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INVITED REVIEW

Sperm capacitation: a distant landscape glimpsed but unexplored

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ABSTRACT: Capacitation is a remarkable process whereby spermatozoa prepare themselves for engagement with the oocyte. Although the existence of this process has been appreciated as a biological phenomenon for more than half a century, its molecular underpinnings still await clarification. We know that some of the major changes involve sterol oxidation and efflux from the plasma membrane, the anterior movement of lipid rafts, changes in the surface expression of a variety of proteins including hyaluronidase and receptors for the zona pellucida, an increase in intracellular cyclic adenosine monophosphate (cAMP), the induction of tyrosine phosphorylation and the expression of hyperactivated motility. These changes are dependent on the presence of bicarbonate, to facilitate cAMP generation, maintain an alkaline intracellular pH and support an optimal level of reactive oxygen species generation and are enhanced by the presence of albumin to provide antioxidant protection to the plasma membrane and promote cholesterol efflux. *In vivo*, the rate at which sperm cells capacitate is carefully controlled in order to ensure that the release of capacitated spermatozoa from a post-insemination reservoir in the isthmic region of the oviduct is synchronized with ovulation. The factors that control these critical events are now being resolved, aided by proteomic studies that are providing critical definitive information on the range of receptors that exist in the sperm plasma membrane and define the manner in which these exquisitely complex cells interact with their environment. Progress in this area has been enhanced by IVF technology pioneered by Bob Edwards and will ultimately facilitate the design of safe, effective culture conditions for optimization of this revolutionary therapy.

Key words: spermatozoa / sperm biochemistry / sperm function / fertilization / acrosome reaction

Introduction

Bob Edwards changed the face of reproductive healthcare forever. The introduction of *in vitro* fertilization and embryo transfer as a form of therapy for human infertility has revolutionized the treatment of this condition allowing millions of couples to have children who would have otherwise been denied this privilege. Less appreciated is the fact that this technology was developed against a tide of negativity created by those who felt, at the time, that it was impossible, unethical or unnecessary. His ultimate triumph over the forces of adversity rightly earned him a Nobel prize—even if it was a decade or so too late.

Throughout the evolution of this technology it was clear that Bob was fundamentally a geneticist who had a particular passion for oocytes and preimplantation embryos and a keen awareness of the potential bound up in stem cell biology (Edwards, 2005). He published seminal works on oocyte maturation and deliberated extensively on the endocrine control of follicular development (Edwards, 1965, 2002). However, to our knowledge he never published a paper on the testes and did not delve deeply into sperm cell biology. To our knowledge his sole experimental excursion into sperm capacitation came in 1968 when he developed a small diffusion chamber that could be inserted into the uterine cavity with a view to exposing human spermatozoa to the secretions of the female reproductive tract (Edwards *et al.*, 1968). Unfortunately this strategy was unsuccessful, possibly because of a localized inflammatory response to the presence of the device itself (Johnson, 2011). Despite such an unpromising start, a solution to the problem of sperm capacitation *in vitro* was rapidly found. Building on the pioneering works of Bunny Austin, MC Chang and Ryuzo Yanagimachi, Bob's prodigé, Barry Bavister, had determined that spermatozoa could be capacitated in readiness for *in vitro* fertilization in a simple defined culture medium (Edwards *et al.*, 1969; Bavister, 1973). Once this practical biological milestone had been achieved, Bob did not give the mechanisms underpinning this process high priority. This is a pity because the processes of sperm transport and capacitation *in vivo* are extremely sophisticated, beautifully controlled biological events, designed to deliver a highly selected subpopulation of spermatozoa to the surface of the oocyte, capable of rapidly and effectively engaging the process of fertilization.

The biological journey to the oocyte begins with hundreds of millions of spermatozoa being inseminated into the female reproductive tract. At the moment of ejaculation these cells instantly express high levels of progressive motility but are otherwise completely incapable of recognizing the egg or engaging in the complex cascade of cell–cell interactions

that culminate in syngamy. During their ascent of the female reproductive system the spermatozoa successfully avoid the gauntlet laid down by the maternal immune system and ignore the large number of other cells with which they make contact on their journey towards the Fallopian tube. However on reaching the isthmic region of oviduct, this behaviour is suddenly reversed as the spermatozoa establish intimate contact with the endosalpingeal epithelium (Suarez and Pacey, 2006). In this location, the bound cells establish a quiescent sperm reservoir and remain in this state until they receive a signal associated with ovulation. At this point, the spermatozoa suddenly break away from their epithelial resting place in a hyperactivated state and migrate rapidly towards the oocyte in a state of readiness for fertilization (Suarez, 2008). By the time the spermatozoa have reached the surface of the oocyte, they are completely transformed cells exhibiting a hyperactivated form of movement (Yanagimachi, 1994), expressing various receptors for the oocyte-cumulus mass on their surface (Reid *et al.*, 2011) and with a plasma membrane that has been primed to initiate the acrosome reaction in response to a calcium transient (Florman *et al.*, 2008). The story of the molecular changes that underpin this complex biological journey is the story of sperm capacitation (Fig. 1). It is a pity that Bob is no longer around to appreciate it.

Cholesterol and changes in membrane fluidity

One of the first changes described in capacitating mammalian spermatozoa was a loss of cholesterol from the sperm plasma membrane (Davis *et al.*, 1979). Sterols such as cholesterol and desmosterol are removed from the sperm surface by proteinaceous acceptor molecules such as albumin, high-density lipoproteins and apolipoproteins (e.g. apoA-1) in the extracellular space. It is for this reason that albumin is an extremely important component of *in vitro* fertilization media, although it may not be mandatory for all species (Choi *et al.*, 2003). Cholesterol is a powerful decapacitation factor that serves to stabilize the plasma membrane of the spermatozoa during epididymal transit and prevent the intermolecular interactions responsible for achieving a capacitated state (Davis, 1980). A small percentage of sperm cholesterol (~6%) is stabilized in the sperm plasma membrane as cholesterol sulfate (Langlais *et al.*, 1981; Sion *et al.*, 2001). As spermatozoa ascend the female reproductive tract and initiate capacitation, sterol sulfatases affect the enzymatic hydrolysis of the sulfate group, thereby increasing the pool of cholesterol available for esterification (Roberts, 1987). The fatty acids required for the esterification of cholesterol are provided via their enzymatic cleavage from membrane phospholipids by phospholipase A. One of the by-products of this process is the creation of highly unstable lysophospholipids that generate increased membrane fluidity and permeability to calcium, both of which should promote capacitation and subsequent acrosomal exocytosis. Cholesterol transfer from the sperm plasma membrane to albumin may involve the mediation of an active cholesterol transporter such as ABCA17 (Morales *et al.*, 2012). However, a major contributor to the cholesterol efflux from the sperm plasma membrane during capacitation is oxidative stress. Thus, recent studies have established that sterols can become oxidized during capacitation and that the increased hydrophilicity of the oxidation products facilitates their transfer to albumin (Brouwers *et al.*, 2011). This process is dependent on the presence of bicarbonate which is, in turn, required to promote reactive oxygen species (ROS) generation by the spermatozoa (Ecroyd *et al.*, 2003; Boerke *et al.*, 2013). Addition of antioxidants such as the combination of vitamin E and C to mammalian spermatozoa inhibits this redox-regulated process and disrupts the capacitation of the spermatozoa (O'Flaherty *et al.*, 1997; Boerke *et al.*, 2013).

Redox regulation

The involvement of ROS in the capacitation of mammalian spermatozoa has been appreciated since the pioneering studies of Claude Gagnon in the 1990s (de Lamirande and Gagnon, 1993a). Which particular ROS is responsible for capacitation has been the subject of some controversy because compelling evidence has been produced to support a key role for hydrogen peroxide (Bize *et al.*, 1991; Aitken *et al.*, 1995, 1996; Rivlin *et al.*, 2004) superoxide anion (de Lamirande and Gagnon, 1993b) and the peroxynitrite anion, generated by the reaction of superoxide anion with another free radical species, nitric oxide (NO) (Herrero *et al.*, 2001; Rodriguez and Beconi, 2009). In reality, the interconversion of these various reactive oxygen and reactive nitrogen species is very rapid and it is probable that several different redox entities are involved in various aspects of the capacitation process. For example, the suppression of tyrosine phosphatase activity is intimately involved in the global elevation of protein tyrosine phosphorylation levels that accompany capacitation. This family of enzymes possesses a key cysteine residue at their active site that must be in a reduced state for phosphatase activity to be expressed. Powerful oxidants generated during sperm capacitation such as hydrogen peroxide and peroxynitrite are both capable of oxidizing this cysteine residue and inactivating tyrosine phosphatase activity (Hecht and Zick, 1992; Takakura *et al.*, 1999). Superoxide is also thought to participate in the direct activation of soluble adenylate cyclase, increasing the intracellular levels of cyclic adenosine monophosphate (cAMP) that, in turn, drive tyrosine kinase activity via a Src-dependent mechanism described in detail below (Zhang and Zheng, 1996; Baker *et al.*, 2006; Ickowicz *et al.*, 2012). Hydrogen peroxide is also thought to enhance adenylyl cyclase activity via the induction of enhanced tyrosine kinase activity (Tan *et al.*, 1995) creating a self-perpetuating cascade involving ROS generation, adenylyl cyclase activation and tyrosine phosphorylation. For its part, peroxynitrite is known to both inhibit tyrosine phosphatases and activate tyrosine kinases of the Src family (Minetti *et al.*, 2002) making it a particularly powerful contributor to the capacitation process.

Further evidence for a positive role for ROS in the molecular mechanisms regulating sperm capacitation can be found in the powerful biological effects elicited by both ROS-specific scavengers and exposure to exogenous ROS of various kinds. For example, the tyrosine phosphorylation surge associated with the capacitation in human spermatozoa can be blocked by the addition of catalase to scavenge all of the hydrogen peroxide generated by these cells (Griveau *et al.*, 1994; Aitken *et al.*, 1995, 1996; Leclerc *et al.*, 1997). Similar results have been reported for hamster, buffalo, mouse and equine spermatozoa (Bize *et al.*, 1991; Baumber *et al.*, 2003; Ecroyd *et al.*, 2003; Roy and Atreja, 2008). In addition, the direct addition of hydrogen peroxide to mammalian spermatozoa has been shown to induce the tyrosine phosphorylation events associated with capacitation in several species (Aitken *et al.*, 1995; Rivlin *et al.*, 2004; Roy and Atreja, 2008) and trigger the acrosome reaction (O'Flaherty *et al.*, 1999). Furthermore, membrane permeant ROS scavengers such as 2-mercaptoethanol have been reported to have a profound inhibitory impact on tyrosine phosphorylation (Aitken *et al.*,

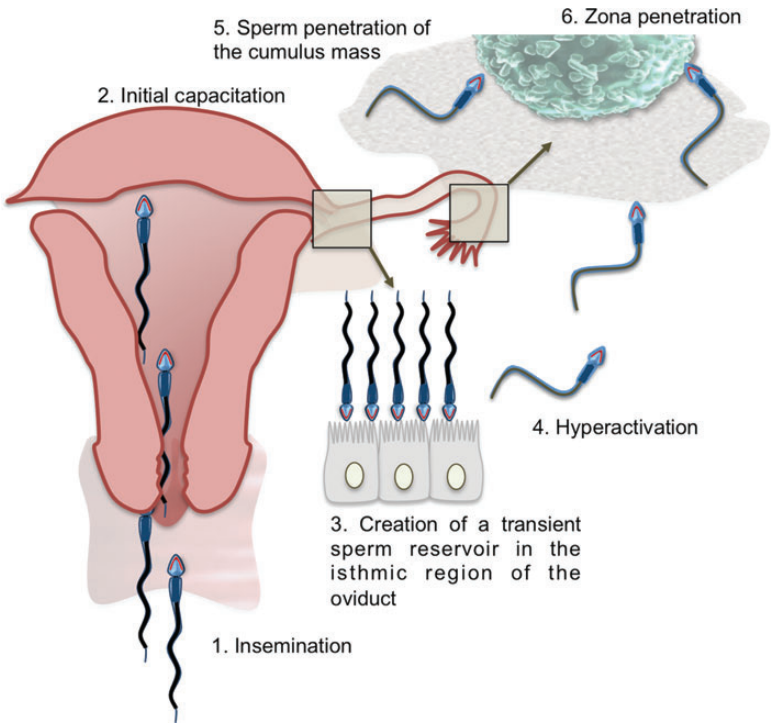

Figure 1 The stages of sperm capacitation *in vivo*. (1) At insemination hundreds of millions of spermatozoa are released into the female tract. At this stage in their life history these cells are progressively motile, yet uncapacitated. (2) As spermatozoa traverse the uterine cavity, the initial stages of capacitation occur characterized by the loss of decapacitation factors, largely acquired from epididymal and seminal plasma, from the sperm surface. (3) Spermatozoa are subsequently thought to establish a reservoir in the isthmic region of the Fallopian tubes (Baillie *et al.*, 1997). While bound to these epithelial cells the spermatozoa become quiescent and are stored in readiness for ovulation. (4) An endocrine signal coincident with ovulation induces a sudden change in sperm biochemistry characterized by an increase in reactive oxygen species (ROS) generation, intracellular cyclic adenosine monophosphate levels and tyrosine phosphorylation. In response to these signals, calcium is released from an intracellular store in the redundant nuclear envelope in a pulsatile manner inducing the expression of hyperactivated motility. (5) In this hyperactivated state, spermatozoa are released from the oviductal epithelium and migrate up the Fallopian tube towards the oocyte where they engage the cumulus mass. (6) Spermatozoa may acrosome react within the cumulus mass or may migrate towards the zona surface and bind to this structure via surface-orientated zona-binding complexes localized within lipid rafts and featuring a number of potential zona-binding molecules including arylsulfatase A (ARSA) and the zona pellucida binding protein (ZPBP2) (Redgrove *et al.*, 2011, 2012).

1998a). In most species, superoxide dismutase (SOD) cannot suppress sperm tyrosine phosphorylation levels suggesting a lack of superoxide involvement in the capacitation process (Baumber *et al.*, 2003). However the late Claude Gagnon found that this enzyme could robustly suppress the capacitation of human spermatozoa induced by fetal chord serum (de Lamirande and Gagnon, 1993a, b). Such results suggest that the latter specifically activates superoxide generation in human spermatozoa leading to metabolic products such as peroxynitrite, which are known to stimulate the capacitation process via the stimulation of tyrosine phosphorylation, the suppression of tyrosine phosphatase activity and, possibly, the induction of cholesterol oxidation as highlighted above. Similarly, for bovine spermatozoa, SOD has been shown to suppress capacitation (O'Flaherty *et al.*, 2003) and in this species too, there is abundant evidence for peroxynitrite as an inducer of sperm capacitation (Rodriguez and Beconi, 2009; Rodriguez *et al.*, 2011).

While the central role of ROS in the induction of sperm capacitation is not in doubt, the molecular source of the free radicals and oxidants that stimulate capacitation is unknown. Although several groups have confirmed that spermatozoa contain nicotinamide adenine dinucleotide phosphate (NADPH) oxidases such as NADPH oxidase 5 (NOX5), there is no definitive evidence for the biochemical involvement of such enzymes in sperm capacitation (Musset *et al.*, 2012). Several studies have demonstrated that the flavoprotein inhibitor diphenylene iodonium (DPI) will inhibit tyrosine phosphorylation during the capacitation of mouse, bovine, human and hamster spermatozoa (Aitken *et al.*, 1995, 1997, 1998a, 2004; Ecroyd *et al.*, 2003; O'Flaherty *et al.*, 2003; Córdoba *et al.*, 2006; Roy and Atreja, 2008). Because DPI is a known and potent inhibitor of NADPH oxidases, such results have been cited as evidence for the involvement of oxidase activity in sperm capacitation. However, DPI is also an inhibitor of mitochondrial ROS generation, so alternative interpretations of these results are possible. The ability of apocynin to suppress sperm ROS generation (Donà *et al.*, 2011) is more convincing because this inhibitor is specific for NADPH oxidases, particularly NOX2. However while apocynin does clearly inhibit ROS generation by human sperm suspensions, the possibility cannot be excluded that such inhibition is a reflection of low-level leukocyte contamination, NOX2 being the major oxidase of phagocytic leukocytes. Nitric oxide synthase has also been proposed as a source of NO in spermatozoa (O'Flaherty *et al.*, 2004; Roessner *et al.*, 2010) although non-enzymatic pathways involving, for example, a direct attack on

arginine by hydrogen peroxide cannot be excluded (Aitken *et al.*, 2004). It is always possible, indeed it is likely, that more than one source of ROS is involved in promoting so critical a process as capacitation, creating a high level of redundancy in the redox regulation of this process. Clearly, the major targets for such redox regulation are the phosphatases and kinases regulating tyrosine phosphorylation; however, the redox activity that drives this process may involve multiple ROS species originating from multiple subcellular sites (Aitken *et al.*, 2003).

Whatever the source of the ROS that drives capacitation, it places these cells on a knife-edge because they are inherently vulnerable to oxidative stress. Indeed it has been argued that sperm capacitation and the entry of these cells into the intrinsic apoptotic cascade are a continuum, the ROS that drive tyrosine phosphorylation, cAMP production and cholesterol efflux from the plasma membrane ultimately inducing a state of apoptosis (Aitken, 2011). It is for this reason that antioxidants such as vitamin E have been repeatedly shown to help preserve the functional integrity of spermatozoa by virtue of their capacity to counteract the oxidative stress associated with apoptotic death (Beconi *et al.*, 1993; Breininger *et al.*, 2005; Silva *et al.*, 2012). During the latter, mitochondrial ROS generation is increased as a consequence of protein adduction within the mitochondrial electron transport chain by cytotoxic lipid aldehydes such as acrolein and 4-hydroxynonenal (4HNE) generated as a result of the lipid peroxidation precipitated by oxidative stress (Aitken *et al.*, 2012b). Senescent, over-capacitated spermatozoa are therefore characterized by high levels of mitochondrial ROS generation, oxidative DNA damage and high rates of 4HNE generation (Aitken and Baker, 2013). These cells also exhibit a significant reduction in their motility, caspase activation and the expression of surface markers of apoptosis such as phosphatidylserine (Koppers *et al.*, 2011). *In vivo*, the latter may be particularly important as a signal to infiltrating leukocytes that the phagocytic process they are about to engage in should be silent, in the sense that no proinflammatory cytokines or ROS must be generated (Aitken *et al.*, 2012a). Given the very high number of dead and moribund spermatozoa that litter the female tract following insemination, it is clearly essential that the phagocytic process that achieves their removal is carefully controlled so that collateral oxidative damage to the female tract is kept to an absolute minimum.

Tyrosine phosphorylation

Visconti *et al.* (1995) were the first to demonstrate that the capacitation of murine spermatozoa was accompanied by a massive increase in tyrosine phosphorylation focused on the fibrous sheath of the sperm tail. Tyrosine phosphorylation has subsequently been shown to be a feature of capacitation in all mammalian species that have been examined including bovine (Galantino-Homer *et al.*, 1997), porcine (Flesch *et al.*, 1999), equine (Pommer *et al.*, 2003), hamster (Visconti *et al.*, 1999), rat (Lewis and Aitken, 2001), mouse (Visconti *et al.*, 1995), human (Aitken *et al.*, 1996) and even wallaby spermatozoa (Bennetts *et al.*, 2004). This process was shown to be promoted by the presence of protein (bovine serum albumin), calcium and bicarbonate in the medium; however, none of these factors are probably essential. As long as the cells are viable, intracellular pH is adequately buffered (Aitken *et al.*, 1998b) and ATP levels are high (Baker *et al.*, 2004; Ecroyd *et al.*, 2004), mammalian spermatozoa will capacitate and exhibit high rates of tyrosine phosphorylation in medium lacking bicarbonate and calcium and in which exogenous protein has been replaced by polyvinyl alcohol (Baker *et al.*, 2004).

The primary kinases involved in triggering this tyrosine phosphorylation cascade are members of the SRC family particularly pp60cSRC and cABL (Baker *et al.*, 2006, 2009). cAMP-mediated activation of protein kinase A (PKA) both directly activates these kinases and simultaneously suppresses an inhibitor of SRC, C-terminal SRC kinase (Baker *et al.*, 2006). The targets of SRC-induced phosphorylation are still being resolved; however, it is possible that this family of kinases drives tyrosine phosphorylation via the phosphorylation-dependent inhibition of a tyrosine phosphatase, which normally keeps PKA-dependent tyrosine phosphorylation under inhibitory control (Krapf *et al.*, 2010; Battistone *et al.*, 2013).

In addition to SRC-mediated tyrosine phosphorylation pathways driven by cAMP there is evidence that capacitation might also involve receptor-activated tyrosine kinases. Specifically, the extracellular signal-regulated kinases (ERKs) represent a specific subset of the mammalian mitogen-activated protein (MAP) kinase family with postulated roles in the induction of capacitation. Claude Gagnon's laboratory was instrumental in demonstrating that the entire ERK pathway is involved in the capacitation of human spermatozoa (de Lamirande and Gagnon, 2002; O'Flaherty *et al.*, 2005, 2006a, b). Receptor tyrosine kinases including fibroblast growth factor receptor, insulin-like growth factor receptor and epidermal growth factor receptor have all been detected in mammalian spermatozoa (Lax *et al.*, 1994; Naz and Padman, 1999; Cotton *et al.*, 2006). These receptor kinases appear to stimulate tyrosine phosphorylation in mammalian spermatozoa by working through the Ras-Raf-MEK-ERK network (Roberts and Der, 2007). There is also some evidence for cross talk between the cAMP/PKA/SRC and MAP kinase pathways in regulating sperm tyrosine phosphorylation during capacitation, although the precise nature of this interaction is not well understood (Luna *et al.*, 2012). In addition, there is evidence that the ERK pathway can also be directly activated by ROS, in the absence of growth factor receptor activation, possibly as a consequence of phosphatase inactivation (O'Flaherty *et al.*, 2005, 2006a, b).

Capacitation and decapacitation factors

The existence of the above-mentioned tyrosine kinase receptors is important since it demonstrates a potential mechanism by which the secretions of the female reproductive tract might control the capacitation process. This is a very poorly understood area of gamete biology. While it is evident that spermatozoa can fertilize an oocyte *in vitro* in a simple defined culture medium, *in vivo*, there are dynamic interactions with the female reproductive tract that carefully regulate the rate at which these cells achieve capacitation so that they are delivered to the surface of the oocyte in a fully primed state, ready for fertilization. These regulatory mechanisms are particularly important in the case of our species because human reproduction is characterized by a lack of synchrony between insemination and ovulation, there being no overt oestrus in our species. If the spermatozoa capacitate too fast, the redox-regulated mechanisms that drove them down the path of capacitation will ultimately create such cellular stress that the cells default to apoptosis (Aitken, 2011). If they capacitate too slowly, they will not be equipped to recognize the oocyte when it arrives in the ampulla of the Fallopian

tube or be able to participate in the intricate cascade of cell–cell interactions that culminate in fertilization.

The regulation of capacitation *in vivo* involves both exposure to a variety of ligands that control the fate of the cell via receptor-mediated mechanisms and the loss of decapacitation factors from the sperm surface. Some of the receptor-mediated interactions are designed to operate late in the sperm capacitation process to prepare the cell for acrosomal exocytosis; progesterone, epidermal growth factor and platelet endothelial cell adhesion molecule would be examples of such late-acting ligands (Nixon *et al.*, 2005; Hunter, 2008; Breitbart and Etkovitz, 2011). Other endocrine factors act early in the capacitation process and are designed to promote cell survival and to impede premature entry of the spermatozoa into the intrinsic apoptotic pathway; prolactin would be a good example of such a factor (Pujianto *et al.*, 2010). A number of other decapacitation factors are associated with the sperm surface in the male tract and also serve to prevent premature capacitation of these cells. Such factors include cholesterol (Davis, 1981), protease inhibitors including the serine protease inhibitor Kasal-type-like protein (SPINKL) (Lin *et al.*, 2008) and serine protein inhibitor (Lu *et al.*, 2011), platelet-activating factor acetylhydrolase (Zhu *et al.*, 2006), phosphatidylethanolamine binding protein 1 (Nixon *et al.*, 2006), NYD-SP27, an isoform of phospholipase C Zeta 1 localized to the sperm acrosome (Bi *et al.*, 2009), HongrES1 (Ni *et al.*, 2009) and mouse seminal plasma protein, SVS2, which interacts with the ganglioside GS1 (Kawano *et al.*, 2008). Capacitation appears to involve the dissociation of such factors from the sperm surface, largely, but not exclusively, as a result of passive diffusion.

Hyperactivation

Hyperactivation is one of the hallmarks of sperm capacitation. It involves a transition in the flagellar wave form from the low-amplitude, symmetrical beat pattern typical of progressively motile cells to a high-amplitude, asymmetrical thrashing of the sperm tail (Yanagimachi, 1994). Hyperactivated spermatozoa display a typical high velocity figure-of-eight pattern of movement that is thought to generate the propulsive forces necessary to pull the spermatozoa away from the oviductal epithelium and penetrate the dense matrix represented by the zona pellucida (Suarez, 2008). In some species such as the hamster, there is an orderly, relatively synchronized, progression towards a hyperactivated form of movement as the spermatozoa attain a capacitated state (White and Aitken, 1989). By contrast, human spermatozoa exhibit brief transient unsynchronized bursts of hyperactivated movement as they become capacitated (Pacey *et al.*, 1997). The underlying biochemistry is still being elucidated but all of the available evidence suggests that this is a cAMP-mediated event involving high levels of tyrosine phosphorylation in the sperm tail (Nassar *et al.*, 1999). One of the consequences of this cAMP-mediated process is to facilitate a pulsatile pattern of calcium release in the flagellum from an intracellular calcium store thought to reside in the redundant nuclear envelop located at the base of the sperm head (Ho and Suarez, 2003; Aitken and McLaughlin, 2007). By eliciting intracellular calcium transients in spermatozoa, progesterone is capable of inducing hyperactivated sperm movement possibly via the mediation of the sperm-specific flagellar calcium channel, CatSper—at least in human spermatozoa (Sagare-Patil *et al.*, 2013; Smith *et al.*, 2013).

Recent data suggest that CatSper-mediated calcium entry into the flagellum does not directly induce hyperactivation (Alasmari *et al.*, 2013). Rather, during capacitation CatSper may be involved in filling the intracellular calcium store, which becomes sensitized to calcium-induced calcium release during capacitation via mechanisms that may involve NO mediated *S*-nitrosylation of ryanodine receptors and/or cAMP-mediated processes. In marsupial spermatozoa we have found that exposure to membrane permeant cAMP analogues induces an immediate burst of hyperactivated motility (M. Lin, unpublished observations). Similar induction of hyperactivation with cAMP has been observed in boar spermatozoa in a manner that paralleled the induction of tyrosine phosphorylation (Harayama *et al.*, 2012). Whether the induction of hyperactivation with cAMP is dependent on tyrosine phosphorylation and, if so, the identities of the proteins phosphorylated in this manner are unknown. Alternatively, tyrosine phosphorylation may simply be an associated phenomenon and the induction of hyperactivated movement with cAMP may involve non-PKA-dependent mechanisms, including exchange proteins directly activated by cAMP (EPACs), in order to facilitate calcium release from the intracellular store (Alasmari *et al.*, 2013). While there may be species-specific differences in terms of the detailed control mechanisms, a general consensus is emerging that calcium and cAMP are the key regulators of hyperactivation and that the ultimate target of their action is the pulsatile release of calcium from an intracellular store located in the redundant nuclear envelope. CatSper is clearly essential for this process, possibly by facilitating the creation of intracellular calcium stores during capacitation (Qi *et al.*, 2007; Alasmari *et al.*, 2013).

Egg receptor expression

As capacitated spermatozoa approach the oocyte, they are primed and in a state of readiness to undergo the acrosome reaction. The induction of acrosomal exocytosis may occur as spermatozoa approach the oocyte in response to soluble factors in the vicinity of the cumulus-oocyte complex such as progesterone (Inoue *et al.*, 2011; Jin *et al.*, 2011). Although these data are incontrovertible in demonstrating that acrosome-reacted spermatozoa can penetrate the zona pellucida and fuse with the oocyte, these observations do not preclude the long-established view that capacitated spermatozoa can also acrosome react on binding to the zona pellucida (Gadella *et al.*, 2008). Indeed, one of the most dynamic properties acquired by capacitating spermatozoa is an ability to recognize the zona pellucida: only capacitated spermatozoa can bind to this structure (Dun *et al.*, 2010). Early attempts to explain the molecular basis of this process focused on the presence of a single receptor species on the surface of capacitating spermatozoa exhibiting an affinity for the zona glycoprotein, ZP3. A number of sperm surface receptors were proposed to mediate this process including zona receptor kinase, mannosidase, sperm protein (SP)56 and beta galactosidase; however, all of these candidates were ultimately discarded when knockout mice lacking each of these putative receptors were shown to be fully fertile (Reid *et al.*, 2011). We proposed an alternative mechanism in 2004 (Asquith *et al.*, 2004), which posited that there is no single receptor for the zona pellucida but rather several candidate molecules, which are assembled into multimeric recognition complexes under the influence of molecular chaperones. In the case of mouse spermatozoa the chaperones associated with this process were identified as HSP90B1 (endoplasmin), HSPD1 (heatshock protein HSP60) and as well as a family of chaperonins belonging to the t-complex (Asquith *et al.*, 2004; Dun *et al.*, 2011). These molecules become surface orientated during

capacitation in a phosphorylation-dependent process (Nixon *et al.*, 2010). They also reside within lipid rafts, microdomains that are moved within the plasma membrane in order to become localized at the anterior acrosomal aspect of the sperm head during capacitation (Nixon and Aitken, 2009; Nixon *et al.*, 2009).

Although we have previously attempted to define the chaperones that might be involved in shepherding human zona receptors to the sperm surface, none of the candidates examined exhibited surface expression, placing the potential role of chaperones in the mediation of human sperm–egg interaction in some doubt (Mitchell *et al.*, 2007). However, by comparing the proteomic structure of spermatozoa from donors who exhibit normal sperm function with patients exhibiting infertility associated with a failure of sperm–zona interaction we have succeeded in identifying a chaperone associated with the presentation of zona receptors to the human sperm surface during capacitation in the form of HSPA2 (Redgrove *et al.*, 2012, 2013). Our analysis of HSPA2 demonstrated that it is primarily localized to the anterior region of the human sperm head (Redgrove *et al.*, 2012), the precise location that mediates zona adhesion. In addition, HSPA2 was found to be a target for capacitation-associated tyrosine phosphorylation (Redgrove *et al.*, 2013), to be recruited into membrane rafts (Nixon *et al.*, 2011) and to form a major component of at least five large molecular mass complexes (Redgrove *et al.*, 2012). The most dominant of these complexes was found to contain HSPA2, in close association with sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA), two proteins that have been implicated in sperm–egg interactions. On the basis of these data we have proposed that HSPA2 is involved in orchestrating the dynamic remodelling of the sperm plasma membrane leading to the surface expression of hyaluronidases such as SPAM1, to enable the spermatozoa to engage with the extracellular matrix surround in the egg and zona receptors such as ARSA as they capacitate. We have even uncovered evidence that the expression of these two molecules is sequential with SPAM1 preceding the surface expression of ARSA, exactly as might be predicted from the chronology of events associated with fertilization. This model is consistent with previous data that have shown that reduced HSPA2 levels are causally linked with defects in zona pellucida adhesion and male infertility (Huszar *et al.*, 1994, 2006, 2007).

Conclusions

In conclusion, our understanding, and our appreciation, of sperm cell biology has increased dramatically as a result of new technologies that permit the high resolution imaging of these cells, their analysis by flow cytometry and a proteomics revolution that has facilitated analysis of the post-translational modifications that are the ultimate determinants of sperm function. Although spermatozoa can perform their functions in simple defined culture medium, *in vivo* they actively interact with a range of physiological regulators during their ascent of the female reproductive tract. We are only just beginning to understand the nature of these regulatory factors and the mechanisms by which they maintain these cells in a viable but uncapacitated state for several days prior to ovulation but then permit the rapid capacitation of these cells at the time of ovulation. The net result of this complex, carefully orchestrated process is to transform the functional competence of these cells such that they approach the oocyte exhibiting a highly specialized, hyperactivated form of movement, expressing receptors for the surface of the zona pellucida and primed to undergo the acrosome reaction. Of the 200 million spermatozoa entering the female tract at insemination only around 50 will successfully complete the journey to the surface of the egg. The molecular attributes of 'the chosen few' and the cellular mechanisms that allow them to attain a capacitated state are not just fascinating from a scientific perspective but also hold the key to understanding the causes of male infertility and possible pathways to male fertility regulation. Understanding the process of sperm capacitation may also help us to develop optimized IVF culture media that support high rates of fertilization while maintaining low levels of DNA damage in both gametes and embryos. Attainment of this goal will enable us to make the revolutionary therapy pioneered by Bob Edwards and Patrick Steptoe as safe and effective as humanly possible.

Authors' roles

R.J.A. generated the first draft of the manuscript which was then refined and edited by B.N.

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