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REVIEW ARTICLE

Molecular markers of human sperm functions

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Summary

Fertilization is a stepwise process that allows two mature gametes to reach each other, fuse and eventually give rise to a new individual. Despite the tremendous importance of reproduction for species development and maintenance, fertility is decreasing worldwide, with peaks in western countries. It is estimated that about 7% of men experiences problems in conceiving a child because of sperm defects. In such a situation, understanding which are the essential sperm players in each of the steps of the fertilization process is essential for the development of new pharmacological strategies to treat the infertile men, for genetic screening of idiopathic male infertility as well as to produce effective male contraceptive agents. The present review will summarize recent evidence for the identification and characterization of molecular markers of sperm functions with emphasis on post-ejaculatory maturation events and the process of sperm–oocyte interaction.

Introduction

Markers of sperm functions: is there still a role in the era of intracytoplasmic sperm injection (ICSI)?

Infertility affects up to 17% of couples and assisted reproduction techniques (ART) are increasingly applied in western countries (Bhasin, 2007). Male factor (pure or in association with female) contributes to about 50% and it is estimated that about 7% of men suffer from infertility in the general population. Pharmacological treatment of the male partner can only be successfully applied to non-idiopathic causes (such as hypogonadropic hypogonadism), whereas for idiopathic infertility, despite many attempts, virtually no treatment is available (Bhasin, 2007).

Intracytoplasmic Sperm Injection (ICSI) was first described in 1992 (Palermo *et al.*, 1992) and consists in forced injection of a sperm into the oocyte by means of a micromanipulator, bypassing all the barriers occurring in natural fertilization. ICSI provides a new hope for couples with severe male factor; however, it has dramatically changed the approach of the clinician to the infertile/subfertile male. If before ICSI the clinician approached the patient also with the intent of identifying the possible cause of infertility or of failure of first level ART applications or in vitro fertilization (IVF), all the efforts now are going on finding a single spermatozoon which could be available for ICSI procedure (Bhasin, 2007). Whether serious birth defects are increased in ICSI babies is still object of debate (Georgiou *et al.*, 2006; Verpoest & Tournaye, 2006). If birth defects after ICSI are present, they are more likely because of unknown genetic/functional problems (of unknown aetiology) of the gametes used for the procedure rather than to the procedure itself. Genetic or epigenetic defects in the spermatozoa used for ICSI could impact on the health and wellbeing of the offspring in several ways, not just by perpetuation of male infertility, and even if a phenotypic change is not present in the first generation it may appear in the future ones. In this light, the identification of molecular markers of each of the sperm functions necessary for the achievement of oocyte fertilization remains a major goal of research in sperm biology even in the ICSI era. Pharmacological intervention to treat male infertility with non-ART based therapies, genetic screening of idiopathic male infertility and development of male contraception, are the three main areas of research which may benefit from the identification/characterization of molecular targets on sperm. As new and highly sophisticated tools are now available for the scientific community, a rapid progress in this field is expected.

Problems in the identification of markers of sperm function

The process of fertilization allows two terminally differentiated cells (a sperm and an egg) to form a zygote, a totipotent cell which may give rise to a new individual. Before and during fertilization, many different steps need to be accomplished by the two individual gametes, and even if one of these steps is not achieved, a failure in the process occurs. Sperm and oocyte quality are equally important in the process; however, it must be considered that the sperm cell is subjected to more stressful events as it needs to 'travel' first in the male and then in the female genital tract to reach the oocyte. Although at first glance it may appear that motility is the main necessary sperm prerequisite to 'travel', the many different obstacles found by the sperm during the journey and the fact that the final steps of maturation (including acquisition of full ability to fertilize) occur during these phases of sperm life, suggests that other requisites are necessary. Conventionally, sperm quality is evaluated by semen analysis, which gives information on concentration, motility and morphology of sperm present in the ejaculate, with criteria for normal semen parameters established by the World Health Organization (1999). Overall, the weak correlations between semen parameters and the rate of natural conception, as well as the existence of couples with unexplained infertility, point out the limitations of this approach. Functional tests investigating the ability of spermatozoa to accomplish each of the single steps leading to fertilization have been developed in the past to overcome such limitations (reviewed in Aitken, 2006), but some of these require expensive equipments and notable technical ability with difficult application for routine purposes.

Studies on the identification of molecular markers of sperm function have mostly employed animal models of male infertility and in vitro experimental conditions mimicking the process of fertilization. Both approaches have limitations. Animal models should be validated in human, as differences in the pathways involved in each sperm function have been observed among species. In addition, studies in the human are mostly performed in ejaculated sperm whereas those performed in animals in epididymal ones, which have a different level of maturation. On the other hand, studies conducted in vitro may suffer from the inappropriate environment. In addition, experimental conditions may vary from one study to another (use of different media, use of demembranated sperm, different incubation times, sperm selection, etc.) making difficult the comparisons of the results.

Molecular markers of post-ejaculatory maturation events

Sperm capacitation

Defined as a process that allows spermatozoa to achieve the final competence to fertilize the oocyte, sperm capacitation is a poorly characterized, highly complex event, occurring, in natural conception, during sperm transit through the female genital tract (De Jonge, 2005). Capacitation is a time-related event initially promoted by loss of decapacitating factors (sperm membrane binding proteins secreted from the epididymis and accessory glands) from the sperm surface (Fraser *et al.*, 2006; Nixon *et al.*, 2006). The timing of the process may vary in vitro in sperm from subfertile subjects (Perreault & Rogers, 1982; Kholkute *et al.*, 1992). For this reason, it may be useful for the clinician to monitor the event with specific markers of the process before application of in vitro fertilization or other ART techniques.

Intracellular pathways activated during capacitation

Results of in vitro studies have documented the activation of many signalling pathways during the process of capacitation in human sperm (Fig. 1), but which pathway is absolutely required for the completion of the process remains to be established and redundancy cannot be excluded. Help in this matter might come from animal models of altered gene function and from proteomic analysis of proteins associated with the specific event. Unfortunately, animal models of altered gene function that result in clear capacitation defects are few and not really conclusive. Such studies indicate a role for the A1 adenosine receptor (Minelli *et al.*, 2004), for platelet activating factor receptor (Wu *et al.*, 2001) and for fibroblast growth factor receptor (Cotton *et al.*, 2006) in the completion of the process. However, sperm from these mice show mild capacitation-related problems, indicating that alternative pathways, equally efficient to reach the capacitation end points are activated. Problems in the ability to undergo capacitation have been observed in mice lacking acid sphingomyelinase (Butler *et al.*, 2002), which are associated with reduced fertilizing ability (Butler *et al.*, 2007). However, sperm from these mice are characterized by multiple defects, including morphological ones, because of alterations of spermatogenesis, which may be responsible for the reduced fertilizing ability. A Knock Out (KO) model where sperm capacitation occurs precociously with respect to wild-type (wt) mice has been also described (Rodeheffer & Shur, 2004). In this model, the lack of the long isoform of β1,4-galactosyltransferase I, a sperm surface protein indicated as a possible ZP3 receptor, prevents binding of de-capacitating factors during epidymal transit

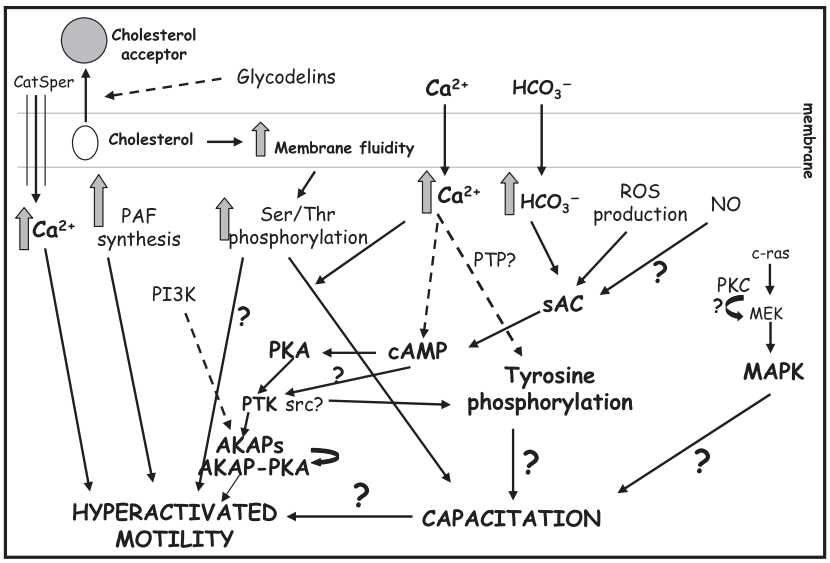

Figure 1 Schematic representation of the main intracellular signalling pathways that are activated during the process of capacitation and development of hyperactivated motility. Early events during the process of capacitation include cholesterol removal from the membrane, a process which is stimulated by an extracellular cholesterol acceptor (physiologically albumin) and inhibited by glycodelins, entry of calcium (Ca^{2+}) and bicarbonate (HCO_{3-}). HCO_{3-} leads to activation of the cAMP/PKA pathway. Autocrine production of reactive oxygen species (ROS), nitric oxide (NO) and platelet activating factor (PAF) has been demonstrated during capacitation. All these pathways converge in an increase of tyrosine phosphorylation of proteins. The increase of intracellular Ca^{2+} limits the increase of tyrosine phosphorylation by a poorly defined mechanism that may involve PTP. ras-MEK/MAPK and PKC activation and serine/threonine phosphorylation of proteins have been shown to occur during capacitation but their role in the process remains to be clearly defined. Pathways involved in development of sperm hyperactivated motility include entry of Ca^{2+} through CatSper calcium channels whose role in motility has been recently disclosed in a KO mouse model, and autocrine synthesis of PAF which exert a positive effect. Hyperactivated motility is stimulated by inhibitors of PI3K through increase AKAP tyrosine phosphorylation and AKAP–PKA interaction. Dotted arrows indicate inhibiting pathways. Interrogation marks indicate lack of definitive proofs of the involvement of a given pathway in the process. sAC, soluble adenylate cyclase; AKAPs, A-kinase-anchoring proteins; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NO, nitric oxide; PAF, platelet-activating factor; PDE, Phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphatydil inositol 3 kinase; PTK, protein tyrosine kinase; PTP protein tyrosine phosphatase; Src, cellular homologue of transforming gene of Rous sarcoma virus; ROS, reactive oxygen species.

of sperm, resulting in accelerated capacitation because of constitutive activation of intracellular pathways such as those mediated by calcium and cAMP. Proteomic analysis is extremely useful for the identification of the proteins that undergo post-translation changes during capacitation or for the identification of the enzymes involved in the process; however, although the advent of proteomics has allowed efficient global profiling of proteins, isolation and separation of plasma membrane proteins or proteins with a large molecular mass still represent the Achilles' heel of these techniques and complementary approaches are often needed to provide a detailed picture. In view of the role played by membrane proteins in sperm functions, a technology able to provide a detailed profile of proteins exposed on the surface of the plasma membrane appears essential to disclose eventual capacitation-related modifications of sperm surface proteins.

Another important point concerns the detailed time course of events depicted in Fig. 1. Loss of membrane cholesterol, driven by albumin present in the extracellular environment, is believed to enhance membrane fluidity and/or to directly affect membrane proteins involved in signalling, enabling capacitation to occur (Travis & Kopf, 2002). Such a view has been disputed by evidence showing that the increase of membrane fluidity drives, rather than being a consequence of, cholesterol redistribution and efflux (Flesch *et al.*, 2001). Cholesterol is concentrated in raft microdomains, which undergo a heavy re-organization during capacitation, leading to a decrease in membrane raft content in human sperm (Cross, 2004). Determining the exact role (mediating cholesterol efflux? Facilitating interaction of signalling molecules? Terminate signalling?), if any, of lipid rafts during capacitation appears to be a complex issue both because of methodological problems in raft isolation as well as because of their variable composition within sperm (Cross, 2004).

Ca^{2+} and bicarbonate increase in the cytoplasm as well as reactive oxygen species (ROS) generation and

activation of a soluble adenylate cyclase (Aitken *et al.*, 1998; Chen *et al.*, 2000; Jaiswal & Conti, 2001) appear to be upstream of tyrosine phosphorylation of proteins. However, whereas bicarbonate and ROS activation of a soluble adenylate cyclase (Aitken *et al.*, 1998; Chen *et al.*, 2000; Jaiswal & Conti, 2001), lead to increased tyrosine phosphorylation of proteins, Ca^{2+} ultimately leads to a limitation of the phenomenon (Carrera *et al.*, 1996; Luconi *et al.*, 1996), although the mechanism remains to be clarified (Carrera *et al.*, 1996; Baker *et al.*, 2004). The central role of soluble adenylate cyclase (sAC) in the process is demonstrated by studies in $sAC^{-/-}$ mice which are infertile and do not display increased tyrosine phosphorylation in response to bicarbonate (Xie *et al.*, 2006).

The rather long list of signalling events demonstrated during sperm capacitation (Fig. 1) is indicative of the complexity of the process. As mentioned above, some level of redundancy may exist. In addition, some of these pathways may be secondarily activated to produce development of hyperactivated motility, a process which is coincident to that of capacitation (see below) but whose dependence on capacitation is discussed. Furthermore, the sperm cell possesses a highly compartmentalized architecture: activated pathways for onset of hyperactivation in the tail are not likely responsible for induction of acrosome reaction in the sperm head.

In such a complex scenario, we decided to focus our review on the two biochemical events that have been better characterized in the last 10 years: intracellular calcium and protein phosphorylation.

Intracellular calcium. An increase in intracellular free Ca^{2+} concentration is characteristic of early phases of capacitation. Because of its fusogenic properties, Ca^{2+} is believed to play an essential role in acrosome reaction (AR) of spermatozoa and in the subsequent process of fusion with the oolemma, but the exact role of the ion in the process of capacitation is less clear. It is possible that the time-related increase of intracellular calcium during capacitation prepares the membrane to the fusogenic process. In addition, in some species, calcium may be involved in stimulation of a bicarbonate- and calcium-sensitive sAC (Hess *et al.*, 2005), which finally results in the increase of tyrosine phosphorylation (Visconti *et al.*, 1995a). In other species including the human, Ca^{2+} has the opposite role (Carrera *et al.*, 1996; Luconi *et al.*, 1996; Kalab *et al.*, 1998; Baker *et al.*, 2004). Ca^{2+} regulation of ATP levels (Baker *et al.*, 2004) and Ca^{2+}-regulated calmodulin activation (Carrera *et al.*, 1996) have been suggested as possible mediators of this effect.

The mechanisms that trigger the spontaneous rise of intracellular Ca^{2+} occurring during capacitation are not completely understood. Hypotheses include inhibition of Ca^{2+}-ATPase (Breitbart *et al.*, 1985), increased permeability to Ca^{2+} because of cholesterol loss from the membrane (Belmonte *et al.*, 2005), or activation of unidentified channels. A large variety of Ca^{2+} channels has been demonstrated in mammalian spermatozoa (for rev see Jimenez-Gonzalez *et al.*, 2006) which are involved in different cellular responses. Evidence exists indicating that voltage-dependent T-type Ca^{2+} channels may be involved in capacitation (Felix, 2005). A large number of voltage-dependent Ca^{2+} channels has been detected in sperm and testis, which could represent an attractive potential target for treatment of infertility and contraception, but a clear documentation of which of these channels are involved in the process of capacitation is currently lacking. Another possible mechanism involves a sperm Ca^{2+}-ATPase which is activated by decapacitating factors in uncapacitated sperm and that become inactivated following their removal from the surface (Adeoya-Osiguwa & Fraser, 1996). The demonstration of IP3-gated internal Ca^{2+} stores both in the head (Walensky & Snyder, 1995) and at the base of the flagellum (Ho & Suarez, 2001) and their involvement in development of hyperactivated motility (Ho & Suarez, 2003; Marquez *et al.*, 2007), suggest that Ca^{2+} entry during capacitation may be also regulated by these Ca^{2+} storage sites, but again, a clear-cut demonstration of their involvement is lacking so far.

In principle, occurrence of capacitation in sperm could be monitored by measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) using fluorescent Ca^{2+} indicators. However, monitoring basal $[Ca^{2+}]_i$ can be difficult as basal levels of Ca^{2+} are often influenced by experimental conditions. An increase in intracellular Ca^{2+} in sperm can be induced by physiological and pharmacological stimuli of AR. Among these, the effects of progesterone (P) and that of zona pellucida proteins (in particular ZP3) have been well documented. The increase of intracellular Ca^{2+} induced by both agonists is because of Ca^{2+} influx and mobilization of Ca^{2+} from internal stores (Bedu-Addo *et al.*, 2007; De Blas *et al.*, 2002; discussed in Sperm acrosome reaction section). The ability of spermatozoa to respond to both stimuli increases gradually during capacitation reaching a maximum after 90 min–2 h incubation in capacitating medium (Baldi *et al.*, 1991; Ward & Kopf, 1993; Garcia & Meizel, 1999). Figure 2A shows intracellular calcium transients in response to P in human spermatozoa incubated 2 h in a capacitating medium compared with a non-capacitating one as measured in fura-2 loaded sperm by a spectofluorimetric method. The difference between the two responses is evidence of the occurrence of capacitation in the sample. However, such an effect can be missed if the experimental conditions are not appropriate. For instance, if initial sperm selection is performed in media containing capacitating constituents (mainly a cholesterol acceptor and bicarbonate) the process of capacitation

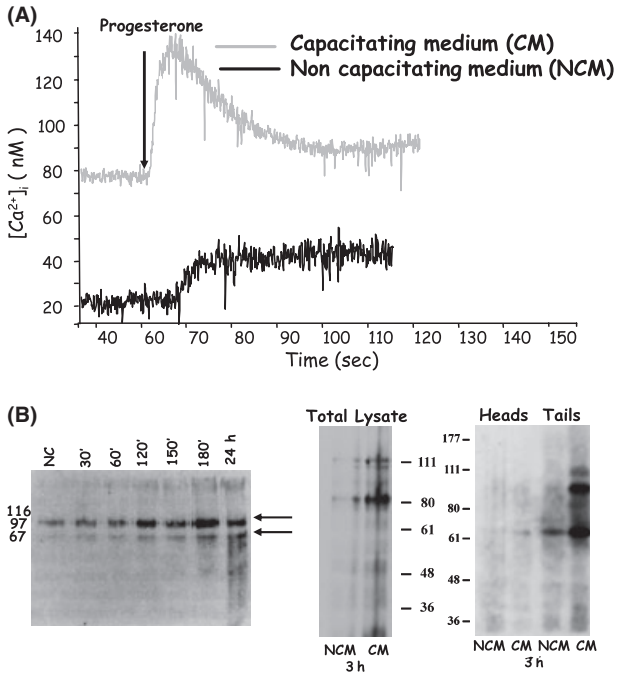

Figure 2 Intracellular calcium increase and tyrosine phosphorylation of proteins as markers of human sperm capacitation in vitro. (A) Spermatozoa incubated in vitro in a capacitating medium (CM) show higher levels of basal and progesterone (10 μm)-stimulated intracellular calcium concentrations respect to incubation in a non-capacitating medium (NCM). (B) Increase in tyrosine phosphorylation of proteins as a function of capacitation time (left blot) and following incubation in a CM or in a NCM (middle blot). The right blot shows tyrosine phosphorylation of proteins in sperm incubated in CM and NCM for 3 h after separation of head and tail fractions. Reproduced from Muratori *et al.*, 2004 and Luconi *et al.*, 1995 with permission.

may occur also in the 'so-called' non-capacitated sperm: as a consequence, the difference in the responses to the stimuli may be lost (see for instance Aitken *et al.*, 1996a). The reason for increased responsiveness to these stimuli during capacitation is unknown, although it is possible that their (not yet definitively identified, see below) 'receptor' become exposed on the sperm membrane during the process of capacitation. Recently, Aitken & McLaughlin (2007) reported that induction of capacitation by pentoxifylline enhanced the proportion of cells exhibiting secondary Ca^{2+} oscillation in response to P because of activation of T-type Ca^{2+} channels as consequence of attainment of the capacitation state.

Protein phosphorylation. Although the increase in tyrosine phosphorylation of proteins during capacitation is well documented, how the process is regulated is poorly understood. As mentioned above, in contrast with other species, tyrosine phosphorylation of proteins in human sperm does not require Ca^{2+} in the extracellular medium (Carrera *et al.*, 1996; Luconi *et al.*, 1996), whereas albumin and bicarbonate are absolutely required. A small decrease of membrane cholesterol content appears to promote the phenomenon, whereas if cholesterol removal become excessive tyrosine phosphorylation is inhibited (Shadan *et al.*, 2004). Despite the demonstration that the process is regulated by a cAMP-dependent pathway, as cAMP analogues greatly enhance the phenomenon (Visconti *et al.*, 1995a,b), the involvement of the serine/threonine kinase protein kinase A (PKA) upstream of the process is not clear (discussed in Sperm motility section), and the downstream tyrosine kinase(s) (TKs) which links the cAMP pathway to tyrosine phosphorylation increase remains elusive. Different cytosolic and membrane-bound TK have been reported in sperm, but so far studies on their role in capacitation are lacking or not convincing. Recent evidence obtained in murine (Baker *et al.*, 2006) and bovine (Lalancette *et al.*, 2006) sperm points to src as one of the kinases which might cover this role. Moreover, at least in murine sperm, PKA has been demonstrated to directly interact and activate src through specific phosphorylation (Baker *et al.*, 2006). However, such an interaction has not been found in human sperm by our group (Varano, G., Lombardi, A., Cantini, G., Forti, G., Baldi, E. & Luconi, M., manuscript in preparation). Evidence exists for occurrence of epidermal growth factor receptor, a receptor TK, in the head of sperm of human and other mammals (Naz & Ahmad, 1992; Damjanov *et al.*, 1993); however, the effect of the growth factor on capacitation has been observed at high concentrations (Furuya *et al.*, 1993), raising questions about its physiological significance. Alternatively and in addition, it is possible that tyrosine phosphatases are inactivated by the cAMP/PKA pathway, thus mediating the increase in tyrosine phosphorylation. Although tyrosine phosphatases have been demonstrated in sperm, whether they are regulated by PKA and/or involved in the increase of tyrosine phosphorylation is presently unknown. Controversy also exists concerning the involvement of protein kinase C (PKC) pathway in the process of capacitation (Bonaccorsi *et al.*, 1998) although a role in ras/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) activation has been suggested (O'Flaherty *et al.*, 2005) .

By phosphoproteome analysis, Ficarro *et al.* (2003) identified 18 tyrosine phosphorylated proteins in human sperm, including ion channels, metabolic enzymes and structural proteins, mainly located in the flagellum. The role of these proteins in capacitation and how tyrosine phosphorylation modifies their activity remains undefined with few exceptions. A sperm protein which becomes highly phosphorylated in tyrosine during capacitation is CABYR, a calcium-binding protein localized in the

principal piece of the tail in association with the fibrous sheath (Naaby-Hansen *et al.*, 2002), which may have a role in the spontaneous increase of $[Ca^{2+}]_i$ during the process. Two members of the extracellular-signal regulated kinase (ERK) family, p42 and p44 ERK, become phosphorylated as consequence of capacitation (Luconi *et al.*, 1998a,c). The two proteins may be actively involved in the completion of the process, as inhibitors of the RAS-ERK pathway inhibit it (Luconi *et al.*, 1998a; De Lamirande & Gagnon, 2002). A-kinase-anchoring proteins (AKAPs) are the main tyrosine phosphorylated structural proteins of the fibrous sheath (Ficarro *et al.*, 2003). Recently, a model of how the increase in tyrosine phosphorylation of one of this protein, AKAP3, is involved in regulation of sperm motility has been proposed (Luconi *et al.*, 2004) (see below). The post-pyruvate metabolic enzyme dihydrolipoamide dehydrogenase becomes tyrosine phosphorylated during capacitation in hamster spermatozoa (Mitra *et al.*, 2005) and is related to induction of hyperactivated motility (Kumar *et al.*, 2006). The enzyme shows an unusual, non-mitochondrial location in sperm and undergoes tyrosine phosphorylation with different time course depending on the location. As phosphorylation induces its activation, this occurs at different times in the different locations. Confirmation of these results in human sperm is awaited possibly shedding new light on how and which metabolic pathways are activated during capacitation. A role for tyrosine phosphorylation of proteins in sperm surface remodelling and sperm–zona recognition has been recently proposed based on the identification of some chaperone molecules among the tyrosine phosphorylated proteins in the mouse sperm acrosome (Asquith *et al.*, 2004). Such results, however, have not been confirmed in human sperm (Mitchell *et al.*, 2007).

Whatever the role of tyrosine phosphorylation in capacitation, its level in human sperm correlates strongly with sperm–zona binding capacity (Liu *et al.*, 2006) and alterations in its increase have been found in subfertile subjects (Buffone *et al.*, 2005) pointing out its physiological role in fertilization. The increase in tyrosine phosphorylation in human sperm maintained in a capacitating medium (CM) [relative to those maintained in a non-capacitating medium (NCM)] (Fig. 2B right blot) is mostly occurring in the tail fraction (Muratori *et al.*, 2004; Buffone *et al.*, 2005; Moseley *et al.*, 2005; Liu *et al.*, 2006), and whether tyrosine phosphorylated proteins are present in the head is questionable.

Although great attention has been given to tyrosine phosphorylation of proteins during capacitation, much less is known about phosphorylation in serine/threonine residues, despite the demonstration that ERKs, which phosphorylate proteins in serine/threonine residues, are present and activated in sperm during capacitation (Luconi *et al.*, 1998a,b,c; De Lamirande & Gagnon, 2002). Recently, an increase in proline-directed serine/threonine phosphorylation during capacitation has been demonstrated in human sperm (Jha *et al.*, 2006) which appears to be independent of bicarbonate/cAMP pathway and dependent on bovine serum albumin (BSA)-mediated cholesterol release from the membrane. Although these data are quite preliminary and need to be substantiated, the increase in proline-directed serine/threonine phosphorylation may represent a marker of membrane modifications during capacitation.

Despite the tremendous advances in the field of protein phosphorylation related to sperm capacitation in the last decade, we are still missing the overall significance of the phenomenon. Whereas identification of each phosphoprotein is essential, more efforts should be put into understanding their role in the phenomenon, having clearly in mind that these proteins may represent ideal targets for therapy or contraception.

Other markers of sperm capacitation

Overall, intracellular Ca^{2+} and tyrosine phosphorylation of proteins represent objective markers to assess the occurrence of human sperm capacitation in vitro. Other possible objective markers of capacitation have been proposed, such as membrane lipid disorder (which can be evaluated using merocyanine 540), cholesterol distribution (which can be evaluated by filipin labelling assays), or membrane phosphatidylserine exposure (which can be measured by annexin V binding) based on studies in different animal species demonstrating changes in membrane lipid organization (Flesch *et al.*, 2001; Baumber & Meyers, 2006) and phosphatidylserine externalization (Gadella & Harrison, 2002; Wang *et al.*, 2004) as consequence of capacitation in vitro. However, results on merocyanine 540 binding and phosphatidylserine externalization in human sperm during capacitation are conflicting (de Vries *et al.*, 2003; Muratori *et al.*, 2004; Martin *et al.*, 2005) and at present, phosphatidyliserine exposure is considered a marker of sperm damage (Muratori *et al.*, 2004; Martin *et al.*, 2005; Barroso *et al.*, 2006; Brugnon *et al.*, 2006). On the other hand, it is possible that the higher cholesterol content of human and bovine sperm membranes relative to murine and porcine may be responsible for the different results obtained with M540 in these species.

Sperm motility

The ability to swim actively is essential for the sperm to reach the oocyte at the site of fertilization and is a unique property of the male gamete. Development of sperm motility is achieved during passage through the

epididymis and a number of different environmental and intracellular factors contributes to its maintenance (Luconi *et al.*, 2006). Besides forward motility characterizing human spermatozoa in the ejaculate and in the first part of the female genital tract, in the proximity of the oocyte, sperm develop a different kind of motility, called hyperactivation, characterized by high velocity, large amplitude and asymmetry of the flagellar waves, which helps the male gametes to detach from the oviductal epithelium, progress in the more viscous oviductal fluid and eventually penetrate the zona pellucida and the other oocyte vestments. The acquisition of hyperactivation can be reproduced in vitro by incubating spermatozoa in defined media supplemented with the same factors that regulate the process of capacitation, enabling analysis of the phenomenon.

Poor or total absence of motility (different grades of asthenozoospermia) in the ejaculate is one of the main causes of male infertility based on sperm factors and it has been recently reported that about 80% of unselected 1085 sperm samples analysed from infertile subjects shows motility defects, with 20% exhibiting asthenozoospermia (Curi *et al.*, 2003). Our present knowledge of the mechanisms and factors concurring to sperm motility reveals that the process is complex (Fig. 1) and involves the presence as well as the correct function and interaction of a plethora of signalling molecules and structural proteins. However, as motility is crucial for sperm, a high redundancy in several of these mechanisms is expected. As widely demonstrated by research using KO mice models, some signalling pathways and some proteins seem to be more decisive than others for the development and maintenance of sperm motility.

Molecular bases of sperm motility

Three groups of proteins are of a pivotal importance for achieving a correct pattern of motility: (i) structural proteins in the tail, such as outer dense fibers, kinase A anchoring proteins (AKAP3 and 4), dyneins; (ii) transducers of activating signals, PKA and serine-threonine-tyrosine kinases/phosphatases and finally, (iii) proteins involved in the generation of energy, such as glycolytic and mitochondrial enzymes.

Structural protein. Among structural proteins, the constituents of the fibrous sheath play a pivotal role in orchestrating sperm motility. Defects in these components, such as outer dense fibers and AKAPs result in several morphological and functional alterations relevant to sperm asthenozoospermia and reduced fertility (Chemes & Rawe, 2003; Francavilla *et al.*, 2006; Luconi *et al.*, 2006). In certain circumstances, correlations have been observed between mutations in specific genes for structural components of sperm flagellum (Inaba, 2003) and disorders of sperm motility. Correlations between gene mutations and specific functions of the encoded proteins in regulating sperm motility have been traced using KO mouse models. Immotile cilia syndrome or primary ciliary dyskinesia (PCD) is because of defects of the axonemal components, such as tektin (Tanaka *et al.*, 2004; Roy *et al.*, 2007) and dyneins (Vernon *et al.*, 2005), resulting in absence of sperm motility in addition to systemic pathological alterations. Fibrous sheath dysplasia (FSD), characterized by thickened and dysmorphic sperm flagella, involves a plethora of defects in different components of the fibrous sheath arising during spermiogenesis (Chemes *et al.*, 1987; Chemes & Rawe, 2003). In particular, AKAP3 and 4 are the main components of sperm flagellum in mouse and human and they act by tethering pivotal kinases, their substrates and inactivating enzymes in the same place, thus allowing the development and the regulation of signalling cascades underlying sperm motility (Moss & Gerton, 2001; Luconi *et al.*, 2004; Bajpai *et al.*, 2006). Therefore, defects in structure and function of such proteins are likely to be associated to defects in sperm motility. Although AKAP4 KO mice show defects in sperm motility and are infertile, in humans, dysplasia of the fibrous sheath does not seem associated with alterations in AKAP3 and 4 genes, in particular in the PKA binding domain (Turner *et al.*, 2001). However, a recent paper from Baccetti's group reported a genetic defect (complete deletion) in AKAP4 gene in a patient with FSD (Baccetti *et al.*, 2005).

Tyrosine phosphorylation in the AKAP3 following treatment with the phosphatidylinositol 3-kinase inhibitor LY294002, which has been shown to enhance motility (Luconi *et al.*, 2001), or physiological stimulation in vitro with bicarbonate, has been recently demonstrated to specifically recruit activated PKA in sperm tails resulting in a significant increase in sperm motility and hyperactivated parameters (Luconi *et al.*, 2001, 2004, 2005), providing an interesting link between tyrosine phosphorylation and function of the protein. Hence, absent or poor tyrosine phosphorylation of AKAP may mark patients with different grades of asthenozoospermia, underlying a possible defect in the ability of the protein to undergo phosphorylation or in the signalling machinery responsible for its phosphorylation (see below).

PKA system and tyrosine kinases. PKA and tyrosine kinases pathways are believed to exert a significant role in development, maintenance and regulation of sperm motility (Fig. 1) by post-translational modifications of the proteins involved in the process. Modulation of compartmentalization of activated/inactivated proteins in sperm (Luconi *et al.*, 2006) is another reported mechanism of modulating motility.

Among the post-translational protein modifications demonstrated to be essential for sperm motility, phosphorylation by soluble adenylate cyclase (sAC)/-cAMP/PKA and the tyrosine kinase systems have been well documented in the last 20 years. Interestingly, such signalling mechanisms are strictly associated and interacting between each other. cAMP is predominantly produced in sperm by sAC, although membrane isoforms have also been described (Baxendale & Fraser, 2003; Wade *et al.*, 2003). However, the lack or minimal effects of stimuli of membrane AC, such as forskolin (Livera *et al.*, 2005; Luconi *et al.*, 2005), has risen doubts about a possible role of these isoforms in the regulation of motility. Inactivation of the AC 3 gene lead to some impairment of fertility in male mice (Livera *et al.*, 2005); however, whether this a direct effect or was indirectly caused by a loss of function early during spermatid differentiation could not be excluded in this report. Precise compartmentalization of sAC in distinct subcellular domains may lead to activation of PKA by locally produced cAMP (Luconi *et al.*, 2005, 2006), in particular in sperm tail, where sAC is predominantly associated with fibrous sheath. KO for sAC results in infertility because of impairment in sperm motility (Esposito *et al.*, 2004). Interestingly, in spermatozoa from this KO model, cAMP administration restores forward (but not hyperactivated) motility, tyrosine phosphorylation defects as well as infertility (Esposito *et al.*, 2004), further confirming the importance of the tyrosine kinase pathway downstream of sAC (Luconi *et al.*, 2005). KO mouse models for different PKA catalytic and regulatory subunits have further complicated this already complex scenario of sperm motility regulatory mechanisms (for rev see Luconi *et al.*, 2006; Burton & McKnight, 2007). Indeed, results of selective KO for different PKA subunits led to controversial results, representing one of the best example of the problems linked to the use of KO animals (Skalhegg *et al.*, 2002; Burton *et al.*, 1997). In particular, selective KO for catalytic PKA subunits results in impaired sperm motility (Skalhegg *et al.*, 2002) whereas, surprisingly, KO for regulatory subunits, while inducing a delocalization of PKA catalytic activity from the fibrous sheath, does not affect sperm motility (Burton *et al.*, 1999). In addition, in all these KO animal models a compensatory increase of the alternative isoforms of PKA subunits occurs. Finally, null mice for the sperm specific PKA catalytic subunit Cα2 display normal ability to initiate motility although bicarbonate failed to induce hyperactivation and tyrosine phosphorylation of proteins (Nolan *et al.*, 2004), pointing out the possible specific role of this sperm specific PKA in hyperactivation.

Tyrosine phosphorylation of sperm proteins represents the main post-translational modification underlying regulation of sperm activation. In particular, the fine equilibrium between tyrosine kinase and phosphatase activity is the central key controlling activation and maintenance of activated and hyperactivated sperm motility (Luconi *et al.*, 2006; Bajpai & Doncel, 2003; Turner, 2006). Reduction in tyrosine phosphorylation of sperm proteins, in particular in the same molecular weight range of AKAP3 and 4, seems to be strictly associated with sperm motility defects in semen of asthenozoospermic subjects compared with normozoospermic and fertile subjects (Yunes *et al.*, 2003; Buffone *et al.*, 2005). The evaluation of tyrosine phosphorylation of AKAPs in subjects with different motility defects is mandatory to establish whether it may represent a valuable marker of sperm motility, although absence or poor phosphorylation may reflect defects at different levels (membrane bicarbonate transport, cAMP production, kinase activation, AKAP, etc.). At the same time, definition of the exact temporal sequence of PKA activation and tyrosine phosphorylation and the exact cross talk between these two signalling pathways is necessary to clarify their role in the development and maintenance of correct sperm motility. Indeed, controversial findings have been reported in the literature about the temporal hierarchy and the dependence of tyrosine phosphorylation on PKA activity (Leclerc *et al.*, 1996; Bajpai & Doncel, 2003; Luconi *et al.*, 2004; Moseley *et al.*, 2005; Baker *et al.*, 2006). Whereas it is clear that an increase of cAMP is upstream of tyrosine phosphorylation, as demonstrated by using cAMP analogues (Aitken *et al.*, 1998; Bajpai & Doncel, 2003; Luconi *et al.*, 2005), controversy exists concerning whether PKA activation is also necessary for tyrosine phosphorylation in human sperm (Aitken *et al.*, 1998; Bajpai & Doncel, 2003; Luconi *et al.*, 2004, 2005). A similar scenario concerns the effects of PKA inhibitors on motility (Aitken *et al.*, 1998; Luconi *et al.*, 2005). Controversy can be explained by the different experimental conditions of sperm (swim up, Percoll) and semen samples (donors, partners of infertile couples, subfertile subjects) selections, which are critical for studies on human spermatozoa. As an example, an identical concentration of H89 produced inhibition of tyrosine phosphorylation but no effect on motility in a study by Aitken *et al.* (1998) performed on Percoll selected sperm from healthy donors, but did not show effects on phosphorylation although inhibited sperm motility in other studies performed in swim up selected sperm from normospermic male partners of infertile couples (Luconi *et al.*, 2005) or normospermic donors (Bajpai & Doncel, 2003). Another problem is that these results have been obtained by the use of so called 'specific' inhibitors of PKA (such as H89) or tyrosine kinase pathways (such as genistein, erbstatin or PP2) which are not so 'selective' for their targets, in particular when used at high doses. Thus, the presence of an inhibitory effect of H89 on tyrosine phosphorylation

may suggest that PKA acts upstream of tyrosine kinases, but may suffer from a direct non-specific effect of the blocker also on TK. In addition, a clear link between cAMP/PKA activation and TK is lacking. As mentioned above, in the mouse, the group of Aitken identified in the pp60SRC, the intermediate PKA-activated tyrosine kinase driving tyrosine phosphorylation during capacitation and hyperactivation (Baker *et al.*, 2006), but our group failed to demonstrate an involvement of this TK in human sperm (Varano, G., Lombardi, A., Cantini, G., Forti, G., Baldi, E. & Luconi, M., manuscript in preparation). In general, most of the researchers agree that H89 is effective in blocking tyrosine phosphorylation induced in sperm by cAMP analogues, while less agreement exists when other stimuli (even those able to increase cAMP, such as bicarbonate) are used. It may be concluded that tyrosine phosphorylation in sperm may be trigger by pathways other than the cAMP one, and/or that cAMP may have PKA independent effects in sperm that are not stimulated by the analogues used so far. Efforts should be carried out in identifying PKA substrates in sperm, as well as cAMP-regulated pathways independently of PKA activation. The recent availability of 2′-*O*-methylated cAMP analogues (also known as Epac-selective cAMP analogues) (Holz *et al.*, 2008), which produce cAMP biological effects that are independent from PKA activation, represents a new interesting tool to better define the effects of cAMP in sperm.

The scenario concerning the role of PI3K in sperm function is also becoming rather complex. Following initial studies demonstrating that the use of the PI3K inhibitor LY294002 induces an increase of motility (Luconi *et al.*, 2001; Aparicio *et al.*, 2005), recent papers, employing wortmanin as PI3K inhibitor, demonstrate that the lipid kinase is involved in induction of AR (Liguori *et al.*, 2005) and capacitation (Etkovitz *et al.*, 2007; Jungnickel *et al.*, 2007) of mammalian sperm. In addition, Nauc *et al.* (2004) have shown that incubation of human sperm with LY294002 induces capacitation and tyrosine phosphorylation of proteins whereas wortmannin had opposite effects. Although our group has shown that incubation of sperm with LY294002 produces a decrease of PI3K activity (Luconi *et al.*, 2004), it is possible that unrecognized non-specific effects of the inhibitor are also involved. Whatever is the mechanism, the effects on motility (Luconi *et al.*, 2001; Aparicio *et al.*, 2005), capacitation (Nauc *et al.*, 2004), tyrosine phosphorylation of proteins (Luconi *et al.*, 2004; Nauc *et al.*, 2004) and AKAP–PKA interaction (Luconi *et al.*, 2004) observed with LY294002 deserve additional efforts to clarify the mechanisms involved as the compound may be the considered as the founder of future pharmacological tools to increase sperm functions in vitro.

Energy sources. Energy sources are of course extremely important for correct sperm motility. ATP supports generation of the sliding force by dynein ATPase in the axoneme finally resulting in the flagellar beat. Although the main source of ATP has long been considered to be the mitochondria in the middle piece, growing evidence of the importance of a local production of energy through glycolysis in the principal piece of sperm tails has more recently been emerging in studies conducted in mouse models. Indeed, mathematical models combining ATP diffusion constant and geometrical architecture of sperm flagellum (Du *et al.*, 1994) suggest that ATP production in the mid piece cannot diffuse efficiently enough along the entire length of sperm flagellum (Turner, 2006). However, the presence of enzymatic shuttles such as adenylate kinase and creatine phosphokinase may help to sustain ATP concentration in the distal region of the tail with no need for diffusion (Ford, 2006). Motility is still present, although reduced, when oxidative phosphorylation is uncoupled in mouse sperm mitochondria (Narisawa *et al.*, 2002), suggesting that the main source of ATP for sperm flagellar beat resides somewhere else. And indeed, fibrous sheath is associated with several glycolytic enzymes (Krisfalusi *et al.*, 2006), many of which, such as LDH, GAPD-S, hexokinase, are present in sperm specific isoforms. KO mice for GAPD-S have demonstrated that glycolysis is necessary for sperm forward motility and fertility because sperm from these mutant mice have impaired motility and fertility associated to extremely low ATP production, with normal mitochondrial oxidative phosphorylation (Miki *et al.*, 2004). Conversely, inhibition of sperm oxidative phosphorylation, does not alter fertilization (Fraser & Quinn, 1981). The ability of sperm from many species including human to remain motile in glucose-free media (Ford, 2006) is not a strong evidence supporting the poor role of glycolysis in motility, as generation of endogenous glucose through glycogen stores and gluconeogenesis cannot be excluded (Albarracin *et al.*, 2004; Aquila *et al.*, 2005). The observation that sperm midpiece increases in volume and mitochondrial membrane potential is higher in species showing sperm competition (Anderson & Dixson, 2002; Anderson *et al.*, 2007), indicates that in species, including human, where competition does not occur, the midpiece and the mitochondrial compartment are less developed and do not play the same role in supporting motility, probably because glycolysis is more important as energy source for the tail beat. Glycolytic production of ATP is necessary not only for forward but also for hyperactivated motility (Urner & Sakkas, 1996).

Calcium

Several signalling pathways and intracellular second messengers are common to the development of activated and hyperactivated motility; however, some are uniquely

essential for hyperactivation (Ho & Suarez, 2003; Qi *et al.*, 2007). In particular, Ca^{2+} is essential for the development of sperm motility and its intracellular levels in the sperm are tightly regulated by several Ca^{2+} channels (Darszon *et al.*, 2005; Publicover *et al.*, 2007). Although Ca^{2+} influx is associated to sperm capacitation and tyrosine phosphorylation of proteins (see above), Ca^{2+} levels must be kept at low levels in order to allow precise timing of sperm activation and ATP to be used for hyperactivation and tyrosine phosphorylation processes (Baker *et al.*, 2004). Catsper, a novel class of tetrameric Ca^{2+} channels expressed in sperm tail, is involved in regulating hyperactivation, as KO mice for genes encoding these channels display absence of such a motility despite normal activated motility, finally resulting in infertility (Qi *et al.*, 2007). Besides the first characterized Catsper 1 and 2 (Ren *et al.*, 2001; Quill *et al.*, 2003), two other related genes, Catsper 3 and 4, showing a similar expression on sperm tail plasma membrane, have more recently been described (Babcock, 2007; Qi *et al.*, 2007). However, the involvement of this class of Ca^{2+} channels in development of hyperactivated motility in human sperm has not been conclusively addressed despite demonstration of expression of mRNA for catsper family members by RT-PCR (Nikpoor *et al.*, 2004; Li *et al.*, 2007). In conclusion, the role of calcium in regulating sperm motility and hyperactivation is quite controversial, because if from one side high Ca^{2+} levels deplete intracellular ATP (which is important for phosphorylation of sperm proteins involved in the process of capacitation and development of motility) (Baker *et al.*, 2004), from the other side, Ca^{2+} influx appears essential for inducing hyperactivation (Qi *et al.*, 2007). Further studies would now seem worth of consideration focusing on the role of external calcium and calcium fluxes in the development and maintenance of motility and hyperactivation, as well as the type of Ca^{2+} channels involved.

Molecular markers of sperm–oocyte interaction

Sperm–zona binding and sperm–oocyte fusion

Interaction between plasma membrane of sperm and oocyte zona pellucida is an important step in the process of fertilization which includes attachment, binding, induction of the AR and penetration of the ZP matrix. The identity of the sperm receptor(s) responsible for the attachment to the zona and initiation of the signal transduction cascade leading to acrosomal exocytosis remains obscure. Many different molecules and many models have been developed in the last decades (for rev see Clark & Dell, 2006), involving different carbohydrates and proteins, but up to now many of the findings remain controversial and more investigations are required to solve the issue.

A similar situation applies for the molecular mechanism of sperm–egg membrane fusion, where many different molecules have been suggested to play a role. The identification, by studies on mice with altered gene function, of Izumo on sperm (Inoue *et al.*, 2005) and of CD9 on oocyte (Kaji *et al.*, 2002) as the two major players in the game represents now a milestone in the field. Evaluation of Izumo protein expression in the sperm membrane and a search for mutations of the gene might reveal alterations in the ability of sperm to fuse. So far, however, no patient negative for Izumo expression (detected by immunocytochemistry) has been identified among 25 affected by severe oligozoospermia or selected for a previous history of fertilization failure (Hayasaka *et al.*, 2007). However, the weakness of this study is reflected in the low number of subjects examined and the choice of oligozoospermic men (who might not be the right population among which searching for *Iuzmo* mutations). The identification of an inactivating mutation of *Izumo* in men with defects of sperm–oocyte fusion is awaited before any definitive conclusion about the involvement of this protein in sperm–oocyte fusion in humans may be drawn.

Sperm acrosome reaction

Acrosome reaction is a central step in the process of fertilization. It occurs following binding to the zona and is mediated by large influxes of Ca^{2+} and activation of kinases (Jimenez-Gonzalez *et al.*, 2006). Physiologically, AR is induced by zona proteins, but in vitro studies have identified many different AR inducers, including steroids, phospholipids, small peptides and growth factors. Recently, the classical view that sperm binding to zona triggers the AR has been disputed by demonstrating, in a murine model, that AR occurs much later following sperm adhesion to zona and hypothesising that mechanosensory signals which develop during zona penetration trigger the process (Baibakov *et al.*, 2007). Such a model, although fascinating, needs to be confirmed in sperm from other species and human in particular.

Until the receptor(s) responsible for sperm–zona binding and induction of AR has been unequivocally characterized, markers of AR are represented by signalling events stimulated by the two physiological AR inducers, i.e. zona proteins and progesterone (P). Zona pellucida from human egg is composed of four glycoproteins, namely ZP1, ZP2, ZP3 and ZP4 (Lefievre *et al.*, 2004; Conner *et al.*, 2005). Studies on human ZP–sperm interaction are restricted because of limited availability of oocytes and of commercially available recombinant human zona proteins. Although recombinant human ZP2, ZP3 and ZP4, produced in different laboratories, have been shown recently to induce AR and promote changes in

motility of human sperm (Chakravarty *et al.*, 2005; Caballero-Campo *et al.*, 2006), few data are available on the glycosylation state of these molecules, which appears relevant for their function (Chakravarty *et al.*, 2008), and it is not known whether they retain full activity. Most of the knowledge on the molecular signalling occurring during AR in human sperm derives from studies employing P. P induces Ca^{2+} influx, modifies the fluxes of other ions (Jimenez-Gonzalez *et al.*, 2006) and induce tyrosine phosphorylation of proteins (Tesarik *et al.*, 1993; Luconi *et al.*, 1995). P is present at micromolar concentration in the follicular fluid (Frederick *et al.*, 1991) and thus sperm are engaged with high levels of the steroid at fertilization. The fact that pre-treatment with P 'primes' sperm to subsequent response to zona proteins (Roldan *et al.*, 1994; Shi *et al.*, 2005) suggests that the steroid may be involved in sensitizing sperm to the AR process while they cross the cumulus matrix. Responsiveness to P is decreased in subfertile men (Tesarik & Mendoza, 1992; Falsetti *et al.*, 1993; Oehninger *et al.*, 1994) and correlates with in vitro fertilizing ability of sperm (Krausz *et al.*, 1995, 1996; Jacob *et al.*, 1998; Allgeyer *et al.*, 2006), indicating a physiological role of the steroid in the process of fertilization. Despite this evidence, whether P is physiologically involved in the process of fertilization is still debated. Identification of the putative P sperm receptor (if any, discussed below) is awaited to give strength to and to provide new tools to demonstrate the physiological involvement of the steroid at fertilization. The involvement of a receptor-mediated mechanism in the action of P has been proposed based on different findings, including increase of responsiveness to the steroid during capacitation, which suggests unmasking of a receptor on the surface, and lack of response to a second challenge with the steroid (Aitken *et al.*, 1996b; Luconi *et al.*, 1998b). The fact that BSA- or POD-conjugated P is also able to induce the effects, has led to the hypothesis of a membrane location of such receptors. However, BSA conjugated steroids are difficult compounds as release of free P cannot be excluded in these experiments. The classic P receptors have not been found in sperm (Castilla *et al.*, 1995; Sabeur *et al.*, 1996; Luconi *et al.*, 1998b), and the antagonist RU496 does not affect P-mediated sperm AR at the concentrations that are effective on classical receptors (Baldi *et al.*, 1991; Blackmore, 1993); hence, its involvement can be excluded. Several candidates have been proposed to cover this role (reviewed in Correia *et al.*, 2007), including a truncated form of the classic P receptor and novel P membrane receptors. Recently, by a detailed proteomic analysis of human spermatozoa, an extragenomic P receptor was identified (Baker *et al.*, 2007), although proofs of its involvement in the P-inducing effects on sperm are still awaited. Despite the numerous papers published on the topic, the identity of the sperm P receptor remains obscure. Given the numerous candidates, the possibility that multiple species of P receptors generate a molecular complex within sperm is attractive (Correia *et al.*, 2007) and should be chased. Despite all the evidence, the possibility that a non-receptor mechanism is involved in P action in sperm cannot be excluded. Evidence against a receptor mechanism include the concentrations (from 0.1 to 10 μM) of the steroid needed to induce the effects, which are highly above those needed for receptor-mediated responses of the hormone, and the membrane perturbating effects of the steroid detected in sperm (Shivaji & Jagannadham, 1992).

Other markers of sperm functions

Other markers identify important sperm functions, in particular for correct post-fertilization events. Among these, sperm DNA integrity is certainly of great value as the primary role of the sperm is to transfer the paternal genome to the eventual new individual. Both the mechanisms that provoke sperm DNA damage (Erenpreiss *et al.*, 2006; Muratori *et al.*, 2006) and the impact of sperm DNA damage on fertility (O'Brien & Zini, 2005; Li *et al.*, 2006) have been extensively reviewed in several recent papers to which readers are referred.

Another important feature is represented by the presence, in mature sperm, of a large spectrum of mRNAs. The function of mRNA transcripts in transcriptionally and translationally silent cells remains obscure. The most accepted explanation of the presence of mRNAs in mature sperm is that they represent remnants of testicular transcriptional activity. With such a view in mind, it has been recently proposed that screening for mutations of candidate infertility genes expressed in mature sperm by reverse transcriptase polymerase chain reaction (RT-PCR) can be a useful approach to diagnose genetic defects in men with different semen pathologies (Yatsenko *et al.*, 2006). Similarly, by using a microarray strategy, the profile of human sperm transcripts was evaluated in infertile and subfertile men, leading to the identification of transcripts that were impaired in teratozoospermic individuals (Platts *et al.*, 2007). Alternative functions for RNAs present in mature sperm have been proposed: Rassoulzadegan *et al.* (2006) suggest the possibility that sperm RNAs are transferred to the oocyte and are translated by the zygote, where they can alter the phenotype of the new individual without affecting the genotype. In addition, a role for sperm mRNA has been recently proposed by Gur & Breitbart (2006). According to this controversial study, nuclear mRNAs would be translated by mitochondrial ribosomes and the corresponding peptides would subsequently be exported out of these organelles. It must be mentioned

that another group failed to detect new mitochondrial protein synthesis in ejaculated human sperm (Diez-Sanchez *et al.*, 2003). In addition, how such transcripts would be transferred from nucleus to mitochondria and how the corresponding peptides would be folded into the native conformation and directed to their extra mitochondrial sites of action remain to be explained.

In addition to mRNA, sperm contain several putative inhibitory RNA (iRNA) that may be involved in inhibition of genes during early embryonic development (Boerke *et al.*, 2007). An emerging marker for sperm function is protein ubiquitination, although at the moment the roles of this protein modification remains obscure and several hypotheses have been proposed.

Sperm ubiquitination

Ubiquitination is a protein modification consisting in the conjugation of proteins to single ubiquitin residues or multi-ubiquitin chains. Such a protein modification is involved in a large variety of cellular processes, the best known being the protein degradation by the 26S proteasome or by lysosomes (Hochstrasser, 1996). Recently, the role of ubiquitin in reproductive and sperm functions (Bebington *et al.*, 2001; Sutovsky, 2003) has been addressed and several roles of sperm ubiquitination have been proposed (Sutovsky, 2003). In particular, it has been hypothesized that a quality control system would act in the epydidymis where defective sperm would be labelled with ubiquitin and subsequently eliminated by phagocytosis (Sutovsky *et al.*, 2001a). In this view, ejaculated sperm with surface ubiquitination are considered cells escaped from such quality control system (Sutovsky *et al.*, 2001a). Accordingly, later on, it has been shown that the global amount of ubiquitination in human semen correlates with poor semen quality (Sutovsky *et al.*, 2004) and unsuccessful outcome of ART (Ozanon *et al.*, 2005). However, both normal and morphologically abnormal sperm are ubiquitinated (Sutovsky *et al.*, 2001b; Muratori *et al.*, 2005; Varum *et al.*, 2007) suggesting that ubiquitination may have additional roles in sperm functions. Indeed, conflicting results were found by our group when ubiquitination in the population formed by only sperm was analysed (Muratori *et al.*, 2005) respect to global semen ubiquitination (Sutovsky *et al.*, 2004). Furthermore, in another study evaluating global semen ubiquitination, no correlation between the latter and sperm apoptotic markers (annexin V binding and DNA fragmentation) was found (Varum *et al.*, 2007). This issue is important in as much as semen ubiquitination is also because of components other than sperm, which may explain the conflicting results obtained when global semen ubiquitination is considered (Sutovsky *et al.*, 2004; Varum *et al.*, 2007). In particular, we showed that M540 bodies, (semen components recently described by our group, see next section, Muratori *et al.*, 2004) are ubiquitinated and that ubiquitination of such elements correlated to poor semen quality (Muratori *et al.*, 2005). On the other hand, when only ubiquitinated spermatozoa were considered, positive correlations with good semen quality were found (Muratori *et al.*, 2005). To make the issue even more complex, in a recent study evaluating sperm head ubiquitination (excluding sperm labelled in other structures) within the population formed by only sperm, an association with unsuccessful outcome of ART was found (Ozanon *et al.*, 2005), suggesting that ubiquitination may have different roles depending on the sperm structures that are marked. It has been proposed that ubiquitination may be involved in elimination of paternal mitochondria following fertilization (Sutovsky, 2003), although such a role needs to be confirmed by further studies. It must be also mentioned that studies on sperm ubiquitination reveal a global protein modification and that the identity of the ubiquitinated proteins and their localization in the different sperm structures are still largely obscure.

In conclusion, whether sperm ubiquitination represents a positive or negative marker of sperm function remains elusive. Future studies are needed to identify the proteins that become ubiquitinated as well as the role of ubiquitination in the different sperm structures.

M540 bodies

In human semen, we have recently described the presence of round bodies, the levels of which appear to be notably higher in subjects with decreased quality of semen (Muratori *et al.*, 2004). As such bodies brightly stain with merocyanine 540 (M540), a compound used to reveal somatic apoptosis, we termed them as M540 bodies. Based on the characteristics found in M540 bodies, we hypothesized and then demonstrated (Marchiani *et al.*, 2007) that they are apoptotic bodies. Indeed, M540 bodies express several markers known to act during programmed cell death, including Fas receptor, p53, caspases activity and DNA fragmentation (Marchiani *et al.*, 2007). The occurrence of M540 bodies in semen has clinical and methodological consequences. Indeed, their presence in semen may be considered a sign of pathological/excessive apoptosis in the male reproductive organs. On the other hand, as M540 bodies have size and density similar to the sperm heads and are (apoptotic) cell remnants, they may represent a confounding factor in studies on sperm utilizing flow cytometry, as already reported for investigation on sperm ubiquitination (Muratori *et al.*, 2005). Similar bias have been found by our group in flow cytometric investigation on sperm DNA fragmentation (Muratori *et al.*, in press).

Table 1 Molecular markers of human sperm function based on available literature

	Motility		Sperm–oocyte interaction			
Capacitation	Activated	Hyperactivated	Chemotaxis	Sperm–oocyte fusion	Sperm AR	Other possible markers
Intracellular concentration of calcium (basal and after P challenge)[a]	**Mithocondrial oxidative phosphorylation**[f]	**sAC activity**[k]	*Odorant receptors*[o]	*Izumo*[q]	**Dynamics of calcium entry in response to stimuli**[s]	*DNA fragmentation*[v]
Tyrosine phosphorylation[b]	**sAC activity**[g]	**AKAP tyrosine phosphorylation**[l]	*Chemokine receptors*[p]	*CRISPs*[r]	**Acrosine activity**[t]	*Sperm ubiquitination*[vw]
PAF synthesis[c]	*AKAP tyrosine phosphorylation*[h]	**AKAP–PKA interaction**[m]				*M540 bodies*[x]
Proline-directed serine/ threonine phosphorylation[d]	*AKAP–PKA interaction*[i]	**Mithocondrial oxidative phosphorylation**[f]			*Tyrosine phosphorylation in response to stimuli*[u]	*Oxidative stress*[y]
Lipid membrane disorder[e]	*Glycolytic enzymes*[j]	*Glycolytic enzymes*[j] *CatSper family members*[n]				*PS expusure*[z]

In bold: well defined markers; in italic: awaiting confirmation markers.

P, progesterone; PAF, platelet-activating factor; sAC, soluble adenylate cyclase; AKAP, A-Kinase-Anchoring Proteni; PKA, protein kinase A; CRISP, cysteine-rich secretory protein; PS, phosphatodyl-serine.

[a]Baldi *et al.*, 1991; DasGupta *et al.*, 1993; Bedu-Addo *et al.*, 2005. [b]Visconti *et al.*, 1995a; Luconi *et al.*, 1995; Buffone *et al.*, 2005; Liu *et al.*, 2006. [c]Baldi *et al.*, 1993; Wu *et al.*, 2001. [d]Jha *et al.*, 2006;. [e]Baumber & Meyers, 2006; Butler *et al.*, 2007. [f]Miki *et al.*, 2004; Narisawa *et al.*, 2002. [g]Esposito *et al.*, 2004. [h]Turner *et al.*, 1999; Luconi *et al.*, 2005. [i]Vijayaraghavan *et al.*, 1997; Luconi *et al.*, 2004; Huang *et al.*, 2005. [j]Urner & Sakkas, 1996;Miki *et al.*, 2004. [k]Xie *et al.*, 2006. [l]Vijayaraghavan *et al.*, 1997; Turner *et al.*, 1999; Luconi *et al.*, 2004. [m]Luconi *et al.*, 2004; Huang *et al.*, 2005. [n]Quill *et al.*, 2003; Qi *et al.*, 2007; Li *et al.*, 2007. [o]Spehr *et al.*, 2003. [p]Isobe *et al.*, 2002; Muciaccia *et al.*, 2005. [q]Inoue *et al.*, 2005. [r]Ellerman *et al.*, 2006. [s]Krausz *et al.*, 1996; Aitken *et al.*, 1996a, 1996b; O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001. [t]Francavilla *et al.*, 1994; Zahn *et al.*, 2002. [u]Luconi *et al.*, 1995; Tomes *et al.*, 1996; Moseley *et al.*, 2005; Bedu-Addo *et al.*, 2005. [v]O'Brien & Zini, 2005; Erenpreiss *et al.*, 2006b. [vw]Sutovsky *et al.*, 2004; Muratori *et al.*, 2005. [x]Muratori *et al.*, 2004; Marchiani *et al.*, 2007. [y]Twigg *et al.*, 1998; Lopes *et al.*, 1998. [z]Taylor *et al.*, 2004; Barroso *et al.*, 2006.

Conclusions

In conclusion, several molecular markers can be proposed for each of the sperm functions necessary for oocyte fertilization (Table 1); however, whether each marker can specifically identify defects in the fertilizing ability of human sperm awaits further studies. Models that define sperm functions such as capacitation and in particular sperm–oocyte interaction are continuously evolving because of genetic approaches and classical biochemical studies that impact this field of research. However, it will be important to translate the knowledge deriving from reproductive phenotype of KO mouse models into human and other animals. Some of the genes which appear to be essential in animal KO models may be unessential or redundant in human. At the same time, knowledge deriving from classical studies, performed mostly in vitro, suffers from the important bias that the natural process occurs in vivo and thus in a likely totally different environment.

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