

Proteomic analysis of mammalian gametes and sperm-oocyte interactions

P. Sutovsky

Division of Animal Sciences and Departments of Obstetrics, Gynecology and Women's Health, University of Missouri-Columbia, S141 ASRC, 920 East Campus Drive, Columbia, MO 65211-5300, USA

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 polyubiquitination 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 (Susor *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 (CYPA), and CCT ϵ , 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, prolactin-inducible protein and precursor, calcium binding protein-S100A9 (2 spots), clusterin precursor, dihydrolipoamide dehydrogenase precursor, fumarate hydratase precursor, heat shock protein-HSPA2, inositol-1 monophosphatase, 3-mercapto-pyruvate sulfurtransferase/dienoyl-CoA isomerase precursor, proteasome subunit-PSMB3 (20S proteasomal 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.

Protein designation	HUGO nomenclature	General function and/or proposed function in reproductive process
Oocyte		
Spermine synthase	SMS	Conversion of spermidine to spermine, a polyamine involved in cell growth and maintenance.
peroxiredoxins	PDX1, PDX2, PDX3	Maintenance of redox potential; support oocyte metabolism, energy balance.
Ubiquitin C-terminal hydrolase L1	UBL1	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 (<i>gad</i> mutant) is subfertile.
Ubiquitin	UBB	Protein turnover/recycling, cell cycle control; also present in oocyte secretome. Mutations are lethal.
Ubiquitin-conjugating enzyme E2D3	UBE2D3	Protein ubiquitination; protein turnover/recycling.
Major vault protein	MVP	Cell protection, drug resistance, nucleo-cytoplasmic transport. Supports embryo development, may protect oocyte and embryo during <i>in vitro</i> culture.
Zona pellucida glycoproteins	ZPA, ZPB, ZPC	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.
Heat shock chaperone proteins	HSP70, HSC71, CYPB, CCT- ϵ	Stress defense, protein folding, prevention of protein aggregation; protein targeting for recycling, protein transport.
Glutathione-S-transferase	GSTM5	Cell-protectant; anti-oxidant; maintains redox balance.
Epidermal fatty acid binding protein	E-FABP	Lipid transport.
Actin	ACTB	Cytoskeletal component.
Clusterin	CLU	Sperm maturation, complement regulation, lipid transport, cell-cell adhesion.
Spermatozoon		
IZUMO		Sperm-olemma adhesion during fertilization; KO mouse is infertile.
ADAM family disintegrins/ fertilins	ADAM2, ADAM3	Sperm-olemma adhesion during fertilization.
Ion channels/transporters	CATSPER1	Mutant mouse is infertile due to lack of hyperactivated sperm motility.
26S proteasome subunits	PSMA1-7 PSMB1-10 PSMC1-6 PSMD1-14	Degradation of ubiquitinated proteins; role in acrosomal exocytosis and sperm-egg coat penetration. Mutations of several subunits are lethal.
Protamines	PRM1, PRM2	Sperm DNA hypercondensation.
Zonadhesin	ZAN	Sperm-zona binding.
Cysteine rich secretory proteins	CRISP1 & 2	Sperm-zona binding; sperm-olemma adhesion.

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Protein designation	HUGO nomenclature	General function and/or proposed function in reproductive process
Outer dense fiber proteins	ODF1, ODF2	Formation of sperm flagellar structures.
Protein kinase A-anchoring proteins	AKAP3, AKAP4	Sperm motility, PKA sequestration in the sperm tail principal piece.
Sperm adhesion molecule 1	SPAM1	Hyaluronidase activity, sperm maturation, storage, sperm penetration through cumulus oophorus; abundant in sperm membrane lipid rafts.
Tubulins	TUBA, TUBB	Cytoskeleton; major component of sperm flagellar axoneme.
Clusterin	CLU	Sperm maturation, sperm protection from stress, microbes, immune attack; complement regulation, lipid transport, cell-cell adhesion.
Alpha-mannosidase	MAN2	Protein deglycosylation, branched sugar trimming, sperm maturation.
Lactoferrin	LTF	Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.
Osteopontin/secreted phosphoprotein 1	SPP1	Cytokine; regulation of fertilization and preimplantation embryo development.
Outer dense fiber proteins	ODF1-4	Formation of sperm flagellar structures.
Alpha-L-fucosidase 2	FUCA2	Protein deglycosylation.
Cathepsin D	CTSD	Proteolysis; lysosomal component.
Bovine seminal plasma proteins	BSP1, BSP3, BSP5	Sperm binding to and detachment from oviductal sperm reservoir, sperm capacitation, maintenance of sperm motility during storage.
Clusterin	CLU	Sperm maturation, Sperm protection from stress, microbes, immune attack, complement regulation, lipid transport, cell-cell adhesion.
Spermadhesin	SPADH2	Sperm binding to oviductal reservoir; sperm-egg binding.
Semenogelin I & II	SEMG1, SEMG2	Add viscosity to seminal plasma; form vaginal plug in rodents; proteolytic cleavage of semenogelins occurs during semen liquefaction.
Osteopontin/secreted phosphoprotein 1	SPP1	Sperm-egg binding, regulation of polyspermy in vitro, recombinant SPP1 improved embryo development in vitro.
Serum albumin	ALB	Blood carrier protein; cholesterol acceptor during sperm capacitation.
Phospholipase A2	PLA2	Lipid regulation, fatty acid hydrolysis, cell signaling.
Fibronectin	FN1-3	Cell adhesion, extracellular matrix formation.
Lactoferrin	LTF	Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.
Laminin	LAM	Cell adhesion, extracellular matrix formation.

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 hexosaminase (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 isoforms (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 phosphoproteins 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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References

- Aitken RJ & Baker MA 2008 The role of proteomics in understanding sperm cell biology. *International Journal of Andrology* 31 295-302.
- Antelman J, Manandhar G, Yi YJ, Li R, Whitworth KM, Sutovsky M, Agca C, Prather RS & Sutovsky P 2008 Expression of mitochondrial transcription factor A (TFAM) during porcine gametogenesis and preimplantation embryo development. *Journal of Cellular Physiology* 217 529-543.
- Arcelay E, Salicioni AM, Wertheimer E & Visconti, PE 2008 Identification of proteins undergoing tyrosine phosphorylation during mouse sperm capacitation. *International Journal of Developmental Biology* 52 463-472.
- Bailey JL, Tardif S, Dube C, Beaulieu M, Reyes-Moreno C, Lefievre I & Leclerc, P 2005 Use of phosphoproteomics to study tyrosine kinase activity in capacitating boar sperm. Kinase activity and capacitation. *Theriogenology* 63 599-614.
- Baker MA, Hetherington L, Reeves G, Muller J & Aitken RJ 2008a The rat sperm proteome characterized via IPG strip prefractionation and LC-MS/MS identification. *Proteomics* 8 2312-2321.
- Baker MA, Hetherington L, Reeves G, Smith N & Aitken RJ 2008b Comparative and phospho proteomics: Identification of protein changes during sperm maturation. In *41st Annual Meeting of The Society for The Study of Reproduction*. Kailua-Kona, Hawaii.
- Baker MA, Hetherington L, Reeves GM & Aitken RJ 2008c The mouse sperm proteome characterized via IPG strip prefractionation and LC-MS/MS identification. *Proteomics* 8 1720-1730.
- Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, Yi YJ & Sutovsky P 2008 Mechanism of extracellular ubiquitination in the mammalian epididymis. *Journal of Cellular Physiology* 215 684-696.
- Berendt FJ, Frohlich T, Bolbrinker P, Boelhaue M, Gungor T, Habermann FA, Wolf E & Arnold GJ 2009

- Highly sensitive saturation labeling reveals changes in abundance of cell cycle-associated proteins and redox enzyme variants during oocyte maturation in vitro. *Proteomics* 9 550-564.
- Bhojwani M, Rudolph E, Kanitz W, Zuehlke H, Schneider F & Tomek W** 2006 Molecular analysis of maturation processes by protein and phosphoprotein profiling during in vitro maturation of bovine oocytes: a proteomic approach. *Cloning and Stem Cells* 8 259-274.
- Boerke A, Tsai PS, Garcia-Gil N, Brewis IA & Gadella BM** 2008 Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology* 70 1188-1196.
- Bohring C & Krause W** 2003 Immune infertility: towards a better understanding of sperm (auto)-immunity. The value of proteomic analysis. *Human Reproduction* 18 915-924.
- Calvert ME, Digilio LC, Herr JC & Coonrod SA** 2003 Oolemmal proteomics-identification of highly abundant heat shock proteins and molecular chaperones in the mature mouse egg and their localization on the plasma membrane. *Reproduction Biology Endocrinology* 1 27.
- Calvete JJ & Sanz L** 2007 Insights into structure-function correlations of ungulate seminal plasma proteins. *Society for Reproduction and Fertility Suppl* 65 201-215.
- Cornwall GA** 2009 New insights into epididymal biology and function. *Human Reproduction Update* 15 213-227.
- Dacheux JL, Gatti JL & Dacheux F** 2003 Contribution of epididymal secretory proteins for spermatozoa maturation. *Microscopy Research Technique* 61 7-17.
- de Mateo S, Martinez-Heredia J, Estanyol JM, Dominguez-Fandos D, Vidal-Taboada JM, Balleca JL & Oliva R** 2007 Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. *Proteomics* 7 4264-4277.
- Ellederova Z, Halada P, Man P, Kubelka M, Motlik J & Kovarova H** 2004 Protein patterns of pig oocytes during in vitro maturation. *Biology of Reproduction* 71 1533-9.
- Essader AS, Cargile BJ, Bundy JL & Stephenson JL Jr** 2005 A comparison of immobilized pH gradient isoelectric focusing and strong-cation-exchange chromatography as a first dimension in shotgun proteomics. *Proteomics* 5 24-34.
- Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF et al.** 2003 Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *Journal of Biological Chemistry* 278 11579-11589.
- Frenette G, Girouard J & Sullivan R** 2006 Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. *Biology of Reproduction* 75 885-890.
- Fung KY, Glode LM, Green S & Duncan MW** 2004 A comprehensive characterization of the peptide and protein constituents of human seminal fluid. *Prostate* 61 171-181.
- Glickman MH & Ciechanover A** 2002 The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological Reviews* 82 373-428.
- Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P & Suarez SS** 2006 Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biology of Reproduction* 75 501-507.
- Hao Y, Mathialagan N, Walters E, Mao J, Lai L, Becker D, Li W, Critser J & Prather RS** 2006 Osteopontin reduces polyspermy during in vitro fertilization of porcine oocytes. *Biology of Reproduction* 75 726-733.
- Hao Y, Murphy CN, Spate L, Wax D, Zhong Z, Samuel M, Mathialagan N, Schatten H & Prather RS** 2008 Osteopontin improves in vitro development of porcine embryos and decreases apoptosis. *Molecular Reproduction and Development* 75 291-298.
- Hermo L & Jacks D** 2002 Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. *Molecular Reproduction and Development* 63 394-410.
- Johnston DS, Wooters J, Kopf GS, Qiu Y & Roberts KP** 2005 Analysis of the human sperm proteome. *Annals of the New York Academy of Sciences* 1061 190-202.
- Katz-Jaffe MG & Gardner DK** 2008 Symposium: innovative techniques in human embryo viability assessment. Can proteomics help to shape the future of human assisted conception? *Reproductive Biomedicine Online* 17 497-501.
- Katz-Jaffe MG, Schoolcraft WB & Gardner DK** 2006 Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertility and Sterility* 86 678-685.
- Kelly VC, Kuy S, Palmer DJ, Xu Z, Davis SR & Cooper CJ** 2006 Characterization of bovine seminal plasma by proteomics. *Proteomics* 6 5826-5833.
- Kickhoefer VA & Rome LH** 1994 The sequence of a cDNA encoding the major vault protein from *Rattus norvegicus*. *Gene* 151 257-260.
- Lefievre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WC & Barratt CL** 2007 Human spermatozoa contain multiple targets for protein S-nitrosylation: an alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics* 7 3066-3084.
- Li SJ, Peng M, Li H, Liu BS, Wang C, Wu JR, Li YX & Zeng R** 2009 Sys-BodyFluid: a systematical database for human body fluid proteome research. *Nucleic Acids Research* 37 D907-912.
- Ma M, Guo X, Wang F, Zhao C, Liu Z, Shi Z, Wang Y, Zhang P, Zhang K, Wang N et al.** 2008 Protein expression profile of the mouse metaphase-II oocyte. *Journal of Proteome Research* 7 4821-4830.

- Massicotte L, Coenen K, Mourof M & Sirard MA 2006 Maternal housekeeping proteins translated during bovine oocyte maturation and early embryo development. *Proteomics* 6 3811-3820.
- Miranda PV, Allaire A, Sosnik J & Visconti PE 2009 Localization of low-density detergent-resistant membrane proteins in intact and acrosome-reacted mouse sperm. *Biology of Reproduction* 80 897-904.
- Moore HD 1981 An assessment of the fertilizing ability of spermatozoa in the epididymis of the marmoset monkey (*Callithrix jacchus*). *International Journal of Andrology* 4 321-330.
- Moura AA, Chapman DA & Killian GJ 2007a Proteins of the accessory sex glands associated with the oocyte-penetrating capacity of cauda epididymal sperm from holstein bulls of documented fertility. *Molecular Reproduction and Development* 74 214-222.
- Moura AA, Chapman DA, Koc H & Killian GJ 2006 Proteins of the cauda epididymal fluid associated with fertility of mature dairy bulls. *Journal of Andrology* 27 534-541.
- Moura AA, Chapman DA, Koc H & Killian GJ 2007b A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. *Animal Reproduction Science* 98 169-188.
- Muñoz-Blanco T, Perez-Pe R & Cebrian-Perez JA 2008 Seminal plasma proteins and sperm resistance to stress. *Reproduction in Domestic Animals* 43 Suppl 4 18-31.
- Nauc V & Manjunath P 2000 Radioimmunoassays for bull seminal plasma proteins (BSP-A1/A2, BSP-A3, and BSP-30-Kilodaltons), and their quantification in seminal plasma and sperm. *Biology of Reproduction* 63 1058-1066.
- Oliva R, Martinez-Heredia J & Estanyol JM 2008 Proteomics in the study of the sperm cell composition, differentiation and function. *Systems Biology in Reproduction Medicine* 54 23-36.
- Paradowska A, Bohring C, Krause E & Krause W 2006 Identification of evolutionary conserved mouse sperm surface antigens by human anti-sperm antibodies (ASA) from infertile patients. *American Journal of Reproductive Immunology* 55 321-330.
- Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC & Memili E 2008 Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC System Biology* 2 19.
- Pelech S, Jelinkova L, Susor A, Zhang H, Shi X, Pavlok A, Kubelka M & Kovarova H 2008 Antibody microarray analyses of signal transduction protein expression and phosphorylation during porcine oocyte maturation. *Journal of Proteome Research* 7 2860-2871.
- Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D & Gygi SP 2003 A proteomics approach to understanding protein ubiquitination. *Nature Biotechnology* 21 921-926.
- Pesin JA & Orr-Weaver TL 2008 Regulation of APC/C activators in mitosis and meiosis. *Annual Review of Cell and Developmental Biology* 24 475-499.
- Pilch B & Mann M 2006 Large-scale and high-confidence proteomic analysis of human seminal plasma. *Genome Biology* 7 R40.
- Rosenberg ME & Silkensen J 1995 Clusterin: physiologic and pathophysiologic considerations. *International Journal of Biochemistry and Cell Biology* 27 633-645.
- Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosawa H, Harada T, Ichihara N, Wakana S, Kikuchi T et al. 1999 Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nature Genetics* 23 47-51.
- Scheffer GL, Schroeijers AB, Izquierdo MA, Wiemer EA & Scheper RJ 2000 Lung resistance-related protein/major vault protein and vaults in multidrug-resistant cancer. *Current Opinion in Oncology* 12 550-556.
- Sekiguchi S, Kwon J, Yoshida E, Hamasaki H, Ichinose S, Hideshima M, Kuraoka M, Takahashi A, Ishii Y, Kyuwa S et al. 2006 Localization of ubiquitin C-terminal hydrolase L1 in mouse ova and its function in the plasma membrane to block polyspermy. *American Journal of Pathology* 169 1722-1729.
- Sirard MA, Dufort I, Coenen K, Tremblay K, Massicotte L & Robert C 2003 The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals. *Reproduction Supplement* 61 117-129.
- Stein KK, Go JC, Lane WS, Primakoff P & Myles DG 2006 Proteomic analysis of sperm regions that mediate sperm-egg interactions. *Proteomics* 6 3533-3543.
- Steiner E, Holzmann K, Elbling L, Micksche M & Berger W 2006 Cellular functions of vaults and their involvement in multidrug resistance. *Current Drug Targets* 7 923-934.
- Strzeczek J, Wysocki P, Kordan W & Kuklinska M 2005 Proteomics of boar seminal plasma - current studies and possibility of their application in biotechnology of animal reproduction. *Reproductive Biology* 5 279-290.
- Susor A, Ellederova Z, Jelinkova L, Halada P, Kavan D, Kubelka M & Kovarova H 2007 Proteomic analysis of porcine oocytes during in vitro maturation reveals essential role for the ubiquitin C-terminal hydrolase-L1. *Reproduction* 134 559-568.
- Sutovsky P, Manandhar G, Laurincik J, Letko J, Caamano JN, Day BN, Lai L, Prather RS, Sharpe-Timms KL, Zimmer R et al. 2005 Expression and proteasomal degradation of the major vault protein (MVP) in mammalian oocytes and zygotes. *Reproduction* 129 269-282.
- Sutovsky P, Manandhar G, McCauley TC, Caamano JN, Sutovsky M, Thompson WE & Day BN 2004 Proteasomal interference prevents zona pellucida penetration and fertilization in mammals. *Biology of Reproduction* 71 1625-1637.
- Syntin P, Dacheux F, Druart X, Gatti JL, Okamura N & Dacheux JL 1996 Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. *Biology of Reproduction* 55 956-974.
- Thimon V, Frenette G, Saez F, Thabet M & Sullivan R 2008 Protein composition of human epididymosomes

- collected during surgical vasectomy reversal: a proteomic and genomic approach. *Human Reproduction* 23 1698-1707.
- Thompson WE, Ramalho-Santos J & Sutovsky P** 2003 Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biology of Reproduction* 69 254-260.
- Vadlamudi RK, Joung I, Strominger JL & Shin J** 1996 p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. *Journal of Biological Chemistry* 271 20235-20237.
- van Gestel RA, Brewis IA, Ashton PR, Brouwers JF & Gadella BM** 2007 Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. *Molecular Human Reproduction* 13 445-454.
- Yi YJ, Manandhar G, Oko RJ, Breed WG & Sutovsky P** 2007b Mechanism of sperm-zona pellucida penetration during mammalian fertilization: 26S proteasome as a candidate egg coat lysin. *Society for Reproduction and Fertility Supplement* 63 385-408.
- Yi YJ, Manandhar G, Sutovsky M, Li R, Jonakova V, Oko R, Park CS, Prather RS & Sutovsky P** 2007a Ubiquitin C-terminal hydrolase-activity is involved in sperm acrosomal function and anti-polyspermy defense during porcine fertilization. *Biology of Reproduction* 77 780-793.