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NEW RESEARCH HORIZON ***Review***

Sperm surface proteomics: from protein lists to biological function

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ABSTRACT: Proteomics technologies have matured significantly in recent years and proteomics driven research articles in reproductive biology and medicine are increasingly common. The key challenge is to move from lists of identified proteins to informed understanding of biological function. This review introduces the range of proteomics workflows most commonly used for protein identification before focusing on the mammalian sperm cell at fertilization as an exemplar for proteomic studies. We review the work of others on entire cells but then argue that proper subcellular fractionation and proper solubilization strategies offers critical advantages to achieving increased biological understanding. In relation to understanding initial gamete recognition events at fertilization (capacitation, zona binding and acrosomal exocytosis) it is imperative to study the sperm surface proteome by using purified plasma membrane fractions. Although this task is challenging there are now strategies at our disposal to achieve comprehensive coverage of the proteins at the sperm surface. Within this context it is also important to understand the milieu of the sperm cell during transit from the testis to the oviduct as proteins (or other entities) from the genital tract epithelia and fluids may also affect the composition and organization of proteins on the sperm surface. Finally the arguments presented for studying the cell plasma membrane proteome to understand the role of the cell surface equally apply to all cell types with important roles in reproductive function.

Key words: fertilization / proteomics / plasma membrane / sperm / surface

Introduction

Proteomics technology

Proteomics (the study of protein products expressed by the genome) has become one of the leading technologies available to researchers in the postgenomic era due to the central role of proteins and protein–protein interactions in cellular function (Cox and Mann, 2007). Functional genomics is focused on understanding the function of genes and their corresponding proteins on a global scale and, like all functional genomic approaches, proteomics is underpinned by the genome sequencing projects. These have continued at a frantic rate to a stage where a number of these are now essentially complete for mammalian model systems (including the human) (for the status of these projects see http://www.ensembl.org). Although DNA/RNA-based functional genomic approaches, for example microarray gene expression analysis, have been used extensively and are clearly important they reveal nothing about the level of protein expression, the protein isoforms that may be produced from each gene or the extent to which proteins are post-translationally modified (Brewis, 1999).

Therefore it is critical to study proteins directly and the comprehensive and systematic identification, quantification and characterization of proteins expressed in cells are fundamental goals to gaining new insights into cellular function. However, this is not a trivial task and there are limits to what can and cannot be achieved. This review will introduce proteomics technology and outline approaches for enriching for cell surface proteins. The mammalian sperm cell at fertilization will be used as an exemplar and we argue that enriching the plasma membrane is critical to map proteins on the cell surface in order to further understand early fertilization events (capacitation, zona binding and acrosomal exocytosis). However, it should be stressed that our arguments for studying the cell plasma membrane proteome to understand the role of the cell surface equally apply to all cell types with important roles in reproductive function.

Proteomics can be described as truly interdisciplinary as it is only made possible by the co-ordinated exploitation and integration of many fields of scientific endeavour. Most importantly genome sequencing, protein separation science, mass spectrometry (MS) and bioinformatics are the four pillars on which the technology stands (Tyers and Mann, 2003). There are many strategies for protein identification

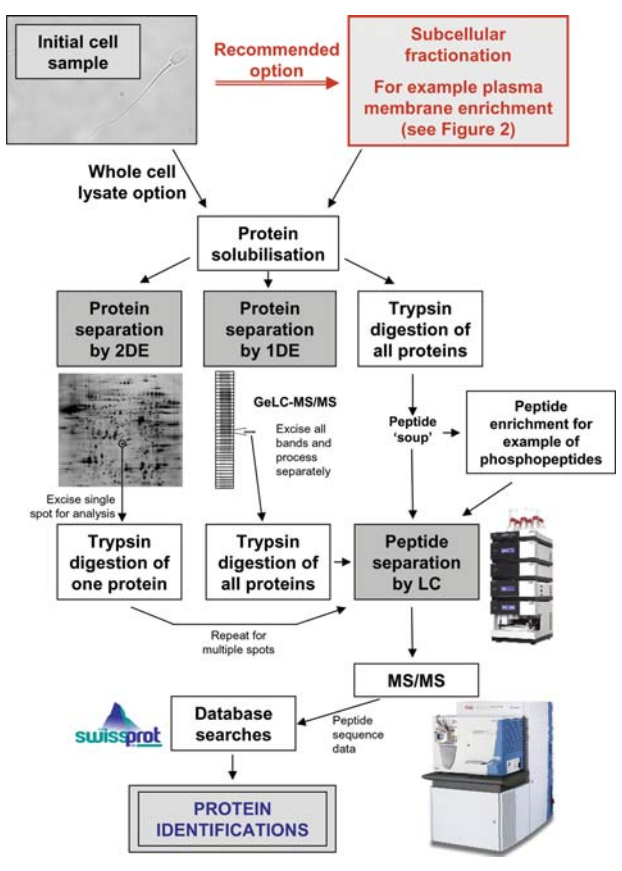

Figure 1 Strategies for the global identification of proteins.

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) (Fig. 2). Following solubilization protein separation may be achieved by two-dimensional electrophoresis (2DE) and this remains popular. 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 this sequence data is 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 commonplace to trypsin digest the solubilized protein mixture to produce a peptide 'soup' of 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 focussing (IEF) as an additional step within the usual LC-MS/MS workflow is also a valid option for increased numbers of IDs.

(ID) on a global scale and the most common ones are summarized in Fig. 1. Proteins first need to be extracted (solubilized) from cells and then separated due to the large numbers of proteins found in cells. This may be done at the protein level by two-dimensional electrophoresis (2DE). An individual separated protein is then removed as a gel plug and is typically digested by the protease trypsin which cleaves at specific site to produce many peptides which on average are 10 amino acids (about 1100 kDa) long. Resulting peptides are then typically separated by nanoscale liquid chromatography (LC) on the basis of relative hydrophobicity and charge.

The amino acid sequence of these separated peptides is then achieved by tandem mass spectrometry (denoted as MS/MS) whereby peptides of the same mass are sequentially fragmented in a collision cell of the MS. This fragmentation most typically occurs at the peptide bond and is inefficient and therefore fragments of different sizes are produced. These can be used to build up the actual peptide sequence as all amino acids (except Leu and Ile) have unique masses. Sequence data from multiple peptides is used to search existing protein sequence or gene/genome sequence databases to achieve a match and therefore a protein ID. Since the early 2000s it has become routine to rely on protein sequence data derived from MS/MS to achieve protein ID. With the complete/near complete nature of a number of mammalian genomes it is now almost always possible to identify at least the gene responsible for encoding the protein in model species. Historically peptide mass fingerprinting (PMF) was employed to achieve protein ID through matching patterns of intact peptide masses from your sample protein with an *in silico* digested database. Although this technique is still used it is not now sufficient in itself for a protein ID in major proteomics/biochemistry journals.

In order to identify many or all separated proteins from a 2D gel it is necessary to excise multiple gel plugs and subject them to this workflow and this can be assisted by robotic solutions. For global identification of proteins it is now more commonplace to instead extract proteins and then trypsin digest the entire sample to produce a peptide 'soup' of all the proteins in the sample (gel-free bottom up proteomics). These peptides are subjected to LC and analysed by extensive MS/MS to identify many (ideally all) of the proteins in the original sample (Aebersold and Mann, 2003). MS technologies in particular have developed enormously over the past decade to facilitate these sorts of workflows and the sensitivity, resolution and mass accuracy of these instruments is now truly staggering (Domon and Aebersold, 2006; Yates *et al.*, 2009). Gel-free workflows have proven to be extremely powerful and some truly remarkable studies have been achieved with amazing contributions to our biological knowledge. However, these technologies are expensive and also require a wide range of expertise in LC, MS and data analysis.

Challenges with proteomics

Although the number of protein-encoding genes in mammals is surprisingly small (humans have 22 000–25 000) (Stein, 2004) there is a general consensus that there are many more proteins than this (certainly at least 300 000 proteins and probably more) (Harrison *et al.*, 2002). This is brought about by alternative splicing and post-translational modifications (for example lipid modification, glycosylation, phosphorylation and proteolytic processing). An individual cell type will only contain a fraction of this number of proteins (perhaps 10 000–12 000) but fluids, such as plasma and serum, will be much more complex. Potentially they will contain degradation products from proteins of all cell types and foreign microbe proteins in

addition to the true (classical) plasma proteins (Anderson and Anderson, 2002).

However, it is not this complexity *per se* that is the fundamental issue in proteomics but instead the difficulty is one of identifying the lowest abundant proteins which may often be the most biologically relevant. This remains the most significant challenge for proteomics as the technologies available still really struggle to deal with dynamic range issues (the difference in abundance between the most abundant detectable proteins and the least abundant detectable proteins). 2DE has 2–4 orders of magnitude of dynamic range and most protein/peptide analytical techniques have broadly similar limits (Gorg *et al*., 2009). The issue is brought into clear focus when one considers that there are typically well over 10 orders of magnitude dynamic range for biological fluids and over six orders of magnitude of dynamic range for cells (Anderson and Anderson, 2002; Wu and Han, 2006).

Another very important challenge is that standard methods for extracting proteins from cells are usually non-specific and will struggle/fail to solublize all proteins. In particular very hydrophobic proteins (such as certain integral membrane proteins) or proteins that exist as multiprotein complexes ('molecular machines') are problematic and may be resistant to all but the most aggressive solubilization procedures (Gingras *et al*., 2007; Josic and Clifton, 2007; Tan *et al*., 2008). Historically the main approach to separate proteins has been 2DE where proteins are separated on the basis of charge (isoelectric focussing, IEF) and molecular weight (SDS-PAGE). Only non-charged detergents can be used to solubilize proteins to be compatible with IEF and the dogma is that this will fail to solubilize multi-spanning integral membrane proteins and certain protein complexes. Although this might not always be the case it is clearly an issue and severely limits the compatibility of 2DE-based approaches for membrane protein and multiprotein complex studies. In addition 2DE also struggles to resolve highly charged or very large proteins (Rabilloud, 2009). Within the scope of this review the shortcomings of membrane protein solubilization for 2DE is well-illustrated in a recently published proteomic study on human sperm. Almost all of the 116 proteins identified were of cytosolic (soluble) origin and only one integral membrane protein was identified (Secciani *et al*., 2009).

There are advantages with the peptide-based (LC) workflows as it is possible, but still not without challenges, to solubilize proteins using more stringent conditions (for example SDS solubilzation). In addition to the standard LC-MS/MS workflows it is also possible to first separate proteins by SDS-PAGE before subjecting individual protein bands to digestion and LC-MS/MS (the so-called geLC-MS/MS workflow). A number of studies have clearly demonstrated that different approaches reveal unique datasets and hence in an ideal world one would perform 2DE, LC-MS/MS and geLC-MS/MS (Ostrowski *et al*., 2002). In addition peptide fractionation by IEF as an additional step within LC-MS/MS workflows is also employed by some leading proteomics groups (Hubner *et al*., 2008).

Sperm cell proteomics

Studying the mammalian sperm proteome is in some respects simpler than is the case with somatic cells as many somatic cell features have been lost. The sperm cell is highly polarised and specialized with a minimal amount of cytosol and organelles (Eddy and O'Brien, 1994; Yanagimachi *et al*., 1994). There is no endoplasmic reticulum, Golgi Apparatus, lysosomes, peroxisomes or ribosomes and therefore the mature sperm has lost the potential for gene expression (both transcription and translation processes are completely silenced) (Boerke *et al*., 2007). This absence of protein synthesis and the reduction of both the number of proteins and probably the dynamic range of protein abundances compared with many other cell types make sperm cells suited to proteomics studies. However, it should still be stressed that, even with this reduction in complexity and dynamic range of abundance these are still major issues. In addition the sperm cell has high membrane content and so relatively more membrane proteins than many cell types. For past reviews on sperm proteomics, the reader is referred to Aitken and Baker (2008), Oliva *et al*. (2008), Aitken and Nixon (2009), Gadella (2009), Oliva *et al*. (2009).

The importance of proteomics in sperm research is also increased by the fact that sperm cells undergo significant post-testicular maturation in the epididymis and reorganization during capacitation in the female reproductive tract in order to confer fertilising ability on the sperm cell (Aitken *et al*., 2007; Boerke *et al*., 2007). As previously stated this happens in the complete absence of gene expression and hence post-genomic profiling approaches (for example differential display, serial analysis of gene expression and microarray analysis technologies) are redundant in characterizing the changes that take place to confer functionality (fertilizing ability) on the mature sperm cell (Barratt *et al*., 2002; Baker *et al*., 2008a). However, this phenomenaon does mean that popular techniques for global protein quantification where labelled amino acids are incorporated into proteins (for example SILAC, stable isotope labelling by amino acids in cell culture) are incompatible with mature sperm (Unwin *et al*., 2006; Boerke *et al*., 2007).

The pioneers of sperm proteomics were John Herr and colleagues in Virginia and their painstaking studies first established a comprehensive humans sperm protein database of ~1400 spots (Naaby-Hansen *et al*., 1997). Other studies have also revealed a similar number of proteins (Pixton *et al*., 2004; Martinez-Heredia *et al*., 2006; Secciani *et al*., 2009) but a recent study using narrow range IEF focussing strips and multiple 2D gels did suggest that this number might be much higher as 3872 distinct protein spots were visualized (Li *et al*., 2007). Whatever this number is it is still smaller than the number of proteins believed to be present in typical somatic cells (Harrison *et al*., 2002). There have now been a number of proteomic studies on mammalian sperm and the most noteworthy are listed in Table I. The greatest numbers of proteins have been identified with whole cell lysates and in particular John Aitken and Mark Baker have led the way with the identification of large numbers of proteins in three impressive bodies of work in the human, mouse and rat (Baker *et al*., 2007, 2008a, b). These recent global analyses are landmark studies and reveal plenty of previously reported proteins but also many proteins hitherto not known to exist in mammalian sperm.

New developments

The importance of subcellular fractionation

There is a clear issue with whole cell lysate studies as no definitive information about the subcellular localization of the protein identified

Table I Summary of the major proteomic studies in mammalian sperm cells

Species	Sample proteins	Separation method	MS approach	Total IDs	References
Human	Whole cell lysate and surface labelled	Protein (surface labelling) and 2DE and then peptide LC	MS/MS	267*	Shetty *et al.* (2001), Domagala *et al.* (2007)
	Phosphorylated	Protein 2DE, peptide affinity and LC	MS/MS	60**	Ficarro *et al.* (2003)
	Whole cell lysate	Protein 2DE, peptide LC	PMF, MS/MS	131	Martinez-Heredia *et al.* (2006), de Mateo *et al.* (2007)
	Whole cell lysate	Protein DDE, 1DE and peptide LC	MS/MS	1056***	Baker *et al.* (2007)
	S-nitrosylated	Protein enrichment and 1DE and then peptide LC	MS/MS	240	Lefievre *et al.* (2007)
	Whole cell lysate	Protein 2DE and peptide LC	PMF, MS/MS	116	Secciani *et al.* (2009)
Mouse	Lipid raft	Lipid raft preparation, protein 1DE and peptide LC	MS/MS	27	Sleight *et al.* (2005)
	Flagellum accessory structures	Protein DDE, 2DE (and peptide LC)	MS/MS	50	Cao *et al.* (2006)
	Sperm acrosome	Subcellular fractionation, protein 1DE and peptide LC	MS/MS	114	Stein *et al.* (2006)
	Whole cell lysate	Peptide IEF and LC	MS/MS	858	Baker *et al.* (2008a)
	Phosphorylated	Peptide enrichment and LC	MS/MS	55**	Platt *et al.* (2009)
	Lipid raft	Lipid raft preparation, protein 1DE and peptide LC, peptide LC	PMF, MS/MS	100	Nixon *et al.* (2009)
Rat	Whole cell lysate	Peptide IEF and LC	MS/MS	829	Baker *et al.* (2008b)
Bull	Cytosolic tyrosine kinase	Subcellular fractionation, protein enrichment and 1DE and then peptide LC	PMF, MS/MS	130****	Lalancette *et al.* (2006)
Boar	Whole cell lysate	Protein 2DE, peptide LC	MS/MS	310	Brewis and Gadella (unpublished data)
	Lipid raft	Lipid raft preparation, then protein 2DE and peptide LC or just peptide LC	MS/MS	34	van Gestel *et al.* (2005), Brewis and Gadella (unpublished data)
	Apical plasma membrane	Subcellular fractionation, then protein 2DE and peptide LC or just peptide LC	MS/MS	63	Van Gestel *et al.* (2007), Brewis and Gadella (unpublished data)

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.

*John Herr, personal communication; **Note these are phosphopeptide sequences not distinct proteins; ***Updated to 1223 in Baker *et al.* (2008a); ****Total number of proteins identified (four were protein tyrosine kinases). Two published studies are excluded from this list. Peddinti *et al.* (2008) claim 5850 IDs on bull whole cell lysates but the presented data does not support this assertion. Johnston *et al.* (2005) report 1760 identifications in human whole cell lysates but the protein IDs and MS/MS data were not reported.

is provided. Although it may be possible to infer localization from the protein sequence or from previous studies (either in sperm or other cells) the ideal would be unequivocal demonstration of protein localization. Although it is entirely feasible to characterize individual proteins one at a time and achieve definitive localization information using antibody based approaches ideally the subcellular localization should already be known. Increasingly proteomics technologists are doing just this and employing extensive subcellular fractionation strategies to achieve localization for an entire proteomic dataset and provide additional insights into biological function (Sadowski *et al*., 2006; Josic and Clifton, 2007; Sprenger and Horrevoets, 2007). In addition these approaches also reduce sample complexity and can assist in the challenges of dealing with the dynamic range of protein abundances.

Although many of the approaches for subcellular fraction are technically challenging and certainly time-consuming the quality of the data produced justifies this effort. In order to now move from lists of identified proteins to informed understanding of biological function, we argue that it is essential to study specific cellular compartments. For example, to more fully understand the early gamete recognition and fertilization events that involve the sperm cell surface it is imperative to study the sperm surface proteome by using purified plasma membrane fractions. Whereas this task is difficult there are now strategies at our disposal to facilitate comprehensive analysis of the sperm surface proteome.

The sperm surface at fertilization

The surface of the sperm head, midpiece and the tail (flagellum) is highly heterogeneous and has a lateral domained ordering that reflects the polar distribution of the main organelles (the acrosome, nucleus and mitochondria) and cytoskeletal elements that lie under the surface (Phelps *et al*., 1988; Gadella *et al*., 1995). In particular different regions (subdomains) of the sperm head surface can be distinguished with separate functions in the fertilization process and these are illustrated in Fig. 2 (Gadella *et al*., 2008). The apical ridge of the sperm head specifically recognizes and binds to the zona pellucida (the extracellular matrix of the oocyte) (Van Gestel *et al*., 2007) and a larger area of the sperm head surface (the pre-equatorial region) is involved in the acrosome reaction which results in the release of acrosome components required for zona penetration (Yanagimachi *et al*., 1994; Flesch and Gadella, 2000). 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 oocyte plasma membrane) in order to fertilize the oocyte (Vjugina *et al*., 2009). Although the surface of the midpiece and tail of the sperm is also heterogeneous the function of these plasma membrane specializations are not yet understood (Kan and Pinto da Silva, 1987). It may well be possible that they are involved in organization of optimal sperm motility characteristics.

Historically sperm surface proteins have been studied using labelling strategies with membrane impermeable tags to facilitate enrichment and identification. In human sperm for instance ^{125}I labelling and biotinylation of surface proteins have been employed to detect immunodominant sperm surface antigens (Naaby-Hansen *et al*., 1997; Shetty *et al*., 1999a, b; Domagala *et al*., 2007; Shetty *et al*., 2008). Other groups have also used biotinylated tags that become covalently

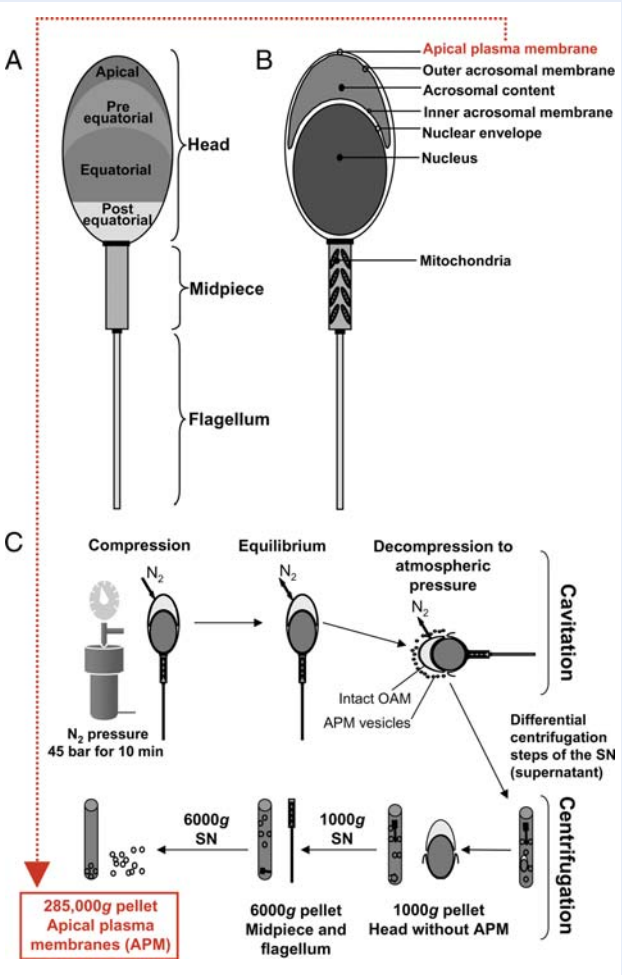

Figure 2 Subcellular fractionation of apical plasma membranes from boar sperm cells.

(**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 pre-equatorial 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 boar sperm cells 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 (Flesch *et al*., 1998).

bound to surface proteins and then a streptavidin immobilized affinity column can be used to isolate the biotinylated proteins and after isolation the tag can be cleaved enzymatically (Holt *et al*., 2005; Stein *et al*., 2006; Fazeli, 2008). These approaches are not completely 'plasma membrane proof' as some intracellular proteins may also be labelled due to damaged cells or cells that deteriorate during preparation and experimentation. Moreover, sperm also contain a small number of endogenously biotinylated proteins. Finally, non-labelled proteins may interact with the biotinylated proteins and thus may also be co-purified with these approaches. It is very difficult to completely rule out those proteins that only interact indirectly with

the sperm surface but are recovered with this technique. Notwithstanding these concerns these are still useful techniques and there are many ways to reduce contamination (Dormeyer *et al*., 2008).

Sperm apical plasma membrane fractionation

Beyond surface labelling strategies it is necessary to purify cell plasma membranes. To achieve this specific sperm disruption methods such as ultrasonication and nitrogen cavitation have been designed (Flesch *et al*., 1998). Sonication gives lower purification and a less defined membrane fraction (Baker *et al*., 2002). After sperm disruption differential centrifugation techniques need to be employed to isolate membranes from insoluble cellular debris and soluble components. The researcher needs to consider whether 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. If the plasma membrane preparation also contains acrosomal contamination one can be sure that secondary (intra-acrosomal) zona binding proteins will be identified as they may overwhelm the amount of primary (plasma membrane) zona binding proteins (Flesch *et al*., 2001a, b; Van Gestel *et al*., 2007). 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 then indicative of the purity of the membrane fraction for surface membranes and therefore also surface proteins.

In our hands an optimized nitrogen cavitation method followed by differential centrifugation turned out to yield a 200 times enriched plasma membrane fraction over possible contaminating membranes with a yield of ~30% of the sperm surface (Flesch *et al*., 1998). This approach is shown is shown diagrammatically in Fig. 2. Ultrastructural analysis of this membrane fraction and of disrupted sperm has shown that the isolated plasma membrane fraction contained resealed plasma membrane vesicles. The vesicles were so called 'right-side outside' unilamellar vesicles with the outer and inner side of the vesicle membranes having the same protein topology as in the intact plasma membrane of sperm. The resealed plasma membrane vesicles also do not have any encapsulated intracellular membranes. Importantly these plasma membrane fractions were also largely from the apical ridge region of the sperm cell where zona binding takes place. These apical plasma membrane (APM) isolates have been instrumental in studying protein–protein interactions relevant for sperm-zona binding (Van Gestel *et al*., 2007) and for the redistribution of membrane microdomains believed to represent lipid rafts (Van Gestel *et al*., 2005). Currently, we also have an unpublished dataset of approximately 60 proteins derived from LC-MS/MS experiments from this APM fraction and we are now engaged in alternative solubilization procedures and geLC-MS/MS strategies in order to maximize the number of proteins we identify.

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 and some marker lectins exclusively bind to the sperm plasma membrane. Therefore, affinity chromatography using immobilized lectins can be used to extract surface proteins (Runnebaum *et al*., 1995) and this method can also be employed on nitrogen cavitated and solubilized sperm plasma membrane material.

There are number of different types of membrane proteins which can broadly be classified as integral or peripheral membrane proteins. In general integral membrane proteins have an extracellular domain and a transmembrane domain whereas peripheral membrane proteins are tethered to integral membrane proteins and/or membrane lipids via electrostatic interactions. Discrimination between these two classes of membrane proteins can be achieved by treating membrane preparations with high salt which destabilizes electrostatic interactions and results in the release of peripheral membrane proteins whereas the integral membrane proteins remain in the insoluble membrane fraction. This high salt strategy can also be used to remove indirectly interacting proteins for instance from the extracellular matrix or the cytoskeleton from membrane preparations (Dormeyer *et al*., 2008).

Sperm lipid raft fractionation

Beyond the purification of APMs another good method of subcellular membrane fractionation that has become increasingly popular in recent years is the isolation of lipid rafts (also known as microdomains or detergent resistant membranes, DRMs). We now understand that these discrete lipid domains in the plasma membrane enriched in cholesterol and sphingolipids lipid rafts are areas on any cell surface where functionally important proteins are co-localized (Sprenger and Horrevoets, 2007). This is also the case in sperm and bicarbonate-mediated capacitation causes the migration and coalescence of these microdomains at the apical region of the sperm head (Van Gestel *et al*., 2005; Boerke *et al*., 2008; Gadella *et al*., 2008). This had led to the hypothesis that it is this process that enables zona binding and the zona-induced acrosome reaction to take place via the assembly of a zona pellucida binding protein complex (Gadella, 2008; Nixon *et al*., 2009). Membrane raft isolation procedures can be employed to isolate microdomains from sperm and most methods use detergents at low temperature (4°C) to isolate the DRM fraction (Asano *et al*., 2009). There have now been two proteomic studies in the mouse with 27 and 100 proteins identified, respectively (Sleight *et al*., 2005; Nixon *et al*., 2009). The latter study is noteworthy as the number of proteins is significant and may even be approaching the total number of proteins that might be present in sperm microdomains.

Glycosyl-phosphatidylinositol (GPI) anchored proteins are membrane proteins that are covalently attached to GPI with just the lipid portion of the anchor contained in the lipid bilayer. These are known to be enriched in lipid rafts and phosphatidylinositol-specific phospholipase C (PI-PLC) can be employed to specifically solublize GPI anchored proteins (Paulick and Bertozzi, 2008). We have identified that the sperm DRM fraction after capacitation becomes highly enriched in GPI anchored proteins and in proteins involved in zona binding and the acrosome reaction (Van Gestel *et al*., 2005; Tsai *et al*., 2007). We also have preliminary data that DRMs from the entire sperm contains surface membrane material but also intracellular (acrosomal) membrane material. The DRM fraction of whole sperm also contains components that can be labelled with marker lectins for the outer acrosomal membrane and hence this is a note of caution in using this fraction. In contrast DRMs from purified APMs did not show any labelling with this lectin and the best explanation

for these results is that the outer acrosomal membrane also contains lipid rafts.

In summary DRMs are a valuable resource for understanding the molecular events that occur in sperm at fertilization. However, one needs use them with caution as not all proteins will certainly be from the sperm surface. Ideally APM fractionation followed by DRM isolation should be performed to ensure that the isolated DRMs are certainly derived form the sperm surface. Finally another note of caution should be associated with all studies on lipid rafts as it is generally accepted that the approach to purify DRMs might not produce fractions that exactly mirror lipid rafts *in vivo* (Waugh and Hsuan, 2009).

Model organism considerations

There are a number of species specific considerations regarding sperm surface proteomics data. In the mouse (and indeed all rodents) sperm are generally obtained by aspirating the epididymis (Aitken *et al*., 2007). Obviously this influences the quality of such a specimen as epididymal sperm may not be fully matured and the amount of sperm collected is not sufficient for proper membrane subfractionation studies. Specific problems related to sperm surface isolation lie in the hook shaped morphology of the mouse sperm head and probably related to this there has only been one attempt describing nitrogen cavitation and no data on the degree of purification of the cavitate (Lopez and Shur, 1987). The other sperm surface isolation method using hypotonic sonication results in only low (4–10 times) purification of mouse sperm plasma membranes (Baker *et al*., 2002). Another approach is to induce the acrosome reaction and collect these acrosomal vesicles and this was employed in the mouse by (Stein *et al*., 2006). In addition they also collected the acrosomal contents and proteomic analysis was performed on both fractions. Although informative ideally one would use a strategy to localize the membrane proteins even more precisely and define the purity of the fractions. The mouse model also has a number of advantages and from a proteomic standpoint there is already a completed genome (Kasukawa *et al*., 2004). It is also the model of choice for generating genetic knockout or silencing phenotypes for validating the function of translation products derived from proteomics or other studies (Cooper *et al*., 2003; Okabe and Cummins, 2007). It has also been the main model organism for fertilization research in part because multiple offspring and relatively short generation times is ideal for obtaining related fertility data (Gadella, 2009).

Considering the human there are also some intrinsic limitations and a key issue is cell heterogeneity. Ejaculated semen from fertile males has a rather high content of grossly abnormal (immature, deteriorated or morphologically aberrant) sperm which can be >40% (Hendin *et al*., 2000; Keel, 2004). This is in contrast to the boar which has <5% aberrant sperm (Gadella *et al*., 1991). According to strict Tygerberg criteria semen only 15% morphologically normal sperm are required for a semen sample to be defined as 'normal' and it is rare that this is higher than 30% (Kruger *et al*., 1986). Again in contrast this morphology score is rarely below 85% in the boar (Gadella *et al*., 1991). The difficulty for sperm surface proteomics is that prepared samples almost certainly also contain intracellular proteins due to the challenges of preparing intact homogeneous samples prior to surface labelling or subcellular fractionation. Another challenge is the issue of cell numbers as nitrogen cavitation and subfractionation of sperm membranes requires larger numbers of cells than present in the human ejaculate. As hypo-osmotic treatment followed by sonication and differential centrifugation does not require such large numbers of sperm it has generally been the method of choice for human sperm surface preparations (for example, Bohring and Krause, 1999). However, the purity of such membrane preparations for sperm plasma membrane material is not well documented and contamination with intracellular membranes is likely.

In practical terms the very high sperm counts of boars and bulls compared with humans makes membrane fractionation, and also other protein purification methodologies, more plausible in boars and bulls (Gadella, 2009). The homogeneity of the sample is also ideal for surface proteomic studies and in both boars and bulls a reliable method has been described for APM isolation (Flesch *et al*., 2001a, b; Lalancette *et al*., 2001). Overall much more reliable surface membrane protein samples can be obtained from these species compared with either the human or the mouse. However, a clear drawback currently is that the genome mapping of these species is not yet as complete as the mouse and human. Although there are well-developed draft sequences for both species that are improving all the time these genomes will always be studied in less detail than the standard mammalian model systems. In addition these species are also not suited to genetic manipulation.

Sperm transit through the male and female genital tracts and the effect on the surface proteome

Membrane proteins and lipids in the sperm head are known to undergo significant reordering under *in vitro* capacitation conditions and this has been extensively studied (Flesch and Gadella, 2000; Gadella and Visconti, 2006; Boerke *et al*., 2008; Gadella *et al*., 2008) The bicarbonate-induced lateral redistribution of membrane components appears to be instrumental for the assembly of a functional sperm protein complex involved in sperm-zona binding as well as for the zona-induced acrosome reaction (Tsai *et al*., 2007; Van Gestel *et al*., 2007; Ackermann *et al*., 2008). Therefore, the researcher interested in the surface proteome of sperm needs to, beyond the composition of sperm surface proteins, consider how these proteins are organized and whether they are functionally complexed for there to be a physiological role in fertilization.

Beyond bicarbonate-induced effects during sperm capacitation it is also important to understand that the milieu of the sperm cell during transit from the testis to the oviduct as proteins from the genital tract epithelia and fluids may also affect the composition and organization of proteins on the sperm surface. This in turn might influence the potential to fertilize the oocyte. These different environments and events include the passage through the epididymis and epididymal maturation, re- and decoating events induced by the accessory fluids combined at ejaculation probably stabilising the sperm for its further journey in the female genital tract, the removal of extracellular glycoproteins (release of decapacitation factors) and further remodelling by (cervical), uterine and oviduct secretions in the female tract which leads to *in vivo* capacitation (Gadella, 2009). In addition sperm interact with ciliated epithelial cells in the oviduct (Sostaric *et al*., 2008) and this probably has a physiological role during capacitation.

Sperm interactions with other ciliated epithelial cells of the female and male genital tract have not been studied extensively but it is possible that such interactions are also important for sperm surface remodelling and function. Finally, the sperm also interacts with cumulus cells and the remaining follicular fluid components surrounding and impregnating the zona pellucida and may even interact with oocyte components in the perivitelline space (Gadella, 2009).

It would be very interesting to assess the effect of each of the events on the sperm surface proteome. At best one can probably only consider each potential process in turn within a defined *in vitro* model system. It may also be very useful to consider the proteomes of the fluid or epithelium being investigated in order to assess the effects on the sperm surface proteome. There is now good proteomic data on epididymal fluid, epididymosomes, prostasomes and seminal plasma (Fouchecourt *et al*., 2000; Gatti *et al*., 2005; Pilch and Mann, 2006; Thimon *et al*., 2008; Dacheux *et al*., 2009). On the female side there have been some excellent studies on the oviduct epithelium and fluid (Georgiou *et al*., 2005; Sostaric *et al*., 2006; Georgiou *et al*., 2007; Fazeli, 2008; Fazeli and Pewsey, 2008). For a more detailed discussion of the possible mechanisms by which the sperm surface might be altered by these epithelia and fluids please, see Gadella (2009).

Implications for future research

Proteomics and male infertility

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 (Shetty *et al*., 1999a, b; Domagala *et al*., 2007; Shetty *et al*., 2008; Shetty and Herr, 2009). 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) (Lefievre *et al*., 2003; Pixton *et al*., 2004; Conner *et al*., 2007; de Mateo *et al*., 2007; Barratt, 2008; Martinez-Heredia *et al*., 2008; Oliva *et al*., 2009). 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.

Proteomic quantification

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 and researchers are strongly encouraged to adopt these approaches in their future research. Difference gel-electrophoresis (DIGE) enables proteins in different samples to be labelled with one of three different fluorescent labels (CyDyes). These are then mixed together (multiplexed) and subjected to 2DE on one gel. Protein spots corresponding to the different samples can then be visualized by confocal laser scanning and detailed software analysis enables relative quantification of the same spots (proteins) from the different samples (Lilley and Friedman, 2004). This approach has clear advantages over the gel-to-gel comparison approach as it is statistically more robust and therefore can reliably detect much smaller changes in expression.

In the case of LC-based workflows quantification can be achieved using reagents that label peptides post trypsin digestion (typically via NHS-ester chemistry on free lysines). The most common approach uses iTRAQ reagents (isobaric tags for relative and absolute quantification) which allow for a 4-plex or even 8-plex comparison. Following peptide labelling up to eight different samples can be multiplexed and subjected to LC-MS/MS. In MS the tags are isobaric (same mass) and co-migrate but relative quantification is revealed by the cleavage of specific reporter ions from the iTRAQ label following MS/MS fragmentation (Unwin *et al*., 2006; Zieske, 2006).

To date there have only been two proteomic studies published in sperm that have used either of these approaches. Baker *et al*. (2005) used DIGE to investigate changes in sperm proteins during epididymal maturation and Asano *et al*. (2009) used iTRAQ to characterize the expression of a small number of proteins in different microdomains. The only other significant quantitative proteomic study in sperm to date used an alternative MS based labelling approach (Fisher esterification of phosphopeptides using differentially deuterated methyl alcohols) to quantify phosphoproteins in capacitated and non-capacitated cells (Platt *et al*., 2009).

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 this phenomena is known to be very important to a number of aspects of sperm function, including epididymal maturation (Baker *et al*., 2005; Aitken *et al*., 2007) and capacitation (Gadella and Visconti, 2006). The first proteomic studies involving both the identification of multiple phosphoproteins and also the sites of phosphorylation were conducted by Pablo Visconti and colleagues on human sperm (Ficarro *et al*., 2003) and the same group has recently published a mouse study (Platt *et al*., 2009). Phosphoproteomic studies are generally performed using peptide affinity based approaches with the enrichment of phosphorylated peptides by immobilized metal affinity chromatography or titanium dioxide and indeed these prefractionation approaches are essential (Thingholm *et al*., 2009).

Another post-translational modification, S-nitrosylation, has also been recently been characterized on a proteomic scale in humans using a biotin switch assay for protein enrichment and provided novel insights on the role of nitric oxide in capacitation (Lefievre *et al*., 2007). It is also possible to combine subcellular fractionation and protein enrichment. The best example in sperm is the use of nitrogen cavitation to produce a cytosolic fraction of bull sperm together with protein enrichment (affinity chromatography with poly-Glu: tyr) to enable the isolation and identification of four tyrosine

kinases that were specifically localized to the cell cytosol (Lalancette *et al*., 2006). This study clearly demonstrates the value of extensive pre-fractionation of the sample of interest even though there were many (126) proteins purified by this approach that were not tyrosine kinases.

Concluding comments

Moving forward we argue that researchers should use as much of the proteomic toolbox as possible in order to address their specific question. In this review, we have given an overview of novel proteomic methodologies to explore the plasma membrane in sperm at fertilization and have argued for a greater focus on the cell surface proteome to achieve greater biological understanding. We have stressed that it is critical to isolate the plasma membrane or a subpopulation of the plasma membrane (for example APMs or lipid rafts). It is difficult to compare studies on the surface proteome of human, mouse and farm animals as each mammalian species has its own technical drawbacks and advantages. In many studies the specificity of labelling methods and sperm surface separation from intracellular and extracellular components have not been analysed or at least not with high enough scrutiny. Finally, the complex and domain-dynamic organization of the sperm surface needs to be considered when studying the protein composition of the fertilising surface of sperm.

We recommend a combination of a sperm surface protein labelling method with a surface membrane isolation method prior to the immobilized affinity column steps. The resulting protein sample should then be solubilized using a range of conditions to distinguish between peripheral and integral membrane proteins. Preferably these solubilized samples should be analysed by LC-MS/MS. Where possible the additional use of geLC-MS/MS should be employed although there are also good arguments for using peptide in solution IEF as well. The advantage of these approaches is that peptides from membrane proteins are prepared using more stringent conditions than is possible with 2DE and should potentially allow the full range of coverage of sperm surface proteins. Researchers should also carefully consider protein or peptide enrichment studies where appropriate. Although this is a tremendous amount of work it should still represent a goal to which workers should aspire.

Although sperm cells have been the focus of this review our arguments for extensive subcellular fractionation apply equally to understanding the role of the plasma membrane in all cell types with important roles in reproductive biology and medicine. The challenge to researchers in the field of reproduction is to harness this technology to a greater extent than at present. Proteomics coupled with other approaches has considerable potential to greatly improve our understanding of the complex cellular processes in reproduction in both health and disease. Beyond this proteomics will undoubtedly be one of the major technologies in the discovery of novel diagnostics and therapeutics for reproductive health in the future.

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