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INVITED REVIEW

Proteomic insights into the maturation and capacitation of mammalian spermatozoa

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Spermatozoa represent the epitome of terminally differentiated, highly specialized cells. They are transcriptionally and translationally silent and yet manage to undergo a complete functional transformation after they leave the testes, entirely fuelled by post-translational modifications occurring during epididymal maturation and capacitation. The latter have been recognized as biological processes for more than half a century. However, the biochemical mechanisms that drive these events have remained elusive, as have the pathological mechanisms that lead to defective sperm function and infertility. In the past decade the combined power of advanced proteomics, biochemistry, and functional genomics has permitted an unprecedented improvement in our understanding of sperm cell biology. We can also predict that a systems-biology approach, in concert with the new tools provided by the 'omics' revolution, will lead to dramatic gains in our understanding in the near future. As a result of such advances, insights will be generated that should ultimately lead to significant improvements in our capacity to diagnose and treat the infertile male.

Keywords epididymis, proteomics, sperm capacitation, sperm maturation, spermatozoa

Introduction

It is often unappreciated how recently proteomics entered the mainstream of biomedical research. It was as late as 1988 that John Fenn (2002 Nobel Prize winner in Chemistry for the development of electrospray ionization; ESI) first demonstrated the ionization of amino acids at an American Society for Mass Spectrometry meeting in San Francisco. These exciting observations engendered belief that a new interface between chemistry and biology was emerging that would ultimately transform our understanding of the relationships between the protein content of a cell and its biology [Griffiths 2008]. Remarkably, only 3 years prior to this event, Franz Hillenkamp and Michael Karas, then of the University of Frankfurt, coined the phrase 'MALDI' (matrix assisted laser desorption ionization) [Karas et al. 1985] as a soft-ionization method that allowed the detection of alanine in the presence of tryptophan. Today these pivotal findings constitute the foundation of modern proteomics. Both MALDI and ESI are the methods of choice for the ionization of peptides and proteins for the bottom up- and top down- sequencing of organic molecules. However, the technology has progressed well beyond the horizons established by Fenn and Karas. Modern proteomic groups are now not only able to identify amino-acid sequences, but can readily determine post-translational modifications within proteins, including phosphorylation [Baker et al. 2010a; Larsen et al. 2005; Thingholm et al. 2006; Thingholm and Larsen 2009]. Although the mass identification of proteins (shotgun/MudPit analysis) was once the method of choice for creating proteomic inventories [Schirmer et al. 2003], this has now given way to more sophisticated methods of quantification. Using the latter, the researcher is no longer primarily interested in the proteomic profile of a given cell. Rather, current emphasis is on the way in which the post-translational modification (PTM) of proteins can modulate the rate and direction of biochemical pathways. Not only is this approach allowing the in-depth analysis of the proteomic composition of target cells, but it is also facilitating a better understanding of the molecular regulation of cell function. This is certainly the case for sperm cell biology.

Spermatozoa

Arguably the most highly differentiated and unique cell type in the body, human males produce spermatozoa at about a rate of 1,000 cells/sec. After their initial morphological differentiation, spermatozoa are released from the germinal epithelium of the testes in a functionally immature condition, incapable of movement or any of the complex array of cellular interactions that are required for fertilization. The acquisition of functionality is driven heavily by PTMs

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that occur during descent of a highly specialized region of the male reproductive tract, the epididymis [Lin et al. 2006], in a process known as epididymal maturation [Aitken et al. 2007; Baker et al. 2003b; Baker et al. 2010a; Baker et al. 2005a]. As a consequence of this maturation process, spermatozoa acquire the potential for fertilization. However, this potential is not realized until these cells have undergone a further round of biochemical and biophysical changes in the female reproductive tract, in a process termed capacitation [Visconti et al. 1995a].

What makes the process of both epididymal maturation and capacitation remarkable is that both occur in the absence of nuclear gene transcription and significant protein translation [Boerke et al. 2007; Engel et al. 1973; Hernandez-Perez et al. 1983]. Thus, PTMs are a major component of the post-testicular processes driving sperm development [Aitken and Baker 2008; Aitken et al. 2007]. These may be canonical PTMs, including phosphorylation [Baker et al. 2003b] as well as non-canonical pathways which include the involvement of reactive oxygen species [Aitken et al. 1989; Aitken et al. 1992; Baker et al. 2003a; Baker et al. 2004; Baker et al. 2005b]. In order to resolve the molecular mechanisms regulating these functional changes, two specialized proteomic approaches have been utilized, including two-dimensional gel electrophoresis (2DE) and more recently, liquid chromatography coupled to mass spectrometry (LC-MS).

Reproductive lessons from the proteome, 2DE

The high resolving power of 2DE is achieved through the combined orthogonal separation of proteins by their isoelectric point and molecular mass. 2DE has several visual representation advantages, not the least of which is the ability to detect post-translational modifications due to the presence of 'charge-train' patterns. However, 2DE has several limitations relative to LC-MS, including an inability to resolve extremely low (<10 kDa), or extremely high (>150 kDa) molecular weight proteins or those that are particularly hydrophobic or insoluble [Cho et al. 2008]. Furthermore, the dynamic range of 2DE (i.e., the ability to see low abundancy proteins in the presence of highly abundant proteins) is extremely limited, even though improvements in fluorescent dyes claim to have increased this range by a log order from 10^3 to 10^4 [Unlu et al. 1997]. In practical terms however, when using large format immobilized pH gradient strips, if the dynamic range of a cell is around 10^9, then, depending on the stain used, only the top <1-5% of the most abundant proteins within the cell will be visualized. This becomes an even more significant problem for blood-based proteomes, which may exhibit a dynamic range in the order of 10^{12} [Simpson et al. 2008]. Despite these limitations, 2DE still remains an excellent choice for the analysis of mammalian spermatozoa, due to the atypically low dynamic range. To demonstrate this, 1,000 protein spots have been identified in normozoospermic samples [Naaby-Hansen et al. 1997], of which, 98 unique protein identifications were ascertained. The best estimate of the human sperm proteome is that it contains around 2,500-3,000 unique proteins [Baker and Aitken 2009], thus 2DE is achieving around 3% sperm proteome coverage [Martinez-Heredia et al. 2006].

Although understanding the proteomic profile of a given cell is helpful, there is a lot of guess-work in deducing which proteins are modified in pathological situations. Hence, in a follow up-study, the same research group used 2DE as a tool to compare the protein profiles of 47 infertile patients to fertile donors. From the 101 spots that were present in at least three replicate gels, 67 qualified as undergoing significant changes in expression. This high number of changes (67%) may indicate that major defects have occurred during spermatogenesis leading to fundamental errors in sperm differentiation as a result of which a large number of proteomic changes were observed relative to normal cells. Alternatively, the high level of gel-to-gel variation seen with 2DE, may have spuriously created the impression that defective sperm function, is associated with a large number of proteomic changes [Cho et al. 2008; Unlu et al. 1997].

In a similar approach, others have reported changes in protein expression using 2DE to compare patients who experienced IVF failure with a cohort of normal donors. Of the 1,087 spots reported, 20 (a mere 2% change) were found to be differentially expressed [Pixton et al. 2004]. Two of the 20 could be identified with confidence and included Secretory Actin-Binding protein and Outer Dense Fiber protein 2. However, it is unclear at this stage how either of these protein's changes is detrimental to sperm function.

In order to overcome the inherent difficulties generated by the spermatozoon's dynamic range, sub-cellular fractionation approaches have been applied to 2DE in an attempt to increase the depth and clarity of the proteome. Along these lines, mouse sperm flagellae were isolated following treatment of the cells with 1% SDS and subjected to 2DE. All the spots detected were then excised for MALDI-TOF-TOF analysis [Cao et al. 2006]. What made this approach interesting was that many of the enzymes involved in glycolysis were shown to be present in these sperm flagellar extracts [Cao et al. 2006]. This finding is consistent with the phenotype of the sperm-specific glyceraldehyde-3-phosphate dehydrogenase KO mice published some years earlier [Miki et al. 2004]. These animals were shown to be infertile due to extremely poor motility, as a consequence of the lack of glycolytically-derived ATP production along the flagella [Miki et al. 2004]. Thus proteomics, in combination with functional genomics, has fashioned our understanding of how ATP is generated in the sperm tail, with the emphasis on glycolysis and not oxidative phosphorylation as previously suspected.

A second sub-cellular fractionation study concentrated on the isolation of sperm nucleoproteins followed by specialized 2DE, which succeeded in resolving a collection of basic nuclear proteins [Yoshii et al. 2005]. Quantification of these spots demonstrated that 2 out of 184 men undergoing male infertility diagnosis, exhibited a higher ratio of

protamine P1 compared to protamine P2, in contrast to the approximate 1:1 ratio observed in healthy men [de Yebra et al. 1998]. These data suggested that the sperm DNA regions with lower amounts of P2, may be more susceptible to DNA damage. Interestingly, reactive oxygen species have been suggested for a number of years to be involved in DNA damage [Aitken and Baker 2002], and these molecules most likely attack susceptible regions along the chromosomes characterized by poor protamination [Aitken 1989; 1994; 1999; Baker and Aitken 2005].

A final quantitation-based analysis of spermatozoa was performed on immature versus mature epididymal rat spermatozoa using 2DE [Baker et al. 2005a] . In this analysis, 60 protein species were significantly changed from approximately 1,500 spots present within the gels (a change of 4.0%). However, from these 60 spots, only 8 could be confidently identified by MALDI-TOF [Baker et al. 2005b]. One of these changes included the β– subunit of the mitochondrial F1-ATPase which underwent serine phosphorylation during epididymal maturation [Baker et al. 2005b]. This may reflect the mitochondrial membrane potential status, which appears to become established as spermatozoa progress through the epididymal lumen [Baker et al. 2005b].

Reproductive lessons from the proteome, LC-MS

A second, more versatile, yet complementary approach to 2DE is that of liquid-chromatography mass spectrometry or LC-MS. In brief, proteins are isolated from spermatozoa then proteolytically digested to enable a mass spectrometer to both detect the peptide(s) and then fragment a selected peptide to detect the daughter ions (known as MS/MS, tandem MS, or MS^2 scan) and enable peptide sequencing. The advantage of LC-MS compared to 2DE, is that both very low and very high molecular weight proteins, together with hydrophobic and insoluble proteins can be analyzed. The inherent problems associated with this analysis are again the dynamic range of the sample. While the latest state-of-the-art mass spectrometers can handle up to 10^5 orders of magnitude, this is again, well below the 10^9 needed for a whole-system approach. Secondly, proteolytic digestion of proteins causes a massive increase in sample complexity. Hence, there is no one method to obtain complete proteomic coverage to date.

Using linear ion trap mass spectrometry we and others have produced large inventories of the proteins present in human [Baker et al. 2007], rat [Baker et al. 2008a], mouse [Baker et al. 2008b], and bovine [Peddinti et al. 2008] spermatozoa. One major issue for proteomic analysis is the purity of the sample. Hence, elucidation of the ejaculated human sperm proteome involved pre-treatment of the samples with anti-CD45 magnetic beads to remove contaminating leukocytes, which are commonly encountered in such material. Furthermore, these cells were purified via Percoll fractionation to isolate only high quality spermatozoa [Baker et al. 2007]. To overcome contamination issues with rodent species, back-flushed caudal epididymal spermatozoa were used, which have a purity of 1 round cell/10^5 spermatozoa (99.999% pure) [Baker et al. 2008a; Baker et al. 2008b].

Due to the dynamic range issue, pre-fractionation of the sperm proteome is necessary to obtain maximal coverage of the proteins present. For this purpose, human sperm proteins were firstly separated by SDS PAGE (two lanes of 1% Triton X-100 soluble and insoluble proteins) [Baker et al. 2007], then excised, digested with trypsin, and run through the LC-MS system. This resulted in 1,056 successful protein identifications. One major lesson obtained from this analysis was a clear indication of how spermatozoa interact with their external environment. Although we knew that receptors must exist on the surface of human spermatozoa, five years ago nobody had definitively characterized any such molecules. Following production of the human sperm proteome, we managed to identify around 20 receptors. While a detailed discussion of these receptors has been written elsewhere [Aitken and Koppers 2011; Baker and Aitken 2009] the major insight generated by this analysis was that the behavior of spermatozoa is profoundly influenced by the molecular composition of the fluids in which they are bathed. While conventional assisted conception protocols involve suspension of spermatozoa in simple balanced salt solutions, *in vivo* these cells are surrounded by complex biological fluids containing factors that significantly influence their survival and potential for capacitation. For example in a follow-up study, it was found that, the addition of pro-survival factors such as prolactin or insulin, to the medium had a major effect on the longevity of these cells [Pujianto et al. 2010]. These effects were mediated by receptors for these growth factors in the sperm plasma membrane which, when ligand bound, promoted the phosphorylation of PI3 kinase and Akt. As a result of these phosphorylation events, downstream targets of Akt's action, including key mediators of apoptosis, are also phosphorylated and, in this state, prevent spermatozoa from defaulting to the intrinsic apoptotic pathway [Koppers et al. 2011]. These results have ramifications for sperm IVF-preparation and the long-term preservation of these cells. They also explain why human spermatozoa maintained in simple culture media for assisted conception show signs of deterioration within 24 hours whereas *in vivo* these cells will survive for up to a week in the female reproductive tract waiting for an egg to arrive. The development of optimized media for the culture and preservation of mammalian spermatozoa will be greatly facilitated by further detailed study of the proteomic structure of the sperm plasma membrane which will in turn provide important clues as to how these cells interact with their immediate surroundings.

A second application for LC-MS in the analysis of sperm function does not just involve creation of an inventory of proteins present in these cells but specifically targets peptide changes occurring within a given pathway. For this purpose, comparative quantification of the peptides is required so that peptide changes associated with the gain or loss of sperm function can be elucidated. Furthermore,

such comparative assessments are facilitated if the analysis is not performed on the entire complement of peptides present in a given sample but rather, is focused on a subset of peptides that are likely to play important roles in the regulation of sperm function. In this context, the primary focus of attention has been on the phosphoprotein composition of these cells. There are several strategies for achieving this aim with LC-MS, however, in the microcosm of sperm research, only two methods have been rigorously applied. The first involves differentially labeling peptides from non-capacitated and capacitated spermatozoa with isobaric substrates, mixing the samples, enriching for phosphopeptides, then running the samples through LC-MS. The advantage of this technique is the ability of the mass spectrometer to distinguish between the 'heavy' and 'light' isotopes. Thus, even in multiplexed samples, one can determine the origin of the peptide. Using this technology, the laboratory of Pablo Visconti has run 2DE on both non-capacitated and capacitated spermatozoa and through the use of anti-phosphotyrosine immunoblots, identified which phosphorylated peptides changed during this process [Ficarro et al. 2003]. The gel plugs of interest were excised and phosphopeptide enrichment performed in association with isobaric labeling of the peptides. Interestingly, 3 sites of tyrosine phosphorylation were found on AKAP3 and AKAP4, which are known regulators of capacitation.

The canonical pathway leading to capacitation includes an influx of bicarbonate (HCO^{3-}), most likely through a sodium-bicarbonate exchanger [Demarco et al. 2003], although the presence of this protein is yet to be definitively demonstrated. Together with calcium and reactive oxygen species, HCO^{3-} activates soluble adenylyl cyclase (sAC), which is essential for capacitation, as demonstrated in the corresponding mouse knockout studies [Aitken et al. 1998; Esposito et al. 2004]. The binding of cAMP generated by sAC to protein kinase A (PKA) leads to the dissociation of PKA catalytic subunit (PKAc) from its regulatory domain and activation of the former. It is here that tyrosine phosphorylation of AKAP3 most likely plays a role in facilitating PKAc release, although this has not been demonstrated experimentally. In addition to this finding, valosin-containing protein demonstrated higher tyrosine phosphorylation in capacitated samples [Ficarro et al. 2003], however it is unclear how this protein is functionally involved in this process. The weakness of combining 2DE with LC-MS is the simplicity of the comparison performed ($n = 1$), and the lack of sensitivity due to peptide losses incurred during sample handling prior to MS/MS identification. Therefore in a follow-up study, phosphopeptide enrichment was performed directly on peptide digests of mouse spermatozoa [Platt et al. 2009]. Using differential isotopic labeling, the authors noted an increase in proline directed serine phosphorylation during capacitation for 18 phosphopeptides. From the sequences obtained it was suggested that ERK1/2 might be the kinase responsible for these changes, however two different ERK pathway inhibitors, U0126 and PD098059, did not inhibit capacitation [Platt et al. 2009], making this prospect unlikely.

In our own studies, we initially tried to understand how post-translational protein modifications could affect sperm function by considering the entire peptide complement of the cells. These studies involved taking spermatozoa in different functional states, digesting the samples with trypsin and then pre-fractionating the peptides on IPG-strips. Following isoelectric focusing the strips were cut into 1 cm fragments, after which the peptides were extracted and subjected to LC-MS analysis. We then used label-free quantification (MS-based) in order to document the peptide changes occurring during epididymal maturation and subsequent capacitation. An example of MS-based quantification is shown in Figure 1. The top panels represent 3 biological replicates of peptides obtained from immature spermatozoa isolated from the caput epididymis, while the bottom panels depict 3 biological replicates of the peptides recovered from mature caudal epididymal spermatozoa. In these survey scans the full length peptides can be seen as the blue/red 'streaks' appearing over time (x axis) at the designated masses (m/z; y axis). Note that the panels presented in Figure 1 depict only a small fraction of the complete analysis since they focus on a narrow range of peptides between 850-900 Da, while the entire survey scan covers peptides from 300-2,000 Da. Label free quantitation involves matching peptides between replicates to account for the intra-treatment variation and then between samples to determine the degree of inter-treatment variation. Since the mass spectrometer 'counts' the amount of peptide present, it is possible to obtain statistics on up/down-regulation, or indeed the total loss/gain of peptides. Three peptides circled in Figure 1 were all found to be significantly up-regulated in immature spermatozoa.

When such techniques were used to compare capacitated and non-capacitated spermatozoa, we found over 52 protein changes occurring [Baker et al. 2008a]. Most of these had never been reported before and included the voltage dependent anion channel 3, Fascin-3, and sorbitol dehydrogenase. Additionally, many enzymes involved in glycolysis were seemingly altered during capacitation, suggesting major regulation of this pathway as spermatozoa enter the capacitated state. The finding that sorbitol dehydrogenase changed during capacitation was supported by directly measuring this enzyme's activity, which was clearly up-regulated during this process [Baker et al. 2008a]. This highlights the importance of the polyol pathway during capacitation and supports the work of others who showed that sorbitol can be used as a fuel source for both mouse sperm motility and protein tyrosine phosphorylation [Cao et al. 2009].

Although these data were of interest, it was evident that we needed to sharpen the focus of our approach in order to deepen our understanding of the molecular regulation of sperm function. As maturation and capacitation are both phosphorylation-dependent events, we chose to focus on the phosphoproteome, using titanium dioxide columns to isolate phosphopeptides and examine their expression in relational to sperm maturation. In one example of this work we examined the phosphoproteomic profile of spermatozoa derived from capacitated and non-capacitated rat

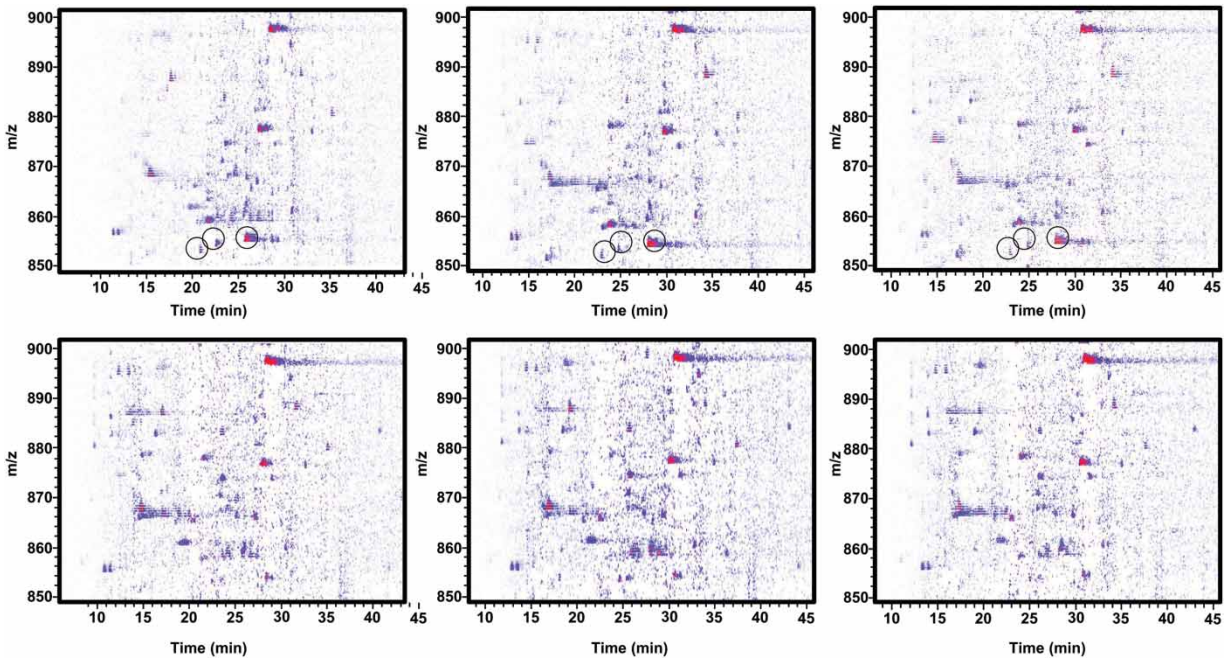

Figure 1. Sperm from the caput (Top panels) or cauda (Bottom panels) epididymides were lysed, digested, and run through nano-LC MS. Following quantification using the software profile analysis, three peptides within the region 850–900 Da were found to be significantly up-regulated in the immature sperm population.

spermatozoa [Baker et al. 2010b]. The rat may not be the ideal model for these studies because capacitation is so difficult to achieve in this species; however it does offer some advantages in that the spermatozoa can be isolated in an extremely pure and synchronized state and one rat yields 50 × more spermatozoa than a mouse. The latter is a critical consideration because phosphopeptides are in low abundance, so high amounts of starting material are necessary. From this analysis, we identified 120 phosphorylated proteins to be present in rat spermatozoa [Baker et al. 2010b]. From these, 15 proteins could be identified as changing in their phosphorylation status during capacitation, including ropporin [Li et al. 2007], the sperm-egg fusion protein Izumo [Inoue et al. 2005], and AKAP3. In a related analysis, we examined the phosphopeptide changes that occur during rat sperm epididymal maturation. In this case we could show 77 phosphopeptide changes, including instances where the entire protein was lost. This list resulted in the identification of around 12 phosphoproteins, some of which exhibited multiple phosphorylations and included ornithine-decarboxylase antizyme 3, heat-shock protein 90α, and testis-lipid binding protein [Baker et al. 2010a].

Notwithstanding our success in identifying proteins in these analyses, it should be emphasized that these definitive identifications represented a small proportion of all the proteins in which phosphopeptide changes were observed. In other words, although we could clearly demonstrate changes in numerous phosphopeptides, when we performed MS/MS on the peptides of interest, the tandem mass spectra could not be interpreted, and hence, we could not report the corresponding protein identification. We have now seen this problem consistently across a number of label-free studies, routinely finding no identification available for 50-70% of the peptides found to change in relation to sperm function.

To overcome our inability to interpret a high proportion of MS/MS spectra, we have incorporated several different strategies into our $MS^{2,}$ studies including the use of different fragmentation strategies (both collision induced- and electron transfer- dissociation) as well as different mass spectrometers, (for example, Q-ToF as well as linear and 3D- ion-trap). This is clearly a major limitation for comparative proteomics that will have to be addressed in the near future.

Reproductive Proteomics for Tomorrow

Although still in its infancy, proteomics, and in particular phosphoproteomics, will play a major role in improving our understanding of sperm function. Through the use of modern genomics, we are quickly gaining insights into the pathway of capacitation. Aside from the initial influx of HCO_{3-} and Ca^{2+} to activate the soluble adenylyl cyclase-PKA pathway, capacitation is associated with an increase in intracellular pH [pH]i [Vredenburgh-Wilberg and Parrish 1995; Zeng et al. 1995] and hyperpolarization of the plasma membrane [Arnoult et al. 1999]. The [pH]i activates the potassium channel SLO3 (also called Ksper) [Navarro et al. 2007], leading to sperm depolarization and activation of the calcium channel CatSper [Ren et al. 2001]. Ultimately, the influx of calcium ions causes the sperm flagella to shift from a low amplitude, high frequency, symmetrical beat pattern, to one characterized by high amplitude, low frequency, asymmetric beats, commonly known as hyperactivation. The regulation of these

conductance channels and transporters must involve intricate changes in the phosphorylation status of key proteins. Thus, inhibition of PKA [Visconti et al. 1995a; Visconti et al. 1999; Visconti et al. 1995b], sAC [Esposito et al. 2004; Xie et al. 2006], and the protein tyrosine kinase, SRC [Baker et al. 2006], all prevent the expression of hyperactivated movement. The involvement of SRC is complex, since this kinase is, in turn, thought to control a protein phosphatase, PP2A, which itself regulates capacitation via a parallel pathway [Krapf et al. 2010]. These advances in our understanding of the biochemical pathways regulating sperm function are recent, heavily dependent on proteomics, and herald the beginning of a new era in sperm cell biology triggered by new advances in mass spectrometry. Such technical developments are obviously critical to the future of the field, although it will be only through a systems biology approach, combining elements of genetics, proteomics, and cell biology that we shall truly appreciate and understand the molecular mechanisms regulating sperm function

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