sperm surface when the spermatozoa reside in the oviduct [40, 52]. Some of them such as  $\gamma$ -aminobutyric acid [GABA] [53] suppress sperm capacitation or premature the AR (e.g. follicular fluid glycodelin-F [54]), and some others promote the interaction between the spermatozoa and oviduct epithelium (e.g. sperm binding glycoprotein [SBG] [55]) and sperm capacitation and subsequent AR (e.g. bicarbonate, free calcium ions, and lipoproteins such as albumin [49, 56, 57]). The higher concentration of bicarbonates and albumin present in the oviduct fluid likely work together to orchestrate the removal of cholesterol from the sperm membrane surface and the activation of the cAMP-mediated signaling pathway [58]. Interestingly, a recently proposed hypothetical model on cholesterol removal from the raft and non-raft membrane domains states that treatment with a lower concentration ( $< 5$  mM) of methyl- $\beta$ -cyclodextrin (MBCD) leads to cholesterol removal from the raft area without affecting

the composition and amount of the phospholipids, while a higher concentration ( $> 10$  mM) of MBCD causes overall lipid depletion and disruption of the membrane raft [59]. Therefore, albumin, a less stringent cholesterol acceptor (when compared with MBCD) present in the female reproductive tract, may serve to remove cholesterol mostly from the non-raft area in the presence of bicarbonates, thus increasing membrane fluidity and allowing free reorganization of capacitation and causing AR relevant proteins to be recruited into the raft membrane domains. Moreover, the late maturation and post-ovulatory modifications of oviduct components such as changes in  $[Ca^{2+}]$  and hormone concentrations can influence the binding properties of the spermatozoa and further regulate the onset of capacitation and AR [56, 60–63]. Oviduct-specific glycoproteins have also been shown to modulate sperm-ZP interaction and to reduce polyspermic fertilization rates in pigs [62, 64]. Interplays between glycoproteins in the sperm membrane surface, oviduct epithelia, and oviduct fluid probably orchestrate sperm activation just around the time of ovulation [65, 66]. This is supported by observations that oviduct-specific glycoproteins (OSG) and osteopontin present in the oviductal fluid can induce sperm capacitation in pigs [55]; the follicular fluid from humans can also induce AR and enhance sperm-oocyte interaction *in vitro* [41, 67]. Interestingly, murine uterine fluids have been shown to contain small membrane vesicles [68], and these particles called uterosomes contain the sperm adhesion molecule SPAM-1 and other GPI-linked proteins that can be exchanged with the spermatozoa [68]. It is likely that these material exchanges between the spermatozoa and the female reproductive tract not only take place at different regions of the oviduct, but also occur in the uterus in some species. Recently, England *et al.* presented evidence indicating that, in canine species, instead of the oviduct, uterine epithelial cells close to the utero-tubular junction can respond to semen deposition and seem to be the sperm reservoir [69, 70]. It is reasonable to believe that components secreted by different regions of the female genital tract can be added onto the surface of the sperm in the uterus or the oviduct, which can enhance the sperm-ZP binding ability. It is therefore possible that ZP itself, besides being a binding target for spermatozoa, can also deliver proteins onto the sperm surface upon sperm-ZP interaction.

Further, sperm surface modification does not cease even when the spermatozoa reach the perivitelline space. Membrane fragments can still be transferred from the oocytes onto the spermatozoa via trogocytosis [71] or through exosomes [72] at this stage of fertilization. Membrane fragments containing CD9, which is essential for sperm-egg fusion, are added onto the sperm surface within the perivitelline space (Fig. 1). The transfer of functional tethering and fusion proteins onto the surface of the spermatozoa would facilitate the entry of the first incoming sperm cell into the perivitelline space for successful fertilization [71, 73]. Intriguingly, Barraud-Lange *et al.* recently showed that the membrane shedding process could be carried out without the presence of the CD9 molecule [74]. It is possible that two distinct populations of oocyte-originated membrane vesicles (CD9-positive and CD9-negative) are secreted by COCs (as proposed in Fig. 1). Therefore, it is reasonable to speculate that the capture of oocyte-originated membrane fragments by the spermatozoa may serve multiple purposes: (1) to increase of the ability of the sperm to fuse with the oocyte (when acquiring CD9-positive membrane



**Fig. 1.** Hypothetical model regarding membrane-shedding process from the COCs. Membrane shedding (either via exosomes or by trogocytosis) from the COCs serves as a material exchange mechanism between the COCs and the spermatozoa and is a continuous but CD9-independent process. (1) The shed materials can be either CD9-positive membrane vesicles (in red, likely can only be derived from the oolemma) or CD9-negative membrane vesicles (in blue, can be derived from parts of the COCs other than the oolemma). (2) When the spermatozoa come in contact with these membrane vesicles, the sperm membrane surface may undergo further remodeling that will favor the subsequent acrosome reaction or interactions of the sperm with the cumulus cells and the intercellular matrix. (3) Processes from step 2 serve to create a path for the binding of other spermatozoa with and for their recognition of the ZP, which this might be independent of whether the sperm cell captures shed materials from the COCs in step 2. (4) Fusion between the sperm cell and the oolemma is regulated by the presence of oolemma-derived CD9. Only when the sperm cell captures CD9-positive vesicles/membrane fragments shed from the oolemma into the perivitelline (PVS) space, can it further undergo fusion with the oocyte, and this 2<sup>nd</sup> capture of materials in the PVS might be independent of whether (4a) or not (4b) the sperm cell captured CD9-negative material before its entry into the PVS.

fragments released from the oolemma), (2) to further modify the surface of the spermatozoa to facilitate functions that may not be directly needed for fertilization, but might be essential for other yet to be identified functions (after acquiring CD9-negative membrane fragments released from parts of the female reproductive tract other than the oolemma). A hypothetical model regarding the membrane shedding process from the COCs is illustrated in Fig. 1.

### Initiation of Sperm AR and the Importance of Cumulus Cells

COCs are ovulated and released together as one structure into

the oviduct and then impregnated with follicular fluid. Remnants of this fluid remain attached to the COCs when the sperm cell is fertilizing the oocyte. Before the spermatozoa reach the ZP of the oocyte, they encounter layers of cumulus cells and their extracellular matrix (ECM)-enriched intercellular space. The classical theory on ZP-induced sperm AR was based on various important *in vitro* observations in the past decades [75–83]. However, this concept has been challenged recently by the use of genetically modified animals in combination with advanced imaging system. Dean's group suggested that sperm-oocyte recognition depends on the cleavage status of ZP2, but the binding at the surface of the ZP is not sufficient to induce sperm acrosome exocytosis in mice [84, 85]. The study of Jin *et al.*

further supported this idea by showing that most fertilizing mouse spermatozoa begin their AR prior to their contact with the ZP of the oocyte [86]. Interestingly, Okabe's group demonstrated that some of the zona-binding sperm cells had an intact acrosome and did not undergo AR after their binding to the ZP of the oocyte [87, 88]. These data suggest that at least in mice, besides the ZP, multicellular layers of the cumulus cells could also be a site for sperm AR. Indeed, defects in the extracellular matrix between the cumulus cells have been shown to reduce fertility rate [89, 90], which indicates the importance of cumulus cells and their intercellular components for fertilization. Moreover, in rodent species, fertilizing spermatozoa mostly initiate their AR in the cumulus mass, and those spermatozoa that initiate their AR at the ZP can rarely fertilize the oocyte [86]. These recent evidences gradually reduce the exclusionary importance of the ZP in the induction of acrosome exocytosis and suggest that sperm-oocyte interactions and AR induction are far more complicated than we expect. The long thought-to-be important factors such as the ZP or the glycosylation or cleavage status of ZP proteins may not be the only elements required to induce AR. Thus, the concept of "zona-induced AR" should be further revised after taking other non-ZP factors such as the cumulus cells into account. One important aspect that should also be considered is the fact that the widely used murine system could be different from that of non-rodent mammals; therefore, it is worthwhile to validate the findings by using other animal models (e.g. pigs).

### Concluding Remarks

Fertilization is a complex process that is tightly regulated by factors from both the male and the female genital tracts. Active and constant membrane surface modifications during sperm transit in the epididymis and the oviduct ensure successful fertilization. Compelling evidences suggest the need for a review of the fertilization processes with additional attention on non-sperm and non-oocyte factors that may have been overlooked in the past (e.g. epididymosomes, seminal fluid components, uterosomes, oviductal fluids, and cumulus cells). Future studies may soon reveal the importance of these non-gamete factors on the regulation of fertility and may provide new opportunities to modulate (reduce or enhance) fertility by regulating the functions or activities of these factors.

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