Proteomic analysis of mammalian gametes and sperm-oocyte interactions

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Proteomic analysis occupies an increasingly important place in gamete and embryo biology as an independent tool of discovery and as a means of follow-up to transcriptional profiling. Proteomics have been and will be increasingly helpful in many areas of reproductive biology, including applied science and technology development. Areas likely to be impacted most rapidly by proteomic knowledge include fertility evaluation in male farm animals, male infertility diagnostics in humans, assessment and optimization of oocyte and embryo culture protocols, selection of fittest oocytes for assisted fertilization and selection of most competent embryos for embryo transfer. Oocyte proteomics will help us understand the process of oogenesis and oocyte maturation, and to discover non-invasive markers of oocyte quality. Sperm proteomics correlate with normal sperm structure and function and can be applied to discover novel biomarkers of farm animal fertility and diagnostic markers of human male infertility. Putative receptors participating in fertilization, as well as proteins acquired onto sperm surface from epididymal fluid and seminal plasma, have been discovered by proteomic analysis. An added level of information is provided by advanced proteomic approaches, capable of identifying posttranslational modifications such as phosphorylation, glycosylation and ubiquitination which play important functions in gametogenesis, fertilization and embryo development. By no means exhaustive, the present paper reviews some of the most interesting proteomic studies of mammalian gametes and embryos published in the last decade.

Introduction

Genomic revolution brought us transcriptional profiling which allowed for the identification of dozens of genes involved in mammalian gametogenesis, fertilization and preimplantation embryo development. The next major challenge facing reproductive biology is to apply meaningful approaches to analyze these gene products at the protein level. Proteomic analyses could therefore be used as a follow up to transcriptional profiling, or as an independent discovery tool. Proteomic follow-up of transcriptional profiling is important because of a lack of consistency and repeatability in microarray data wherein multiple levels of verification are necessary to confidently confirm differential expression. Also, some of the identified transcripts could be products of untranslated pseudogenes, or their transcription levels may differ from their translation levels. Other transcripts could represent non-coding RNAs with
distinct biological functions that are not to be translated. Finally, protein half life varies greatly and can be a cause of discrepancies between mRNA and the protein level of a given gene product in a given cell type.

Cataloging of the oocyte proteome will be instrumental in studies of the process of oogenesis and oocyte maturation. In addition, proteomic approach can be used to identify non-invasive markers of oocyte quality in the oocyte proteome and oocyte secretome. Quality control of oocyte maturation is of importance for farm animal embryo transfer technology and, in particular, for human assisted fertilization. If sensitive biomarkers are identified in the oocyte secretome, human oocytes or fertilized eggs could be cultured individually in media drops and the oocyte-exposed media could be collected and evaluated for the presence or abundance of protein biomarkers associated either with normal or deviant oocyte quality. Based on such a test, oocytes or zygotes with highest developmental potential could be selected for fertilization or embryo transfer, respectively.

Sperm proteomics correlate with normal sperm structure and function and can also be used for comparison of normal and subfertile/infertile sperm samples. Furthermore, the carryover of proteins translated during the haploid phase of spermatogenesis, the spermiogenesis, can be informative of the mechanisms involved in spermatid differentiation into a spermatozoon.

During fertilization, sperm structures such as the acrosome are lost but their remains (e.g. the acrosomal shroud or ghost) can be collected from the egg coats of the fertilized oocytes. By tagging spermatozoa with biotin before fertilization, sperm proteins that interacted with the egg coat can be purified and identified by proteomics. This can lead to the identification of sperm surface receptors involved in sperm-zona pellucida interactions during fertilization. Of equal importance are the proteomics of seminal plasma and epididymal fluid, since it is in the epididymis where many sperm surface proteins are acquired that convey sperm fertilization-potential. Advanced proteomic approaches, capable of identifying posttranslational modifications such as phosphorylation, glycosylation and ubiquitination, pick up where conventional proteomics leave off, adding a new layer of information to straight protein identification in gamete and embryo biology.

While not all inclusive, the present paper reviews a select group of recent reports on proteomes of mammalian gametes, with particular emphasis on reports relevant to sperm fertilizing ability and oocyte developmental potential. Readers are referred to other recent review articles to complement the information reviewed here (Sirard et al. 2003; Aitken & Baker 2008; Katz-Jaffe & Gardner 2008; Oliva et al. 2008). Some proteins related to the ubiquitin-proteasome pathway are discussed in more detail, as this pathway is the main focus of research in our laboratory.

**Oocyte secretome**

The importance of assessing oocyte secretome, i.e. the protein composition of culture media enriched by oocyte-secreted or oocyte-leaked proteins, lies in its potential for identifying molecular biomarkers of good or poor oocyte competence for fertilization and embryo development. Thus far, ubiquitin was reported as the only informative marker of mouse and human embryo quality (Katz-Jaffe et al. 2006). Ubiquitin is the central protein of the substrate-specific, proteolytic ubiquitin-proteasome pathway (Glickman & Ciechanover 2002). Ubiquitin can occur as a free protein (as seems to be the case of secretome in good quality oocytes and embryos), or covalently linked to other proteins in form of a monomer (monoubiquitination), dimer (diubiquitination), tetramer (tetraubiquitination) or polymer (polyubiquitination). Tetra and polyubiquitiation serve as signals for protein degradation by the 26S proteasome, an event
central to a number of physiological cellular mechanisms (e.g. cell cycle regulation, antigen presentation by immune system cells) and pathologies (e.g. Alzheimer’s, AIDS, liver cirrhosis). The housekeeping function of ubiquitin-proteasome pathway, i.e. the recycling of outlived and damaged proteins is certain to play an important role in oocyte and embryo metabolism.

**Oocyte proteome**

Mass spectrometry of protein spots excised from two dimensional PAGE gels of porcine oocytes before and after meiotic maturation revealed 35 abundant oocyte proteins, including zona pellucida proteins, cytoskeletal proteins, redox proteins and ubiquitin-system proteins (Ellederova et al. 2004). Relative abundance of these proteins was compared to that of actin, revealing that spermine synthase, peroxiredoxin and ubiquitin C-terminal hydrolase L1 (UCHL1) were extremely abundant in the porcine oocyte proteome, even more so than actin (Ellederova et al. 2004). Among these proteins, UCHL1 amount doubled between the germinal vesicle (GV) and metaphase II (MII) stage of oocyte maturation (Ellederova et al. 2004).

The role of UCHL1, and related UCHs and ubiquitin-specific proteases (USP) is to remove ubiquitin from substrate proteins (deubiquitination) and regenerate the available monoubiquitin pool by disassembling the multi-ubiquitin chains. A detailed study of porcine fertilization revealed that UCHL1 is concentrated in the oocyte cortex, where it may regulate the events of sperm oolemma-fusion and sperm incorporation (Yi et al. 2007a). Based on these studies, a role of UCHL1 and a related enzyme UCHL3 was proposed in the regulation of anti-polyspermy defense. Remarkably, electrofusion of a donor cell fibroblast during somatic cell nuclear transfer (SCNT) produced an UCHL1 free cortex area on the oocyte pole to which the donor cell was fused (Yi et al. 2007a). The cortical accumulation of UCHL1 is also observed in bovine (PS, unpublished) and murine (Sekiguchi et al. 2006; Yi et al. 2007a) oocytes. The gracile axonal dystrophy (gad) mutant mice expressing a truncated form of UCHL1 (Saigoh et al. 1999) were subfertile in mating studies and appeared to produce many polyspermic zygotes by in vitro fertilization (Sekiguchi et al. 2006). It is not clear whether the in vivo produced gad mutant zygotes are also polyspermic.

A follow up study confirmed the high abundance of UCHL1 in porcine oocytes and provided evidence that this enzyme participates in the regulation of oocyte meiosis-I, particularly at metaphase-anaphase transition (Sisor et al. 2007). Such a role is consistent with the requirement of ubiquitin-dependent proteolysis for metaphase-anaphase transition in somatic cells, wherein the ubiquitin conjugating and deubiquitinating enzymes are constituents of the anaphase promoting complex (APC) (Pesin & Orr-Weaver 2008). In summary, UCHL1 is likely important for protein turnover in the oocyte and embryo, but it also has more specific functions in the cell cycle control during meiosis, and in the regulation of oocyte cortex and anti-polyspermy defense during fertilization. Remarkably, UCHL1 was also identified as one of ten most abundant proteins in the bovine oocyte proteome (Massicotte et al. 2006). Besides UCHL1, ubiquitin conjugating enzyme E2D3, chaperone proteins HSP70, HSC71, cyclophilin A (CYPAT), and CCTs, glutathione-S-transferase GSTM5 (anti-oxidant), 2,3-bisphoglycerate mutase (2,3-BPMG; glycolytic pathway enzyme), epidermal fatty acid binding protein E-FABP (lipid transport protein), and two actin isoforms were identified (Massicotte et al. 2006).

The major vault protein (MVP; lung resistance-related protein/LRP) was also revealed to be an abundant protein in the pig oocyte proteome (Ellederova et al. 2004). A more recent study demonstrated that MVP, which is the major ribonucleoprotein component of the cytoplasmic vault particle (Kickhoefer & Rome 1994), accumulated during oocyte maturation in porcine ova and showed aberrant accumulation patterns in morphologically abnormal human...
oocytes (Sutovsky et al. 2005). High MVP levels would appear to be desirable in oocytes and preimplantation embryos as this drug resistance-related protein is thought to have a cell protecting activity (Scheffer et al. 2000). High content of MVP protein could have positive influence on oocyte maturation and preimplantation development; the MVP containing vault particles protect cells from stress and conveys resistance to chemotherapy drug treatment in cancer cells (Steiner et al. 2006). The turnover of MVP in porcine oocytes and zygotes is regulated by ubiquitin-dependent proteasomal proteolysis (Sutovsky et al. 2005). Translation and turnover of MVP appears to be misregulated in cloned porcine embryos and could contribute to their reduced developmental competence (Antelman et al. 2008).

Proteomes of immature and mature bovine oocytes were compared using saturation labeling (2-D DIGE)(Berendt et al. 2009). Ten proteins were identified as significantly different in their amount between immature and mature oocytes. These included the Ca(2+)-binding protein/translationally controlled tumor protein, enzymes of the Krebs and pentose phosphate cycles, clusterin, 14-3-3 epsilon signaling protein, elongation factor-1 gamma, and redox enzymes including GST Mu 5 and peroxiredoxin-3 (Berendt et al. 2009).

Large scale proteomic profiling of mouse metaphase II oocytes identified 380 proteins, including 53 putative phosphoproteins (Ma et al. 2008). According to gene ontology annotation, most abundant categories included protein metabolism, protein binding, and hydrolase activity.

Sperm proteomics

Comprehensive analysis of the human sperm proteome was undertaken as part of a contraceptive target identification project at Wyeth Research, an effort that also generated a valuable depository of testicular and epididymal transcriptomes and sperm protein sequences (Johnston et al. 2005). A total of 1,760 proteins were identified, of which 1,350 proteins were associated with the soluble sperm fraction, 719 with the insoluble fraction, and 309 identified in both fractions. The high content of insoluble fraction-associated proteins in the sperm proteomes is likely a result of a high level of disulfide bond cross-linking and detergent resistance observed in the sperm accessory structures. Dominant ontologies by protein function included, in decreasing order of identified protein number, protein folding & degradation, cytoskeletal function, cell growth/maintenance and metabolism in the soluble fraction and cytoskeleton, protein folding/degradation, and cell growth/maintenance in the insoluble fraction (Johnston et al. 2005). Twenty seven different subunits of the 26S proteasome, a multi-subunit protease implicated in fertilization process (Sutovsky et al. 2004), were identified.

Baker et al. (2008a) identified 829 rat sperm proteins by using prefractionation of sperm extracts with narrow range immobilized pH gradient (IPG) gel strips. This technique, described by (Essader et al. 2005), can increase the chance of identifying low abundance proteins. Identified sperm proteins were clustered according to subcellular localization, molecular function, and biological processes in which they were known to participate. By protein annotation/localization, 82% of identified proteins were intracellular, 29% were mitochondrial proteins, and only 14% were annotated as cytosolic proteins, which is understandable due to low abundance of cytoplasm in spermatozoa. Molecular function was attributed to 600 proteins, of which 69% possessed a binding domain and 66.5% contained a catalytic domain. Among proteins of interest to gamete and fertilization biologists were superglobulin family protein IZUMO and ADAM family disintegrin-proteins implicated in sperm-oolemma fusion during fertilization, ion transporters, 26S proteasome subunits implicated in both spermatogenesis and fertilization, proteins related to spermatid elongation/differentiation and proteins related to sperm maturation.
The same group also analyzed 858 proteins in the mouse sperm proteome and found some of the same proteins as described above for rat sperm proteome. As an example of this conserved proteome composition, they reported that 26 of the 42 identified proteases were subunits of the 26S proteasome (Baker et al. 2008c). Other proteins of interest included previously characterized sperm proteins IZUMO, zonadhesin, CatSper4, and ODF1 & 2.

Proteomic analysis can be used to compare protein composition of fertile and infertile/defective spermatozoa, potentially identifying novel biomarkers of male fertility. In such comparisons of high and low fertility bull sperm proteomes, 125 differentially expressed proteins have been identified, including proteins involved in sperm-oocyte interactions such as IZUMO, CRISP and ADAM-family proteins including fertilin alpha (Peddinti et al. 2008). Proteins involved in metabolism, cell signaling, spermatogenesis and cell motility were upregulated in the high fertility group. In particular, proteins involved in EGF signaling, PDGF signaling, oxidative phosphorylation, and pyruvate metabolism pathways were upregulated in the high fertility group (Peddinti et al. 2008).

For human infertility research, proteomes of normozoospermic men and asthenozoospermic patients were compared, and 17 differentially expressed proteins were identified (Oliva et al. 2008). They were actin-B, annexin-A5, cytochrome C oxidase-6B, histone H2A, prolatin-inducible protein and precursor, calcium binding protein-S100A9 (2 spots), clusterin precursor, dihydrodihydroamide dehydrogenase precursor, fumarate hydratase precursor, heat shock protein-HSPA2, inositol-1 monophosphatase, 3-mercapto-pyruvate sulfurtransferase/dienoyl-CoA isomerase precursor, proteasome subunit-PSMB3 (20S proteosomal core subunit beta 3), semenogelin 1 precursor and testis expressed sequence 12.

In a separate study from the same group, the relative abundance of 58 identified proteins correlated significantly with the expression of other proteins in sperm samples from 47 infertile patients and 10 fertile donors (de Mateo et al. 2007). Several proteasomal subunits were identified, with inter-correlated expression patterns. The 26S proteasome has been increasingly implicated in several steps of mammalian fertilization (Yi et al. 2007b). Eight proteins, including proteasomal subunit PSMA6 and mitochondrial membrane protein prohibitin correlated positively with sperm DNA fragmentation revealed by TUNEL assay (de Mateo et al. 2007). Prohibitin also showed increased levels in sperm samples with abnormally low ratios of protamine 1 to protamine 2, an indicator of sperm chromatin structure. Of importance, prohibitin ubiquitination in the bull sperm mitochondria has been implicated in the mechanism of targeted degradation of paternal mitochondria after fertilization (Thompson et al. 2003).

Proteomics have been applied to identify sperm antigens that could induce autoimmune infertility in humans (Bohring & Krause 2003). Human antisperm antibodies were found to recognize 18 major antigens in the preparations of human sperm plasma membrane and acrosomal membrane proteins, including heat shock proteins HSP70 and HSP70-2, disulfide-isomerase-ER60, caspase-3 and two different proteasomal subunits (Bohring & Krause 2003). Major proteins most frequently identified by human antisperm antibodies in the proteomic screen of mouse sperm extracts included apo lactate dehydrogenase (LDHC4), voltage-dependent anion channel (VDAC2), outer dense fiber protein ODF2 and glutathione S-transferase mu5 (Paradowska et al. 2006).

**Epididymal fluid and sex accessory gland**

Studying epididymal fluid (EF) proteome, which represents the secretome of epididymal epithelial cells and proteins released from epididymal spermatozoa, is of importance to gamete and fertilization biology. It is during epididymal maturation and storage when spermatozoa
Table 1. Listing and proposed functions of most abundant and reproductively essential proteins identified in mammalian gametes and reproductive tissues or fluids by proteomic analysis.

<table>
<thead>
<tr>
<th>Protein designation</th>
<th>HUGO nomenclature</th>
<th>General function and/or proposed function in reproductive process</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oocyte</strong></td>
<td></td>
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<tr>
<td>Spermine synthase</td>
<td>SMS</td>
<td>Conversion of spermidine to spermine, a polyamine involved in cell growth and maintenance.</td>
</tr>
<tr>
<td>Peroxiredoxins</td>
<td>PDX1, PDX2, PDX3</td>
<td>Maintenance of redox potential; support oocyte metabolism, energy balance.</td>
</tr>
<tr>
<td>Ubiquitin C-terminal hydrolase L1</td>
<td>UBL1</td>
<td>Protein deubiquitination, disassembly of multiubiquitin chains, maintenance of stable pool of unconjugated ubiquitin. Proposed to have a function in oocyte meiosis, fertilization and anti-polyspermy defense. Female mutant mouse (gad mutant) is subfertile.</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>UBB</td>
<td>Protein turnover/recycling, cell cycle control; also present in oocyte secretome. Mutations are lethal.</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2D3</td>
<td>UBE2D3</td>
<td>Protein ubiquitination; protein turnover/recycling.</td>
</tr>
<tr>
<td>Major vault protein</td>
<td>MVP</td>
<td>Cell protection, drug resistance, nucleo-cytoplasmic transport. Supports embryo development, may protect oocyte and embryo during in vitro culture.</td>
</tr>
<tr>
<td>Zona pellucida glycoproteins</td>
<td>ZPA, ZPB, ZPC</td>
<td>Structural proteins of the egg coat, zona pellucida; oocyte protection, sperm receptor activity during sperm-zona binding, induction of sperm acrosomal exocytosis during fertilization. KO mice are infertile.</td>
</tr>
<tr>
<td>Hsp70, Hsc71, Cypa, Cct-e</td>
<td>HSP70, HSC71, CYP, CCT-e</td>
<td>Stress defense, protein folding, prevention of protein aggregation; protein targeting for recycling, protein transport.</td>
</tr>
<tr>
<td>Glutathione-S-transferase</td>
<td>GSTM5</td>
<td>Cell-protectant; anti-oxidant; maintains redox balance.</td>
</tr>
<tr>
<td>Epidermal fatty acid binding protein</td>
<td>E-FABP</td>
<td>Lipid transport.</td>
</tr>
<tr>
<td>Actin</td>
<td>ACTB</td>
<td>Cytoskeletal component.</td>
</tr>
<tr>
<td>Clusterin</td>
<td>CLU</td>
<td>Sperm maturation, complement regulation, lipid transport, cell-cell adhesion.</td>
</tr>
<tr>
<td><strong>Spermatozoon</strong></td>
<td></td>
<td></td>
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<tr>
<td>IZUMO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM family disintegrins/fertilins</td>
<td>ADAM2, ADAM3</td>
<td>Sperm-oolemma adhesion during fertilization; KO mouse is infertile.</td>
</tr>
<tr>
<td>Ion channels/transporters</td>
<td>CATSPER1</td>
<td>Sperm-oolemma adhesion during fertilization.</td>
</tr>
<tr>
<td>26S proteasome subunits</td>
<td>PSMA1-7, PSMB1-10, PSMC1-6, PSMD1-14</td>
<td>Mutant mouse is infertile due to lack of hyperactivated sperm motility.</td>
</tr>
<tr>
<td>Protamines</td>
<td>PRM1, PRM2</td>
<td>Degradation of ubiquitinated proteins; role in acrosomal exocytosis and sperm-egg coat penetration. Mutations of several subunits are lethal.</td>
</tr>
<tr>
<td>Zonadhesin</td>
<td>ZAN</td>
<td>Sperm DNA hypercondensation.</td>
</tr>
<tr>
<td>Cysteine rich secretory proteins</td>
<td>CRISP1 &amp; 2</td>
<td>Sperm-zona binding; sperm-oolemma adhesion.</td>
</tr>
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<tr>
<td><strong>Gamete and embryo proteomics</strong></td>
<td></td>
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<tr>
<td>Outer dense fiber proteins</td>
<td>ODF1, ODF2</td>
<td>Formation of sperm flagellar structures.</td>
</tr>
<tr>
<td>Protein kinase A-anchoring proteins</td>
<td>AKAP3, AKAP4</td>
<td>Sperm motility, PKA sequestration in the sperm tail principal piece.</td>
</tr>
<tr>
<td>Sperm adhesion molecule 1</td>
<td>SPAM1</td>
<td>Hyaluronidase activity, sperm maturation, storage, sperm penetration through cumulus oophorus; abundant in sperm membrane lipid rafts.</td>
</tr>
<tr>
<td>Tubulins</td>
<td>TUBA, TUBB</td>
<td>Cytoskeleton; major component of sperm flagellar axoneme.</td>
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<tr>
<td><strong>Epididymal fluid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine</td>
<td>CLU</td>
<td>Spnr maturation, sperm protection from stress, microbes, immune attack; complement regulation, lipid transport, cell-cell adhesion.</td>
</tr>
<tr>
<td>Alpha-mannosidase</td>
<td>MAN2</td>
<td>Protein deglycosylation, branched sugar trimming, sperm maturation.</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>LTF</td>
<td>Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.</td>
</tr>
<tr>
<td>Osteopontin/secreted phosphoprotein 1</td>
<td>SPP1</td>
<td>Cytokine; regulation of fertilization and preimplantation embryo development.</td>
</tr>
<tr>
<td>ODF1-4</td>
<td></td>
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</tr>
<tr>
<td>Alpha-L-fucosidase 2</td>
<td>FUCA2</td>
<td>Protein deglycosylation.</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>CTSD</td>
<td>Proteolysis; lysosomal component.</td>
</tr>
<tr>
<td><strong>Seminal plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine seminal plasma proteins</td>
<td>BSP1, BSP3, BSP5</td>
<td>Sperm binding to and detachment from oviductal sperm reservoir, sperm capacitation, maintenance of sperm motility during storage.</td>
</tr>
<tr>
<td>Clusterin</td>
<td>CLU</td>
<td>Sperm maturation, Sperm protection from stress, microbes, immune attack, complement regulation, lipid transport, cell-cell adhesion.</td>
</tr>
<tr>
<td>Spermadhesin</td>
<td>SPADH2</td>
<td>Sperm binding to oviductal reservoir; sperm-egg binding.</td>
</tr>
<tr>
<td>Semenogelin I &amp; II</td>
<td>SEMG1, SEMG2</td>
<td>Add viscosity to seminal plasma; form vaginal plug in rodents; proteolytic cleavage of semenogelins occurs during semen liquefaction.</td>
</tr>
<tr>
<td>Osteopontin/secreted phosphoprotein 1</td>
<td>SPP1</td>
<td>Sperm-egg binding, regulation of polyspermy in vitro, recombinant SPP1 improved embryo development in vitro.</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>ALB</td>
<td>Blood carrier protein; cholesterol acceptor during sperm capacitation.</td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>PLA2</td>
<td>Lipid regulation, fatty acid hydrolysis, cell signaling.</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>FN1-3</td>
<td>Cell adhesion, extracellular matrix formation.</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>LTF</td>
<td>Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.</td>
</tr>
<tr>
<td>Laminin</td>
<td>LAM</td>
<td>Cell adhesion, extracellular matrix formation.</td>
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acquire their fertilizing potential through posttranslational modifications of testicular origin—sperm proteins and addition of new epididymis-secreted proteins onto sperm surface. The acquisition of fertilizing capacity is first observed between the distal caput and proximal corpus epididymis, suggesting that secretory proteins of these epididymal regions are of particular importance for sperm function (Moore 1981). In recent years, the understanding of epididymal function expanded well beyond sperm storage function (Cornwall 2009). Most abundant epididymal proteins are those governing sperm maturation, epididymal protein turnover and sperm storage. Early biochemical and proteomic studies established that clusterin alone represents ~20-40% of total epididymal fluid protein in various mammalian species (Dacheux et al. 2003). Clusterin is a glycoprotein found in a number of tissues and biological fluids. Besides sperm maturation, clusterin has been implicated in complement regulation, lipid transport, cell-cell interactions and various diseases (Rosenberg & Silkensen 1995). Other abundant EF proteins include cytosine rich secretory protein CRISP implicated in sperm-oocyte interactions, lactoferrin, mannosidase and hexosoaminase (Syntin et al. 1996).

Proteins of the EF are secreted by epididymal epithelial cells via specialized membrane vesicles, apical blebs and epididymosomes, allowing for apocrine secretion of cytosolic proteins that in other cell types do not enter secretory pathways (Hermo & Jacks 2002, Baska et al. 2008, Cornwall 2009). Proteomics of human epididymosomes revealed 146 proteins, including enzymes (27%), adhesion molecules (14%), transporters and protein trafficking molecules (13%) and signal transducers (12%) (Thimon et al. 2008). Analysis of bovine caput and cauda epididymis epididymosomes by liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC-QToF-MS/MS) identified 10 proteins, including aforementioned clusterin and also aldose reductase, an enzyme involved in steroid hormone metabolism (Frenette et al. 2006). Cauda epididymal fluid proteins have also been analyzed with regard to fertility of dairy bulls. A total of 118 differential spots were identified in 2D gels of the EF fluid from high versus low fertility bulls. Amounts of alpha-L-fucosidase 2 and cathepsin D were more than two fold greater in high-fertility bulls, while prostaglandin D-synthase was upregulated in low fertility bulls (Moura et al. 2006). In another study, the same group used in vitro fertilization to assay the effect of seminal plasma from high and low fertility sires on sperm ability to penetrate the oocytes. Proteins identified as beneficial to fertilization included seminal plasma glycoproteins BSP A1/A2, BSP A3 and BSP 30 kDa, and also clusterin, albumin, phospholipase A(2) and osteopontin (Moura et al. 2007a). Osteopontin has been used as an additive to porcine in vitro fertilization medium, to improve embryo development and reduce the rates of polyspermic fertilization (Hao et al. 2006; Hao et al. 2008).

Proteomic data on boar seminal plasma have been reviewed previously (Strzezek et al. 2005). Most abundant proteins include spermadhesins, cell protectants with antioxidant properties, immune suppressors, sperm decapacitating factors and proteins implicated in the regulation of sperm motility via protein phosphorylation such as phosphotyrosine protein acid phosphatase.

Bovine seminal plasma proteins BSP-A1/A2, BSP-A3 and BSP-30 kDa represent 40-57% of bovine seminal plasma proteins (Nauc & Manjunath 2000). Spermadhesins, clusterin and osteopontin are also highly abundant (Moura et al. 2007b). The BSP proteins coat the sperm head and have been implicated in various aspects of sperm function, including sperm capacitation, maintenance of sperm motility within female reproductive system and sperm binding to and release from the oviductal sperm reservoir (Gwathmey et al. 2006). Detailed analysis of bull seminal plasma by 2D SDS-PAGE and MS/MS revealed 99 proteins, including BSP proteins, clusterin, spermadhesin 2 and osteopontin, but also 49 minor proteins that were not previously reported in seminal plasma of any species (Kelly et al. 2006). Seminal plasma
is one of eleven human body fluids covered by a recently established proteomic database (Li et al. 2009). Human seminal plasma was found to contain 923 proteins, with most abundant proteins being semenogelins, fibronectin, lactoferrin, laminin and serum albumin (Pilch & Mann 2006). Seminal plasma proteins display a wide range of posttranslational variants (Fung et al. 2004). The many roles of the above seminal plasma proteins include adding viscosity to seminal plasma, protecting spermatozoa from stress, microbes and immune cells, providing energy substrates, preventing premature sperm capacitation and mediating the interactions between spermatozoa and oviductal epithelial cells (Calvete & Sanz 2007, Muino-Blanco et al. 2008).

**Sperm-oocyte interaction proteomics**

During fertilization, sperm structures undergo irreversible changes, and some of them are lost or partially retained on the egg vestments. Proteomic studies profited from these irreversible and relatively stable binding reactions for identification of sperm proteins involved in interactions with oocyte surface.

van Gestel et al. (2007) isolated, purified and solubilized plasma membranes from boar spermatozoa and incubated such preparations with porcine zona pellucida fragments. They resolved the complex sperm and ZP proteins on 2D gels and identified the potential ZP-binding sperm proteins by using Q-Tof Nanospray MS/MS. They identified four dominant proteins in these ZP-bound sperm membranes, including spermadhesin AQN-3, lactcadherin/p47, fertilin beta and peroxiredoxin 5. Except for peroxiredoxin 5, all three remaining proteins have been implicated previously in sperm-oocyte interactions. Other sperm membrane-associated proteins included eight ADAM family disintegrins, ion channels and transporters, enzymes, secretory pathway associated proteins, acrosomal surface proteins and various plasma membrane receptors.

Sperm head tail-separation and subcellular fractionation of the sperm head components were applied to identify mouse sperm head proteins potentially involved in different steps of fertilization (Stein et al. 2006). To distinguish the sperm surface proteins from the rest, spermatozoa were surface biotinylated and membrane surface, membrane vesicle and acrosomal matrix fractions were recovered. Of the identified proteins, one third were products of genes for which the mutations have been shown previously to cause subfertility or infertility. Identified acrosomal proteins included glycolytic and proteolytic enzymes and enzyme inhibitors, receptor proteins for zona pellucida, secretory pathway proteins and proteins involved in protein folding (Stein et al. 2006). Identified proteins with a proposed function in fertilization included those implicated in sperm zona interactions (e.g. SP38/IAM38/ZPBP2), sperm-oolemma binding (ADAM-family proteins) and various acrosomal enzymes.

Biotinylated oolemmas were harvested to identify mouse oocyte surface proteins potentially involved in fertilization and zygotic development. The putative surface-labeled proteins identified by biotinylation included heat shock proteins HSP70 and HSP90a, chaperone proteins GRP94 and GRP78, oxygen regulated protein ORP150, calreticulin, calnexin and protein disulfide isomerase (Calvert et al. 2003).

Some proteins involved in sperm capacitation and acrosome reaction assemble within designated membrane microdomains, the lipid rafts (Boerke et al. 2008). Some of these proteins were identified in the mouse detergent-resistant membrane fractions (Miranda et al. 2009). Among the most abundant proteins were those previously implicated in fertilization process, including IZUMO and SPAM1. Other proteins of interest were membrane raft-associated proteins caveolin 2 and flotilin 2, TEX101 and hexokinase 1.
Posttranslational modification proteomics in gamete research

Phosphoproteomics are particularly important for sperm studies due to the importance of sperm protein phosphorylation for the process of sperm capacitation (Bailey et al. 2005). In spite of intensive research, relatively few capacitation-related phosphoproteins have been identified. A predominant one is the tyrosine phosphorylated p32 antigen/group of antigens migrating at 32 kDa in boar sperm extracts. These are acrosomal proteins including acrosin-binding protein sp32 and two triosephosphate isomerase isosforms (Bailey et al. 2005).

Proteins phosphorylated on Tyrosine residues were detected by 2-D Western blotting with anti-phosphotyrosine antibodies in non-capacitated versus capacitated mouse spermatozoa and identified by MS/MS. Among proteins identified were 20S proteasomal core subunit alpha 6 (PSMA1), VDAC, tubulin, PDHE1 beta chain, glutathione S-transferase, NADH dehydrogenase (ubiquinone) Fe-S protein 6, acrosin binding protein sp32-precursor and cytochrome b-c1 complex (Arcelay et al. 2008). Findings from an earlier study of capacitated human sperm phosphoproteome by the same group zeroed in on phosphorylated proteins in the sperm tail principal piece that are likely to convey signals for hyperactivated sperm motility observed in capacitated spermatozoa, including the A-kinase anchoring proteins (AKAP3 and AKAP4). Also identified was the valosin-containing protein (VCP) involved in substrate presentation to 26S proteasome (Ficarro et al. 2003). This study used Fe3+ immobilized metal affinity chromatography (IMAC) to enrich phosphopeptides generated by enzymatic digestion of sperm phosphoproteins prior to MS/MS. Preliminary data from a large scale proteomic study of sperm phosphoproteome have been presented recently, but not yet published as a peer-reviewed article (Baker et al. 2008b).

Oocytes have also been subjected to phosphoproteomics. Phosphoprotein profiling of bovine oocyte maturation identified 40 proteins belonging to various protein families such as protein kinases, cell cycle regulators, chaperone proteins, and cytoskeletal proteins (Bhojwani et al. 2006). Identified proteins included some found in other oocyte proteome studies discussed above, such as MVP, heat shock proteins and peroxiredoxin 2. Proteins differentially phosphorylated during oocyte maturation included beta-tubulin, beta-actin, cyclin E2, aldose reductase and UMP-synthase, protein disulfide isomerase 2 and peroxiredoxin 2.

We have been particularly interested in posttranslational modification of gamete proteins by ubiquitination. Ubiquitinated proteins can be purified by using the agarose-immobilized recombinant UBA domain of ubiquitin binding-protein p62 (Vadlamudi et al. 1996). Such proteins can be resolved on PAGE and excised for proteomic identification. Using this approach, we have identified three abundant, ubiquitinated proteins in porcine oocytes: MVP (Sutovsky et al. 2005), UCHL1 (Yi et al. 2007a), and TFAM (Antelman et al. 2008). Ubiquitin binds covalently to an internal Lys-residue of its substrate proteins through its C-terminal residue Gly-76, backed by Gly-75 and Arg-74. Consequently, trypsin digestion of ubiquitinated proteins around the ubiquitinated substrate Lys-residue produces a unique fingerprint-peptide with Gly-Gly adjunct and a mass increased by 114 Da (Peng et al. 2003). This “Gly-Gly” modification can be accounted for in proteomic database searches and used to identify the position of the ubiquitinated Lys-residue on the substrate protein. Studies are in progress to identify such ubiquitin-modified sites in several sperm and oocyte proteins that participate in fertilization.

Protein S-nitrosylation is a covalent posttranslational modification that results in the formation of an S-NO bond on a thiol group of a cysteine residue of the modified protein. Nitrosylation was examined in human spermatozoa by Lefievre et al. (2007). Sperm proteins in which nitrosylation was identified included proteins that were shown to be nitrosylated in other cell types (e.g. tubulin, GST and several heat shock proteins), as well as sperm/seminal plasma proteins such as the A-kinase anchoring proteins AKAP3 and AKAP4, and semenogelin 1 and
2. Similar to other sperm proteomes discussed above, subunits of the 20S proteasomal core were represented prominently.

Conclusions

Table 1 summarizes the proposed functions of the most prominent proteins found in mammalian gametes and secretions of the male sex accessory glands. Altogether, the present review illustrates positive impact of proteomics on our understanding of mammalian gametogenesis, fertilization and preimplantation embryo development. An outstanding challenge in basic gamete and embryo research is the optimization of strategies for a high throughput proteomic validation of transcriptional profiling data. Antibody arrays are being developed by several companies. Recent examples of such arrays applied to the study of mammalian oocyte and embryo is the screening of 400+ signal transduction proteins in porcine maturing oocytes by using Kinex antibody microarray (Pelech et al. 2008). The problem with antibody arrays is that at the present time only a small portion of changes observed in protein expression and posttranslational modification seem to be repeatable and validated by immunoblotting (Pelech et al. 2008). The large number of oocytes necessary for proteomic analysis is another limitation, particularly in studying rodent and human oocytes. This issue will likely be mitigated by an increased use of large animal models in basic gamete research and by steadily increasing sensitivity and decreasing total protein requirement of novel proteomic protocols. Differential isobaric tagging of a protein sample, akin to the differential probe labeling employed by microarray technology, will allow for the accurate comparison of protein composition between samples.

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