Sperm Surface Proteomics

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Abstract

This contribution will focus exclusively on the total (global) protein composition (the proteome) of the sperm surface. Immune responses directed towards sperm surface proteins may cause infertility since functionally intact sperm are under immune attack. The immune attack can be achieved directly by deteriorating sperm or by antibody blocking of a sperm surface protein with a specific function in the fertilization process. Antibodies that bind to the sperm surface proteins could also impair the fertilization potential of sperm more indirectly by causing lateral redistribution of the sperm surface proteins and/or by hindrance of the assemblage of functional membrane protein complexes involved in fertilization. Currently the information about the sperm plasma membrane proteome is increasing but has only led to limited understanding of the functionality that is related to the complex ordering and processing of this specific cell surface. New proteomic data and new strategies designed for complete coverage of the surface proteome of mammalian sperm will significantly increase our understanding of how fertilization is accomplished but also how immune responses may frustrate this process. This information will become highly relevant for studying immune infertility. An overview is provided about the current knowledge of the sperm

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surface and how this structure should experimentally be approached for proteomic studies. Comparative analysis of different mammalian species is covered as this will provide better understanding for the possibilities and limitations of analyzing the surface proteome of human sperm.

3.1 Introduction

3.1.1 The Sperm Surface

The sperm is a highly polarized cell with a minimum of cytosol and organelles [54, 157]. The sperm head has two organelles namely the nucleus that houses the male haploid genome which is highly condensed to protamines, and a large secretory granule called the acrosome which is oriented over the anterior area of the sperm nucleus. At the distal part of the sperm head, the flagellum sprouts. In the mid-piece of this flagellum, mitochondria are spiraled around the microtubules of the flagellum. In the tail part, specific cytoskeletal elements are surrounding the microtubules of the flagellum. The surface of the sperm head, mid-piece, and the tail parts of the sperm is heterogeneous [66, 118] and reflects the polar distributed organelles that lie under the surface. The sperm head surface is particularly heterogeneous, and at least four different regions can be distinguished with separate functions in the fertilization process. In general the sperm has lost many somatic cell features and does not house an endoplasmic reticulum, Golgi, lysosomes, or peroxisomes and has lost ribosomes. Primarily due to this, the sperm has lost the potential for gene expression (both transcription as well as translation processes are completely silenced [27]). In fact due to this the sperm has lost the de novo protein synthesis capacity as well as vesicle endocytosis/exocytosis-mediated cycling of the sperm surface. Related to this, the endogenous sperm surface proteins (that is, those not added postspermiation) are prone to very complex posttranslational modifications, which normally would be eliminated and are a challenge for proteomic detection [10]. The sperm has also lost almost the entire cytoplasm. The cell has a typical ordering of the remaining organelles and cytoskeletal elements, and probably this polar ordering is reflected into the lateral domained ordering of the sperm surface [67].

3.1.2 Function of Sperm Membrane Domains at Fertilization

The subdomains of the sperm head area have particularly diversified functions in the series of processes that are involved in fertilization. The apical ridge area of the sperm head specifically recognizes and binds to the zona pellucida (the extracellular matrix of the oocyte) [151], and a larger area of the sperm head surface (the preequatorial domain) is involved in the acrosome reaction, which results in the release of acrosome components required for zona penetration [58, 157]. The equatorial segment of the sperm head remains intact after the acrosome reaction and is the

specific area that recognizes and fuses with the oolemma (the egg plasma membrane) in order to fertilize the oocyte [154]. Although the mid-piece and tail surface of the sperm cell are also heterogeneous, the function of these surface specializations is not yet understood [82]. It is quite possible that they are involved in organization of optimal sperm motility characteristics. When studying sperm surface proteins, the researcher has to keep in mind that a rather complex surface is under study, and especially for studying the processes of fertilization in which the sperm head surface is involved (zona binding, acrosome reaction, and fertilization), this specific surface area needs first to be separated and purified.

3.1.3 Sperm Surface Dynamics Before Fertilization

The domained surface of sperm is already apparent in spermatids before spermiation in the seminiferous tubules of the testis [54]. The molecular dynamics involved in the establishment of surface specialization upon spermatogenesis is largely unknown. Moreover, once liberated in the lumen of the seminiferous tubule, the sperm will start its travel through the male and female genital tract and will meet a sequence of different environments. During this voyage, surface remodeling takes place most likely at any site of the two genital tracts: (1) upon somatic maturation in the epididymis major changes in the sperm proteome are reported [2, 8, 11, 69, 92, 132], (2) by re- and decoating events induced by the accessory fluids combined at ejaculation probably stabilizing the sperm for its further journey in the female genital tract [74, 75, 77, 95], (3) after their deposition in the female genital tract which is followed with the removal of extracellular glycoprotein coating (release of decapacitation factors) and further remodeling by (cervical) uterine and oviduct secretions are activating the sperm to meet the oocyte (in vivo capacitation) [72, 87, 104, 123, 138, 161, 162], and (4) sperm also interacts with cumulus cells and remaining follicular fluid components surrounding and impregnating the zona pellucida [71, 73] and even in the perivitelline space (that is, the fluid filled space between the zona pellucida and the oolemma) with components [21, 22]. All these changing environments may cause surface remodeling to the sperm and thus may influence its potential to fertilize the oocyte [43].

The possible mechanisms of altering the sperm surface are reviewed earlier [62] and are schematically drawn in Fig. 3.1. Note that recently proteomic studies have elucidated the protein composition of extracellular vesicles/exosomes from diverse origin such as in the male genital tract secreted by the epididymis and prostate [53, 70, 139] and female genital tract secreted by the uterus, the oviduct, or even the oocyte perivitelline space [3, 21, 22, 37, 106]. It has been demonstrated that at least epididymosomes, but probably also the other extracellular vesicles/exosomes can deliver certain proteins to the sperm surface. Therefore, the exact role of extracellular vesicles/exosomes in sperm surface physiology will become more and more relevant. Beyond this it is likely that the redox balance at both the extracellular and the intracellular side of the sperm surface is also subjected to changes which will cause thiol changes, which may relate to altered folding and even complexing of proteins [10, 11, 34].

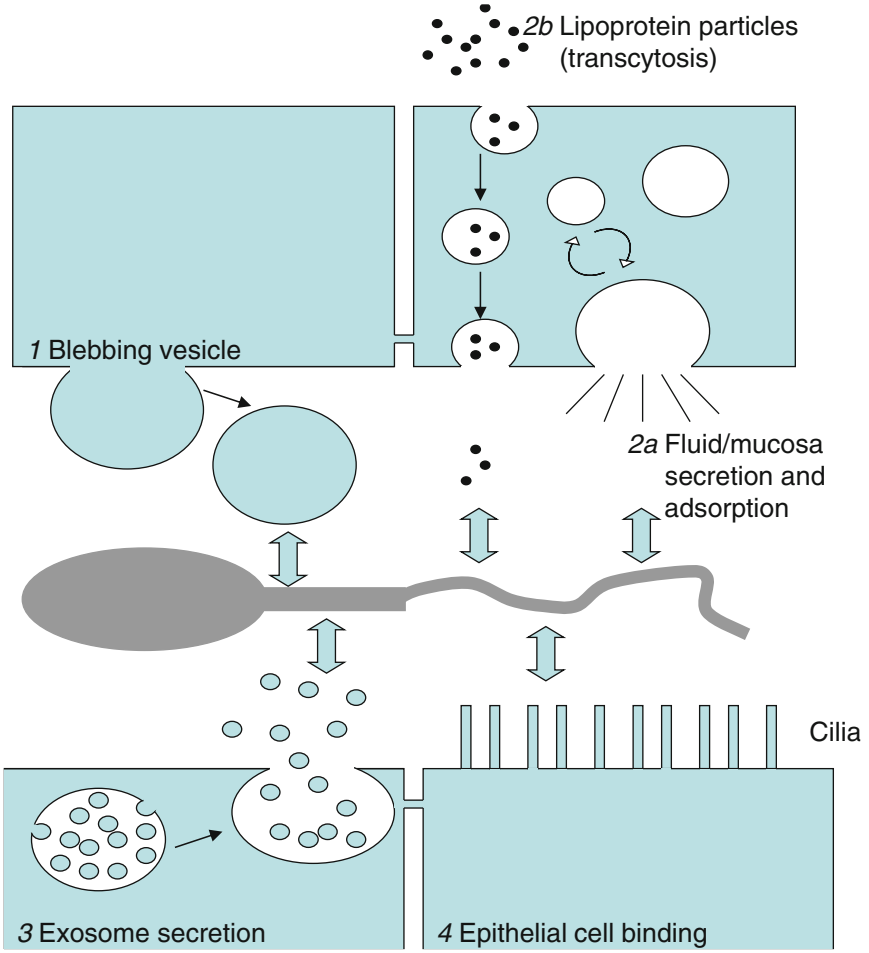


Fig. 3.1 Possible alterations at the surface of sperm due to somatic modifications in the lumen of the male and female genital tract. Possible interactions of male and female genital tract components with the sperm surface. (*1*) From the diverse epithelia of the male and female genital tract blebbing vesicles containing cytosol may be released into the genital fluids. Such vesicles may interact and exchange surface components with sperm. It is highly unlikely that such vesicles fuse with the sperm as this would dramatically increase the volume of sperm (which has been reduced to a minimum in order to obtain an ergonomically designed cell optimally suited for fertilization). (*2a*) Serum components can be released into the genital fluids by transcytosis [45]. Interestingly lipoprotein particles may invade the surrounding of sperm and may facilitate exchange of larger particles and the sperm surface. (*2b*) Fluid phase secretion and adsorption of either fluid or mucosa may directly alter the ECM of sperm. (*3*) Apocrine secretion of exosomes has been suggested to alter the sperm surface and sperm functioning. Exosomes have been demonstrated to be secreted by the epididymis (epididymosomes) and by the prostate (prostasomes) [72, 144] but likely are also secreted throughout the female genital tract. Interestingly exosomes may provide sperm with tetraspanins which are a group of membrane proteins involved in tethering of proteins into protein complexes. Recently the addition of CD9 onto the sperm surface by membrane particles has been described to occur even when sperm reaches the perivitelline space [21, 22]. (*4*) Sperm interacts with ciliated epithelial cells, which has been observed in the oviduct [135] and probably has a physiological role during in situ capacitation. Sperm interactions with other ciliated epithelial cells of the female and male genital tract have not been studied extensively. It is possible that such interactions are important for sperm surface remodeling and for sperm physiology (Adapted from Gadella [62])

It is very difficult to study the above-described sperm surface alterations in situ. However, for many mammalian species, including human, specific sperm handling and incubation media have been optimized for efficient in vitro fertilization purposes. In general mammalian sperm are activated in a medium that compares with the oviduct in that it contains the capacitation factors such as high concentrations of bicarbonate, free calcium ions, and lipoproteins such as albumin [58]. In some species specific glycoconjugates [105] or phosphodiesterase inhibitors are added for extra sperm activation [19]. All strategies are designed to evoke capacitation in vitro. This implies that the researcher can observe the relevant sperm surface reorganization primed under in vitro conditions for fertilization. The membrane composition as well as ordering of membrane components can be compared with control conditions (media without the capacitation factors) or with the membrane ordering of sperm at collection time. Sperm can be collected at ejaculation for human, boar, stallion, bull sperm but needs to be aspirated from the cauda epididymis for murine species (rat, mouse, guinea pig), which can also be the case under certain clinical conditions from male subfertile patients in the IVF clinic. In particular the surface reordering of membrane proteins and lipids in sperm head has been studied extensively under in vitro capacitation conditions (for reviews see [58, 64, 68, 95, 96]). It is important to stress the importance of the sperm surface reordering and changes in composition of membrane components by diverse extracellular factors. The induced lateral redistribution of membrane components appears to also be instrumental for the assembly of a functional sperm protein complex involved in sperm-zona binding as well as for the zona-induction of the acrosome reaction [1, 146–148, 151]. Therefore, the researcher interested in the surface proteome of sperm needs, beyond the composition of sperm surface proteins, to consider how these proteins are organized and whether they are functionally complexed for their physiological role in fertilization. In this light, it is also important to stress that sperm surface protein reordering can be imposed by processing semen for instance during density gradient washing, cryopreservation, or sex-sorting in a flow cytometer [63, 95, 153].

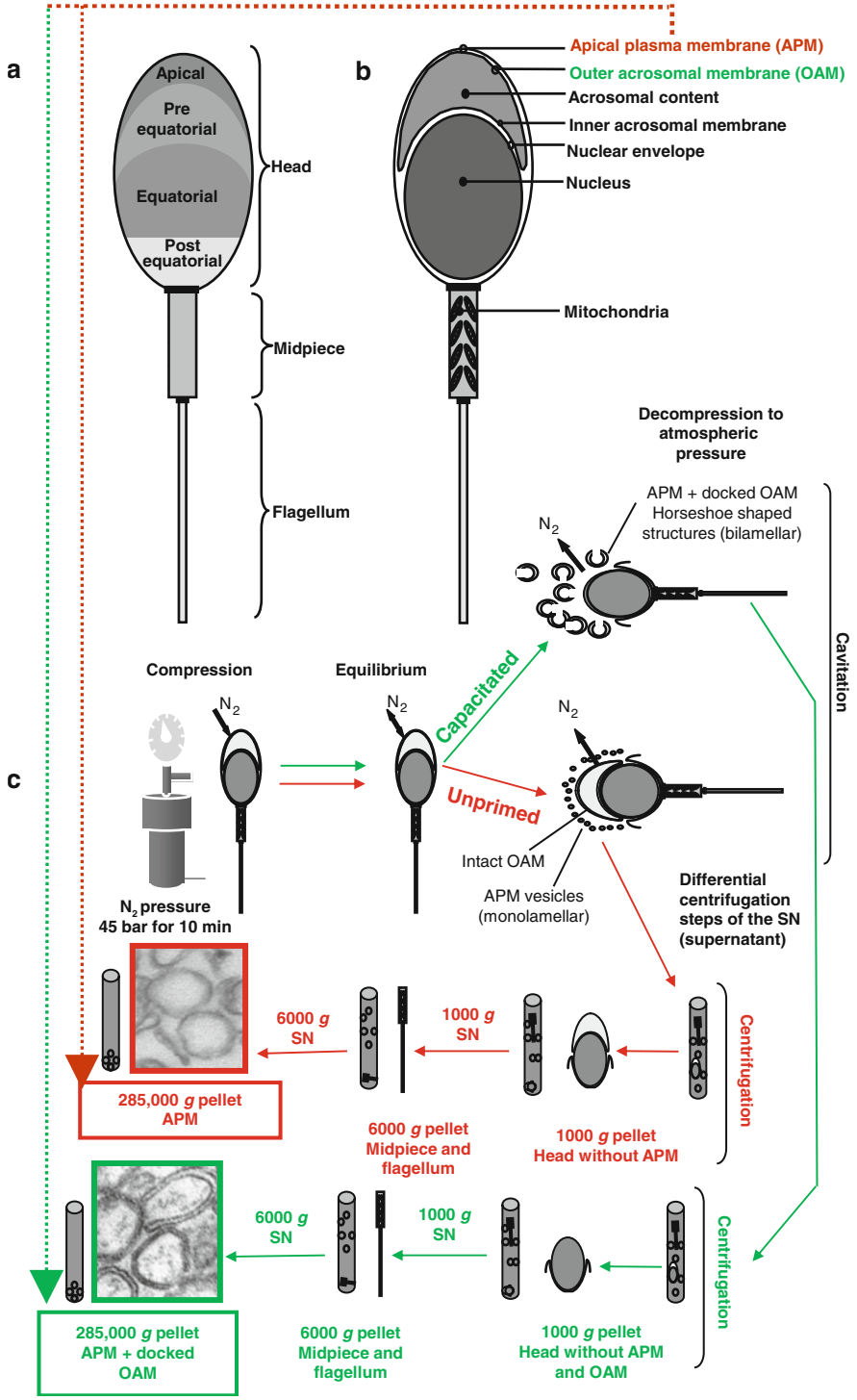
3.2 Isolation of Sperm Surface Proteins

Membrane proteins can be classified as integral membrane proteins and peripheral proteins. Most integral membrane proteins have an extracellular domain and a transmembrane domain (often an alpha helix region with the hydrophobic part exposed to the fatty acid moieties of the phospholipid bilayer). However, other integral membrane proteins interact by covalent lipid anchors such as glycosylphosphatidylinositol (GPI), acylation, and other modifications [52]. Peripheral proteins have electrostatic interactions with the integral membrane proteins or with the lipids of the membrane. Discrimination between these two types of membrane proteins can be done by treating membrane preparations with high salt which destabilizes the electrostatic interaction and results in the release of peripheral membrane proteins, while the integral membrane proteins remain in the insoluble membrane fraction. In general, to study the sperm surface proteins, one has to isolate the sperm membrane

from soluble proteins and insoluble nonmembrane material (such as cytoskeletal components and the condensed nuclear chromatin). Furthermore, researchers need to give particular attention to the indirect interactions of nonsurface material to the membrane extract.

To this end, specific sperm disruption methods such as ultrasonication and nitrogen cavitation (see Fig. 3.2) have been designed [59]. Sonication gives lower purification and less defined membrane fraction [17] although good results were obtained on bovine sperm [38]. After sperm disruption, differential centrifugation techniques need to be employed to isolate sperm membranes from insoluble cellular debris and soluble components. The researcher needs to consider that the disruption method as well as the isolation protocol is really delivering sperm plasma membrane or also intracellular membranes. This is especially relevant for studying proteins involved in zona recognition. When the plasma membrane preparation also contains acrosomal contamination, one can be sure that secondary (intraacrosomal) zona binding proteins will be identified and will possibly overwhelm the amount of primary (plasma membrane) zona binding proteins [60, 151]. To this end, the specific abundance of marker proteins or specific activities of marker enzymes of plasma membrane and intracellular membranes need to be quantified. The relative purification is indicative for the purity of the membrane fraction for surface proteins. In our hands, an optimized nitrogen cavitation method turned out to yield a 200 times enriched plasma membrane fraction over possible contaminating membranes with a yield of approximately 30 % of the sperm surface [59]. Moreover, ultrastructural analysis of this membrane fraction and of disrupted sperm showed that the isolated plasma membrane fraction contained resealed plasma membrane vesicles. The vesicles were so-called right-side outside unilamellar vesicles (see Fig. 3.2) implicating that the outer and inner side of the vesicle membranes had the same protein topology as in the intact plasma membrane of sperm and that the resealed plasma membrane vesicles have not encapsulated intracellular membranes.

Fig. 3.2 Subcellular fractionation of apical plasma membranes from boar sperm cells (Adapted from Brewis and Gadella [24]). (**a**) A schematic of the surface of the sperm cell showing the main subdomains in the head. The apical ridge specifically recognizes and binds to the zona pellucida, and a larger area of the preequatorial region is involved in the acrosome reaction. The equatorial segment of the sperm head remains intact after the acrosome reaction and is the specific area that recognizes and fuses with the oolemma in order to fertilize the oocyte. (**b**) A sectional view of the sperm cell. Note that all solid lines represent membrane bilayers. (**c**) Procedure to isolate apical plasma membranes (*APM*) from unprimed boar sperm cells (*red arrows*) using nitrogen cavitation and differential centrifugation. This results in a 200 times enriched apical plasma membrane fraction with the outer acrosomal membrane (*OAM*) remaining intact and represents an exceptional resource for further understanding zona binding and the acrosome reaction. Note that boar sperm capacitation (see *green arrows*) leads to acrosome docking which as a result leads to the isolation of APM and the outer acrosomal membrane (*OAM*) (see also [147]). As mentioned in the text, this phenomenon should be carefully considered as an artifact when interpreting proteomics data on sperm surface proteins of capacitated versus noncapacitated sperm samples)


a
Apical
Pre equatorial
Equatorial
Post equatorial
Head
Midpiece
Flagellum
b
Apical plasma membrane (APM)
Outer acrosomal membrane (OAM)
Acrosomal content
Inner acrosomal membrane
Nuclear envelope
Nucleus
Mitochondria
c
Decompression to atmospheric pressure
APM + docked OAM
Horseshoe shaped structures (bilamellar)
N2
Cavitation
Compression
Equilibrium
Capacitated
Unprimed
Intact OAM
APM vesicles (monolamellar)
N2 pressure
45 bar for 10 min
Differential centrifugation steps of the SN (supernatant)
6000 g SN
1000 g SN
Centrifugation
285,000 g pellet APM
6000 g pellet Midpiece and flagellum
1000 g pellet Head without APM
285,000 g pellet APM + docked OAM
6000 g pellet Midpiece and flagellum
1000 g pellet Head without APM and OAM

This membrane preparation turned out to be instrumental to study protein-protein interactions relevant for sperm-zona binding [151] and for the redistribution of membrane microdomains believed to represent lipid rafts [152]. Interestingly, multiple proteins known from the literature as being zona binding protein candidates were identified by proteomics on the isolated boar apical plasma membrane preparations [151] which were extremely enriched in the DRM fraction of sperm. Most notable were fertilin beta, P47, carbonyl reductase, and the sperm adhesion AQN3. Interestingly, proteomics revealed a possible role of chaperone proteins in formation of functional protein complexes involved in zona binding [35, 36, 122], which will be discussed later. The folding and grouping of sperm surface proteins is relevant for the observed capacitation-induced redistribution of sperm surface proteins. This phenomenon also allows the plasma membrane to firmly dock to the outer acrosomal membrane exactly at the area where the characteristic lipid ordered microdomains were clustering. A trans trimeric SNARE complex (containing VAMP, syntaxin, and SNAP proteins) was formed at multiple sites of the apical sperm surface and was stabilized in the trans configuration by complexin [148]. In fact the isolation of the vesicles that were formed after a calcium-ionophore-induced acrosome reaction showed the declipping of complexin and the trans- to cis-configuration of the trimeric SNARE protein complex which coincided with the hybird vesicles formed after multipoint fusions between the apical plasma membrane and the docked outer acrosomal membrane [148].

Note that the isolated hybrid vesicles are an interesting source for proteomic analysis with a number of interesting proteins identified such as synaptotagmin-4 involved in cis-configuration of the trimeric SNARE complex [148] but also of a number of acrosome-specific and surface-specific proteins involved in zona binding zona penetration. The identified proteins that were not observed in the apical plasma membrane preparations derived from noncapacitated sperm cells are noteworthy. On membranes isolated from capacitated sperm, the emergence of spermatid-specific heat shock protein 70 and arylsulfatase A as well as the acrosomal proteins acrosin, acrosin inhibitor, acrosomal vesicle protein 1, IAM 38, SP10 was reported [148], which may all derive from the acrosomal membrane. Thus, the possibility that membrane preparations from capacitated or acrosome reacted sperm samples contain larger proportions of acrosomal membrane proteins should be checked with robust ultrastructural techniques such as those shown in Fig. 3.2. In general we believe it is of crucial importance to take care on interpretations of changes in the capacitation sperm surface as isolation nitrogen cavitation (Fig. 3.2) or detergent resistance membrane fractions (Fig. 3.3) or any other membrane isolation method will likely result in a more substantial co-isolation of the docked outer acrosomal membrane [147, 148, 150]. In this light manuscripts, describing the emergence of proteins at the capacitating sperm surface [12, 35, 36, 48, 88, 110, 122, 143, 147, 160] should be interpreted with care.

Human sperm surface preparations are usually made after a hypo-osmotic incubation followed by sonication and differential centrifugation (see for instance [30]). The purity of such membrane preparations for sperm plasma membrane material is not well documented, and contamination with intracellular membranes is likely.

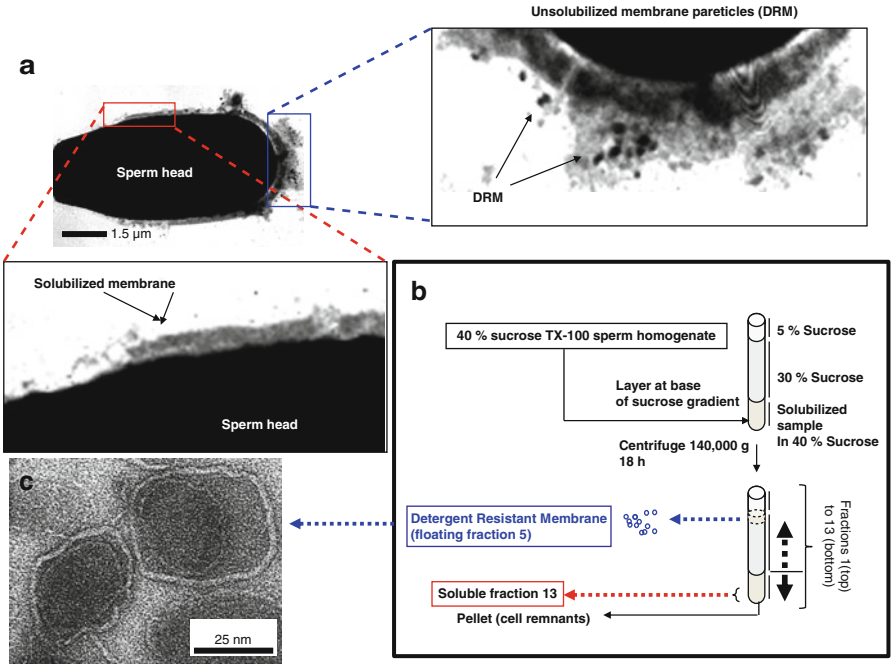


Fig. 3.3 Procedure to isolate the detergent-resistant membrane fraction (DRM) from porcine sperm cells. (**a**) The *upper left panel* shows the sperm cells head imaged by transmission electron microscopy. The *boxes* in this panel refer to either solubilized membrane (*lower zoom panel*) or nonsolubilized membrane (*upper right panel*). (**b**) Separation of the solubilized or the DRM fraction is achieved by layering the solubilized sperm sample in Triton X-100 in a final concentration of 40 % sucrose; on top of this a layer of 30 % sucrose is added and the last layer consists of 5 % sucrose. After centrifugation for 18 h at 140,000 g, the DRM is present in the interface between 5 and 30 % sucrose (Adapted and extended from introduction chapter of the PhD thesis of Dr. A. Boerke [26]). (**c**) Note that it is likely that due to stable acrosome docking the DRMs isolated from capacitated sperm contain more acrosomal material when compared to DRMs isolated from unprimed sperm samples. In fact a negative staining micrograph of a DRM fraction 5 isolated from capacitated sperm cells shows beyond the multilamellar outside membrane (cf. what was observed for DRMs isolated from unprimed sperm and on DRMs derived from two epithelial cell lines [150]) also the inclusion of an additional unilamellar membrane

Note also that in contrast to, for instance, porcine semen (typically with > 95 % life and fully matured normal morphology and motility spermatozoa), human semen is of much poorer quality with a large number of defective and contaminating cells. The high sperm surface purification by a factor in the hundreds is not to be expected for human semen samples under any conditions.

Another method to isolate surface proteins is to make use of lectins immobilized to beads. Lectins can bind to specific sugar residues at the extracellular domain of integral membrane proteins. Some marker lectins exclusively bind to the sperm plasma membrane. Therefore, affinity chromatography using immobilized lectins can be used to extract surface proteins [124]. A comprehensive profiling of

accessible sperm surface glycans using a lectin microarray has been described recently [156]. These methods can also be employed on nitrogen cavitated and solubilized sperm plasma membranes. Noteworthy is also the phenomenon of (de-)glycosylation which takes place on sperm surface proteins during sperm maturation ejaculatory transport and in the female genital tract [42].

Finally membrane raft isolation procedures can be employed to isolate microdomains from sperm (see Fig. 3.3 [6, 28]). Most methods use detergents at low temperature (4 °C) to isolate the detergent-resistant membrane fraction. Our group has identified that this DRM fraction after capacitation becomes highly enriched in GPI anchored proteins and in proteins involved in zona binding and the acrosome reaction [79, 146, 149]. With the use of phosphatidylinositol-specific phospholipase C GPI anchored proteins can be cleaved of the DRM (enriched in these proteins) or in untreated sperm [29, 79]. Possibly the treatment of sperm with such lipases may result in the liberation of a very specific subclass of integral sperm plasma membrane proteins, and clearly such proteins play an important role in capacitation-specific membrane surface alterations related to sperm-zona binding [151, 152] as well as the induction of the acrosome reaction [146–148, 158]. We have data that DRM from entire sperm contains intercellular (acrosomal) membrane material beyond the surface membrane material [26, 29, 63, 148]. The DRM fraction of whole sperm contains components that could be labeled with marker lectins for the outer acrosomal membrane. DRMs from purified plasma membranes did not show any labeling with this lectin. The best explanation for these results is that the outer acrosomal membrane also contains lipid rafts, which may explain the results of [35, 36, 111, 114] or that this membrane is stably docked to the capacitating raft aggregating sperm surface as discussed earlier [147]. Ultrastructural studies on the DRM fraction indeed showed that this insoluble membrane fraction appears as multilamellar membrane vesicles [150] and that DRMs derived from capacitated sperm show additional mono-lamellar membrane inclusions (see Fig. 3.3c).

As stipulated above, in general we advise any researcher working on sperm surface-specific proteins to have appropriate ultrastructural controls regardless of what type of membrane isolation technique is used. This will enable the exclusion of intracellular membranes especially when working on surface protein changes that may occur during sperm capacitation as this coincides with multiple synaptic docking of the outer acrosomal membrane (see also Fig. 3.2).

3.3 Detection of Sperm Surface Proteins

3.3.1 Tagging of Sperm Proteins and Peptides

Other chapters in this book describe a number of protein separation and mass spectrometric techniques mentioned that are key or of relevance for detecting and identifying amino acid sequences of peptides and proteins of sperm samples [101, 127]. The most popular present day proteomics approaches are summarized in Fig. 3.4,

and these and other approaches are reviewed more extensively [23]. Here, we will focus on strategies for the proteomics analysis of surface sperm-specific proteins. First of all it is important to clarify that a number of proteomics protocols studying differential expression of proteins in biological specimens under experimentally manipulated conditions are not possible with sperm. Specifically those techniques that make use of the fact that cells are fed with amino acids that are used for translation are not possible in sperm as sperm are transcriptionally and translationally silent (the translational machinery has shut down in the last phase of spermatogenesis) [27]. The most common approach involves control cells cultured with normal amino acids, while the experimental conditioned cells are fed with stable isotope label tags (SILAC; stable isotope labeling by amino acids in culture) and uses labeled hydrogen, carbon, or nitrogen in a number of amino acids [23]. Most of these techniques can also be used to detect translational capacities of cell extracts in vitro.

The lack of transcription and translation in sperm implies that variations in surface protein composition are either due to the changing environments the sperm faces en route to fertilizing the oocyte (Sect. 3.1.3) or due to aberrations in the sperm formation process in the testis. While approaches such as SILAC are not feasible in sperm, a number of surface labeling techniques have been used for proteomics analysis of sperm surface proteins. In human sperm, for instance, ^{125}I labeling of sperm surface proteins or biotinylation of surface proteins has been employed to detect immunodominant sperm surface antigens [128, 130]. This method turned out to be not completely "membrane proof" as some intracellular proteins were also iodinated.

Beyond SILAC there are a range of labeling approaches in general use for quantification in proteomics workflows. iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tags) are the two most extensively used proprietary methodologies [23, 159]. These approaches rely on peptide labeling post trypsin digestion. The tags are isobaric and have an amino-specific protein reactive group which will label all peptide fragments and enable detection of differential peptide (and hence protein) expression in 4–10 samples depending on the product. These approaches could be used on the isolated and solubilized membrane protein fractions and might be useful to detect changes in protein composition of sperm surface under various physiological and in vitro conditions (for instance, the release of decapacitation factors during in vitro fertilization treatment or alterations of sperm surface proteins of sperm collected at different regions of the epididymis). To date several studies have been published using either iTRAQ or TMT on whole cell lysates, but none have been reported on membrane fractions [5, 6, 9, 98].

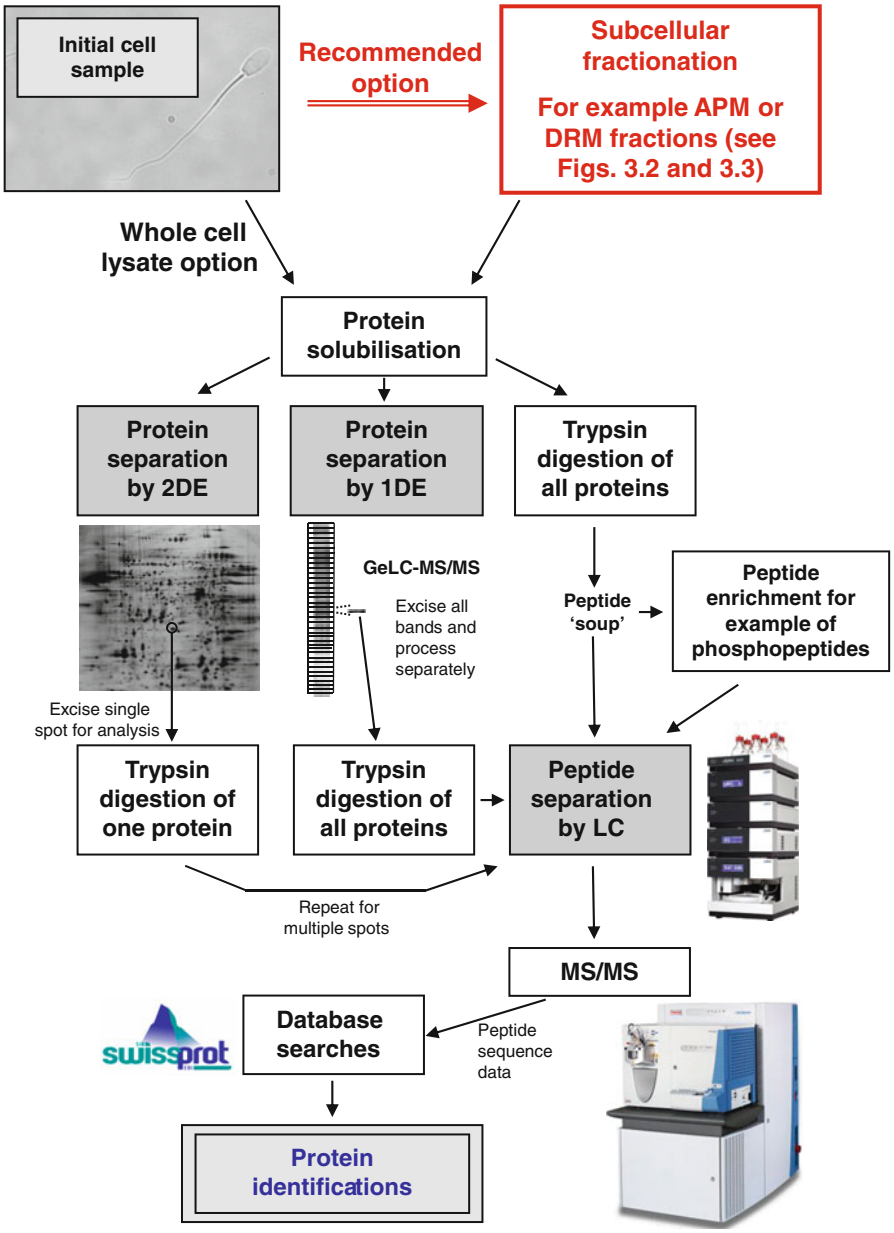
Posttranslational changes of the sperm surface membrane can also be detected [7, 57, 119]. In another study, a biotin-switch assay was employed to detect protein S-nitrosylation in human sperm [97] which provided fundamental new insights in NO-mediated sperm signaling under in vitro capacitation conditions. The modifications of these proteins take place intracellularly, and only a surface-specific membrane protein preparation can provide insights into surface posttranslational proteins.

3.3.2 Surface-Specific Considerations for Sperm Proteomics

Section 3.3.1 dealt with the applicability of a number of proteomics strategies to study sperm proteins. However, a number of additional considerations have to be taken into account when studying sperm surface proteomes. The researcher should be sure about the surface topology of the proteins under investigation. A first step into this direction is to isolate the membrane of interest (see Sect. 3.2). However, additional scrutiny is needed in ruling out the high amount of proteins that only interact indirectly with the sperm surface since they can easily become co-isolated and identified. To this end, sperm can be labeled with membrane impermeable tags prior to membrane subfractionation. Most commonly a biotinylated tag is used which is covalently bound to the sperm surface proteins [78, 85, 131, 136]. A streptavidin immobilized affinity column can be used to isolate the biotinylated proteins. After isolation the tag can be cleaved enzymatically, and the proteins can be digested into peptides for MS/MS analysis. Importantly this technique has some drawbacks as one has to be sure that only surface-oriented proteins are labeled. If sperm cells deteriorate during biotinylation, intracellular proteins will become biotinylated because they are accessible for the tag. For extracellular matrix components, this will always be the case even for intact sperm. Moreover, sperm also contains a certain number of endogenously biotinylated proteins. Finally, nonlabelled proteins may interact with the biotinylated proteins and thus may also be immobilized into the streptavidin columns. Indeed, many studies using immuno-purified surface-labeled membrane samples report the identification of a large number of nonmembrane proteins. There are

Fig. 3.4 Strategies for the global identification of proteins. Traditionally proteins are solubilized from entire cells to produce whole cell lysates, but subcellular fractionation is strongly recommended to enrich for proteins of particular biological interest and to achieve localization information. One option is the preparation of sperm apical plasma membranes (APMs; see Fig. 3.2) or isolation of the detergent resistance membrane fraction (*DRM*; see Fig. 3.3) of sperm). Following solubilization, protein separation may be achieved by two-dimensional electrophoresis (2DE), and this remains popular in low throughput studies. An individual separated protein is removed as a gel plug, trypsin digested, and the resulting peptides are separated on the basis of charge and relative hydrophobicity by nanoscale liquid chromatography (*LC*). Amino acid sequence of these peptides is then determined by tandem mass spectrometry (*MS/MS*), and these sequence data are used to search existing protein databases to achieve a match and therefore a protein identification (*ID*). In order to identify many (or all) of the separated proteins, it is necessary to excise and process multiple gel plugs from the 2D gel. For global analysis, it is more appropriate to trypsin digest the solubilized protein mixture to produce a peptide "soup" from all the proteins in the sample. Peptides are then separated by LC before extensive MSMS and database searches to identify many (ideally all) of the proteins in the original sample. Beyond this it is also possible to first separate proteins by one-dimensional electrophoresis (*1DE*; SDS-PAGE) before subjecting individual protein bands to digestion and LC-MS/MS (the so-called geLC-MS/MS workflow). It is also possible to enrich for peptides of a particular type, for example phosphopeptides, to study a particular group of proteins. In addition to the workflows illustrated, there are many other options. Protein rather than peptide enrichment may be used and peptide isoelectric focusing (IEF) as an additional step within the usual LC-MS/MS workflow is also a valid option for increased numbers of IDs (Adapted and modified from Brewis and Gadella [24])

many ways to reduce the amount of this contamination. For reviews around this topic see [52]. Besides the two steps mentioned here (labeling of the sperm surface and subsequent membrane isolation), the resulting preparations need to be treated with high salt media to get rid of adhering extracellular matrix and cytosolic components. The resulting membrane sample is highly enriched in integral membrane proteins.



Another important issue for integral membrane proteins (that is, those with (multiple) alpha helices spanning the membrane or with beta sheet barrels) is that such proteins have highly hydrophobic domains. This property of a major portion of membrane proteins often prevents solubilization under conditions compatible with 2D electrophoresis. A number of reviews provide an excellent overview of techniques that can be employed to identify these integral membrane proteins [52, 142]. Those workflows that first rely on trypsin digestion of protein mixtures overcome many limitations by digesting a specific isolated sperm surface protein fraction and analyzing the derived peptides with LC-MS/MS.

3.4 Comparison of Sperm Surface Proteomics in Different Species

A number of considerations for studying the sperm surface proteome have been summarized in this chapter. They need to be carefully considered in order to make proteomics databases of sperm surface protein composition more useful or meaningful. In this section, more emphasis is put on how existing sperm proteomics libraries should be interpreted and where appropriate some comments will be made on the suitability or originality of approaches used to decipher protein compositions of the sperm surface.

A number of groups have successfully analyzed the sperm proteome in a range of different species using either whole cell lysates or different fractions. Table 3.1 summarizes those studies that are the most noteworthy either from the point of view of the high numbers of proteins identified or the rigor of the sample preparation. From the perspective of this review, it is noteworthy that the majority are on whole cell lysates and that very few proteomics studies have focused on the sperm surface or membrane fractions. When browsing through such data, one needs to be critical in how the protein samples were prepared in order to understand how meaningful the proteomics libraries generated actually are for the sperm surface. (1) Sperm membranes are often isolated by the method of [30] in which sperm are first incubated in a hypo-osmotic environment followed by sonication and differential centrifugation. However, the purification for plasma membrane marker proteins over possible contaminating intracellular membranes is not tested convincingly for human sperm. (2) Indirect reacting proteins for instance from the extracellular matrix or the cytoskeleton may also be identified when the isolated membrane preparations were not subjected to high salt [52]. (3) Other groups use surface modification techniques to study sperm surface membrane proteins [85, 128]. The labeled proteins are supposed to originate from the sperm surface, but this approach can lead to the iodination or biotinylation of many intracellular proteins. (4) The isolated or labeled proteins are routinely solubilized and subsequently separated using protein gel-electrophoresis. The drawback of this technique is that an important group of integral membrane proteins due to their hydrophobic properties is not suitable for 2D gel-electrophoresis [52] and other approaches, such as geLC-MS or peptide IEF, may be required to enable full surface proteome coverage [23, 55].

Table 3.1 Summary of the major proteomics studies in mammalian sperm cells

Species	Sample proteins	Separation method	Total IDs	References
Boar	Whole cell lysate	Protein 2DE, peptide LC	310	(Brewis and Gadella, *unpublished data*)
	Lipid raft	Lipid raft preparation and protein 2DE or peptide LC and peptide LC or just peptide LC	34	[152] (Brewis and Gadella, *unpublished data*)
	Apical plasma membrane and docked outer acrosomal membrane	Subcellular fractionation and protein 2DE or peptide LC	63	[14, 148] (Brewis and Gadella, *unpublished data*)
Bull	Cytosolic tyrosine kinase	Subcellular fractionation, 1DE and peptide LC	130[a]	[94]
	Membrane fraction	Peptide LC	419	[38]
Human	Whole cell lysate and surface labeled	Surface protein labeling, 2DE and peptide LC	267[b]	[51, 129]
	Whole cell lysate	Protein DDE, 1DE and peptide LC	1056[c]	[15]
	S-Nitrosylated	Protein enrichment, 1DE and peptide LC	240	[97]
	Nuclear extract	Protein 2DE, 1DE and peptide LC	403	[49]
	Whole cell lysate	Peptide LC	348	[98]
	Whole cell lysate	Peptide LC	1157	[4, 5]
	Whole cell lysate	Peptide LC	1975	[102]
Mouse	Flagellum accessory structures	Protein DDE, 2DE and peptide LC	50	[39]
	Sperm acrosome	Subcellular fractionation, protein 1DE and peptide LC	114	[136]
	Whole cell lysate	Peptide IEF and LC	858	[14]
	Lipid raft	Lipid raft preparation, protein 1DE and peptide LC, peptide LC	100	[11]
	Whole cell lysate	Peptide LC	2850	[41]
	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1234	[132]
Rat	Whole cell lysate	Peptide IEF and LC	829	[13]
Rhesus macaque	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1247	[133]
Stallion	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1130	[141]

This table is adapted from Brewis and Gadella [24] and has been updated and modified to include the most noteworthy studies. *Key*: *DDE* differential detergent extraction, *IDs* protein identifications, *LC* liquid chromatography, *MS/MS* tandem mass spectrometry, *1DE* one-dimensional electrophoresis, *PMF* peptide mass fingerprinting by MALDI-TOF MS, *2DE* two-dimensional electrophoresis, *geLC-MS* current terminology for IDE and peptide LC. All studies are based on MS/MS data except for [94] and [11] which additionally includes PMF data

Three published studies with high numbers of IDs are excluded from this list. Peddinti et al. [116] report 2814 IDs on bull whole cell lysates, but the presented data do not support this assertion. Johnston et al. [81] report 1760 identifications in human whole cell lysates, but the protein IDs and MS/MS data were not reported. Wang et al. [155] report 4675 IDs in human whole cell lysates, but the inclusion criteria used were not sufficiently robust

[a]Total number of proteins identified (4 were protein tyrosine kinases)

[b]John Herr, personal communication 2015

[c]Updated to 1223 by Baker et al. [14]

These points of attention are valid for sperm surface proteomics studies independent of the mammalian species under study. However, there are also a number of species-specific advantages and disadvantages in studying the human, mouse, and porcine or bovine sperm surface, which will be dealt with the next sections for these species.

3.4.1 Human Sperm Proteomics

Referring to Table 3.1, it is clear that there have been many studies on human sperm, and indeed there are many smaller studies not included in this table. For a very elegant summary of human sperm proteomics, the reader is referred to [5] which reported a total of 6198 proteins predominantly from studies on whole cell lysates. However, focusing on the sperm surface proteome, some specific limitations that are intrinsic to human sperm need to be considered and perhaps they explain why there has been relatively little focus on this region of the cell in humans. (1) Humans (and some primate species) produce semen with a rather high content of abnormal sperm (immature, deteriorated, or morphologically aberrant). Even in the ejaculate of fertile men, the proportion of deteriorated sperm is >40 % [76, 86], whereas the ejaculate of a fertile boar (male porcine) has only <5 % aberrant sperm [65]. When assessing human sperm with the strict Tygerberg criteria, in semen only 15 % morphologically normal sperm is the value for normal fertilization rates and morphology scores rarely were higher than 30% for most fertile men [89]. In stark contrast in porcine sperm, this morphology score is rarely below 85 % [65]. The problem with human sperm is that the surface of aberrant sperm is also labeled and/or isolated following the above-mentioned methods (Sects. 3.2 and 3.3). Therefore, the resulting protein mixtures will contain more proteins from malfunctional sperm and intracellular labeled proteins compared with porcine or mouse sperm. On the other hand, the relative abundance of abnormal sperm in ejaculates from males with reduced fertility characteristics are of use for diagnostic proteomics comparisons [51, 113]. With respect to the theme of this book, sperm antigens have been detected and characterized by comparing sperm proteins from healthy and infertile men. (2) For proper sperm surface isolation, one needs to have large amounts of sperm cells. This is not the case for the commonly used method to isolate total sperm membranes using the hypo-osmotic treatment followed by sonication and differential centrifugation. For sperm cavitation and subfractionation of sperm membranes, one needs much more starting material. However, the amount of sperm released in a human ejaculate (from a healthy fertile donor) is less than 200 million sperm [99], while for porcine (and bovine) sperm this number is approximately 100–200 times higher [31, 100].

3.4.2 Mouse Sperm Proteomics

Proteomics data obtained from mouse sperm need to be viewed with extra care. (1) When mouse sperm is collected by (electro-stimulated) ejaculation, they will almost immediately deteriorate due to the spermicidal coagulation plug in which the

sperm become entrapped during collection (in contrast to the in vivo situation where the sperm remain separated from the coagulation plug). Therefore, mouse sperm for IVF purposes or for studying sperm surface are routinely obtained by aspirating the epididymis [134]. Obviously this influences the quality of such specimen as epididymal sperm may not be fully matured and the amount of sperm collected is not sufficient for proper membrane subfractionation studies. (2) Specific problems to sperm surface isolation are related to the hook-shaped morphology of the mouse sperm head. Probably related to this, only one attempt has been described to strip the plasma membrane from mouse sperm with nitrogen cavitation [103] without data on the purification degree of the cavitate. The other sperm surface isolation method of blunt hypotonic sonication resulted in only low purification of mouse sperm plasma membranes 4–10 times [17]. (3) Obviously the mouse species also has specific advantages over human and porcine species for sperm surface proteomics. Like for human, the complete genome and proteome of mouse are available [84]. (4) Because the mouse is an important laboratory animal model, species-specific genetic breeding lines are available. When compared to human (also valid to some extent for porcine samples [124]), the advantage is that within a specific breeding line relative low biodiversity exists which will result in much more repeatable data [83]. (5) Of course the mouse is also a model of choice for generating genetic knock out or silencing phenotypes for validating the function of certain translation products identified in proteomics [44, 112]. Due to the fact mouse give birth to nests (multiple off spring) and have a relatively short generation time, this laboratory species is very well suited for obtaining fertility data that can be related to proteomics data bases to verify the functionality of certain proteins in fertilization. Genotypic manipulation of humans is of course not permitted.

3.4.3 Porcine and Bovine Sperm Proteomics

The major potential of porcine and bovine sperm is noteworthy. (1) Each ejaculate contains an overwhelming amount of mature and morphologically intact functional sperm [31, 100]. (2) Moreover, for both species a reliable method has been described for purification of the apical plasma membrane (or further subfractionation to obtain surface specific of membrane microdomains) [60, 93]. Therefore, much more reliable surface membrane protein samples can be obtained from these species compared to human and mouse. (3) Both in porcine and bovine species, most offspring is produced by artificial insemination. Over the past decade or more, all large AI-industries have set up huge fertility data sets of individual male animals, collection time, female animals inseminated, nonreturn rate, birth rate, and litter size (for pigs) [32, 126]. The enormous amounts of data for each sperm-producing animal can be used to get very relevant correlations between sperm characteristics and fertility potential. In collaboration with the AI-industries, these data sets can become accessible to correlate the presence of certain sperm surface proteins in certain sperm donors to the fertility performance of the boar or bull [25, 33, 115]. To a lesser extent, this is also possible for equine sperm [80]. (4) The equine and bovine

species are mono-ovulatory and therefore have a reproductive physiology that resembles the human reproductive physiology more than the laboratory animals or pigs which are poly-ovulatory mammals [50, 108]. (5) Porcine and bovine breeding is performed on a very large-scale worldwide. The offspring is of course relevant for delivery of milk for dairy products and for our need for animal food and animal-derived materials from those animals. At a certain moment, animals will be slaughtered to harvest these materials. For veterinary scientists, it is possible to obtain fresh materials from those animals at the slaughter line continuously. This enables the researcher to obtain materials of >6000 animals per day. In our setting, we were for instance able to isolate 5000 ovaries with ovulatory follicles from adult pigs in one collection session [59]. From this material, we isolated 500,000 oocytes with a mature diameter size and a functional zona pellucida. We were able to isolate zona ghosts that were not contaminated with other proteins as was verified on solubilized zona material on 2D electrophoresis [151]. This zona material was used to identify isolated apical plasma membrane proteins. A number of integral membrane proteins originating from the testis (such as fertilin beta) and GPI anchored proteins attached to the sperm surface when traveling through the epididymis (spermadhesins) were identified [28]. Although a number of proteins were not identified, this direct primary zona binding approach could not have been carried out with mouse or human material as such an amount of purified mature and prefertilization zona ghost material cannot be prepared from these species. In addition, due to their larger size farm animals are easier to approach for internal genital tract processing of the sperm surface. Examples are of epididymal surface remodeling or of in vitro manipulation of the sperm surface in the oviduct [47, 80, 137]. (6) Although technically possible, it is very expensive to perform genotypic silencing of farm animal species. This is due to the larger size of these animals compared to laboratory animals: Both the housing of animals and the relatively long generation time in larger animals make these types of studies less suitable. We should note here that fertility data from molecular manipulated mouse experiments can only to a limited manner be extrapolated to other mammalian species. This has to do with the fact that proteins involved in reproduction show a very rapid evolutionary diversification. There is a lot of redundancy in proteins within one species (in porcine sperm there are >10 zona binding proteins [150]) and between species; completely different sets of proteins are involved in the same processes related to fertilization due to rapid evolutionary diversification of proteins [77, 149]. For this reason, phenotypically altered mice may not always provide insights to understand the role of sperm surface proteins identified in other mammalian species.

3.5 Implications for Future Research

3.5.1 Proteomics and Male Fertility

Much of the research on mammalian sperm that has benefited from proteomics technology has been interested in better understanding molecular events and how they

affect the biological function of the sperm cell. Proteomics has also been used closer to the clinic to investigate potential human sperm defects that contribute to infertility. John Herr's group has been interested for many years in characterizing immunogenic surface epitopes to further understand the role of antisperm antibodies in infertility and to potentially provide insights for the development of contraceptive vaccines. For a recent overview in the use of "omics" for human male infertility, see [40]. Other studies have used proteomics to characterize functionally defective sperm (sperm that fail to fertilize at IVF, are asthenozoospermic, or are correlated with DNA damage/protamine content) [18, 20, 48, 49, 56, 107, 109]. Candidate proteins that are differentially expressed in patient samples compared with normozoospermic samples have been identified, but much work still needs to be done to properly validate these early candidates. Some may prove to be protein biomarkers of specific male infertility (sperm dysfunction) phenotypes, but in all likelihood much more rigorous analysis needs to be undertaken before such biomarkers are realized [117, 121]. Recently, sperm proteomics data have also been used to relate fertility properties of male animals (in pigs, horses, and cows), and both proteins were assigned to relate with higher fertility and with infertility characteristics [46, 61, 90, 91, 98, 140].

3.5.2 Quantification of the Proteome

The sperm research community has been slow to adopt the now gold standard approaches for relative protein quantification in proteomics. Such approaches will be key to the discovery of protein biomarkers of male infertility and in further understanding sperm dysfunction and function at the molecular level. In the past there have been some useful studies using difference gel-electrophoresis (DIGE) (fluorescently tagged samples are multiplexed, separated by 2D electrophoresis, and quantified with confocal laser scanning) [16, 125]. This approach has been superseded by the previously mentioned iTRAQ or TMT tagging workflows. To date there have been very few studies published in sperm that have used either of these tagging approaches and these have generally been on whole cell lysates [4, 9, 98]. One interesting exception to this is the study of Asano et al. [6] who have used iTRAQ to characterize the expression of certain proteins in different microdomains. Liu et al. [102] have used a label-free approach, which is a newer tagging-free mass spectrometric quantification approach to study changes involved in asthenozoospermia on whole cell lysates. Finally it is also possible to quantify phosphorylation on a larger scale using an alternative MS-based labeling approach (Fisher esterification of phosphopeptides using differentially deuterated methyl alcohols), and this was employed in an elegant study comparing capacitated and noncapacitated cells [61].

3.5.3 Protein and Peptide Enrichment for Proteomic Studies

As an alternative to subcellular fractionation, another option is to enrich for protein types of interest from a whole cell lysate. Several studies on sperm have investigated

protein phosphorylation on a proteomic scale as the this phenomenon is known to be very important to a number of aspects of sperm function, including epididymal maturation and capacitation. The first proteomics studies involving both the identification of multiple phosphoproteins [145] and the sites of phosphorylation were conducted by Pablo Visconti and colleagues on human sperm, and the same group has published widely in a number of species. For a recent review on sperm phosphoproteomics, see Porambo et al. [120]. Currently phosphoproteomics studies are generally performed using peptide affinity-based approaches with the enrichment of phosphorylated peptides by immobilized metal affinity (IMAC) chromatography or titanium dioxide, and indeed these prefractionation approaches are essential.

Conclusions

Antigens at the surface of sperm are of considerable interest compared with intracellular antigens as the latter are only accessible for immune responses when the integrity of sperm is compromised. When immune responses are elicited towards the sperm surface of intact sperm the fertilization potential of such sperm may be altered by the immune response. Thus, proteomics studies that focus exclusively on sperm surface material are very relevant for immune infertility studies. A number of considerations have been dealt with in this chapter to ensure that only the proteins of sperm surface membranes are isolated or labeled. Very few of the noteworthy proteomics studies to date have focused in the cell surface, and this remains a key challenge for this field. It is difficult to compare the surface proteome of human, mouse, and farm animals as the sperm surface proteome is highly species specific, and each mammalian species has its own drawbacks and advantages for studying the sperm surface proteome. The functional relevance of genotypic silencing experiments of mouse sperm proteins for human reproduction is therefore also questionable to a certain degree. The major drawbacks for studying the human surface proteome are the limited amount of material that is present in an individual ejaculate, the high incidence of aberrant sperm (both are no issues for farm animal species). Another drawback is that genetic manipulation of man is not permitted (this is not an issue for murine species and it is possible but very expensive and time consuming for farm animals).

Finally in many studies, the specificity of labeling methods and sperm surface separation from intracellular and extracellular components have not been analyzed or at least not with high enough scrutiny. For functional sperm surface proteomics, it will be of fundamental interest to have specific sperm surface protein preparations. In addition, the interacting structures should be purified to a satisfactory level. Somatic cells and fluids from the male and female genital tract are involved in the relevant surface modifications to achieve fertilization. Finally the complex and domain-dynamic organization of the sperm surface needs to be considered when studying the protein composition of the fertile surface of sperm. With this respect, it is noteworthy that sperm membrane proteins form complexes at different places on the sperm surface with specific functions in mammalian fertilization.

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