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First Author: Maria João Freitas
Corr. Author:

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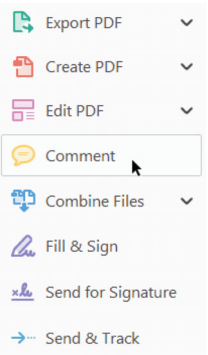

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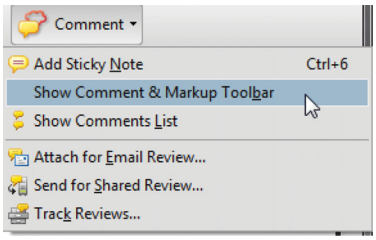

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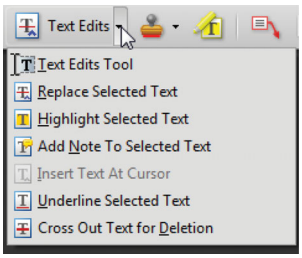

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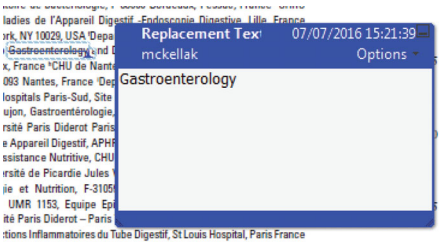

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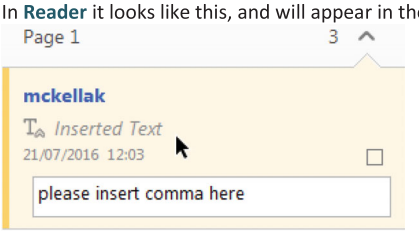

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Signaling mechanisms in mammalian sperm motility†

Maria João Freitas[1], Srinivasan Vijayaraghavan[2] and Margarida Fardilha[1,*]

[1]Laboratory of Signal Transduction, Institute for Research in Biomedicine, Medical Sciences Department, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal and [2]Department of Biological Sciences, Kent State University, Kent, Ohio, USA

Q2 *Correspondence: ~~Laboratory of Signal Transduction, Institute for Research in Biomedicine, Health Sciences Program, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal,~~ E-mail: mfardilha@ua.pt

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Abstract

The goal of sperm is to fertilize the oocyte. To achieve that purpose, it must acquire motility in the epididymis and hyperactivated motility in the female reproductive tract. Motility is only achieved when the sperm presents a fully functional flagellum, is capable of producing energy to fuel the movement, and suffers epididymal maturation and capacitation. Since sperm is a transcriptionally silent cell, motility depends on the activation and/or inhibitions of key signaling pathways. This review describes and discusses the main signaling pathways involved in primary and hyperactivated motility, as well as the bioenergetic mechanisms necessary to produce energy to fuel sperm motility. Although the complete human sperm motility process is far from being fully known, we believe that in the upcoming decades extensive progress will be made. Understanding the signaling pathways behind sperm motility can help pinpoint the cause of male infertility and uncover targets for male contraception.

Summary Sentence

Sperm motility depends on energy availability, intact flagellum and the crosstalk of several signaling pathways that lead to an increase of tyrosine phosphorylation of key proteins.

Key words: sperm motility, signaling pathways, phosphorylation, epididymis, female reproductive system.

Introduction

Human spermatozoon is one of the most differentiated cell types and the only that must leave the male body where it is produced and achieve its goal in the female reproductive system [1]. To fertilize an egg, the sperm is formed in the testes, in a process called spermatogenesis. At the end of spermatogenesis, sperm are morphologically complete but functionally immature and incapable of fertilizing an egg. To be functional, sperm cells must undergo (i) maturation in the epididymis, (ii) capacitation, and (iii) acrosome reaction in the female reproductive system [2]. These events are co-dependent since acrosome reaction does not occur if capacitation is impaired and capacitation depends on functional maturation of sperm in the epididymis. Motility acquisition is essential for human sperm function and ultimately male fertility. In 2011, Paoli defined sperm motility as a propagation of transverse waves along the flagellum in a proximal-distal direction producing an

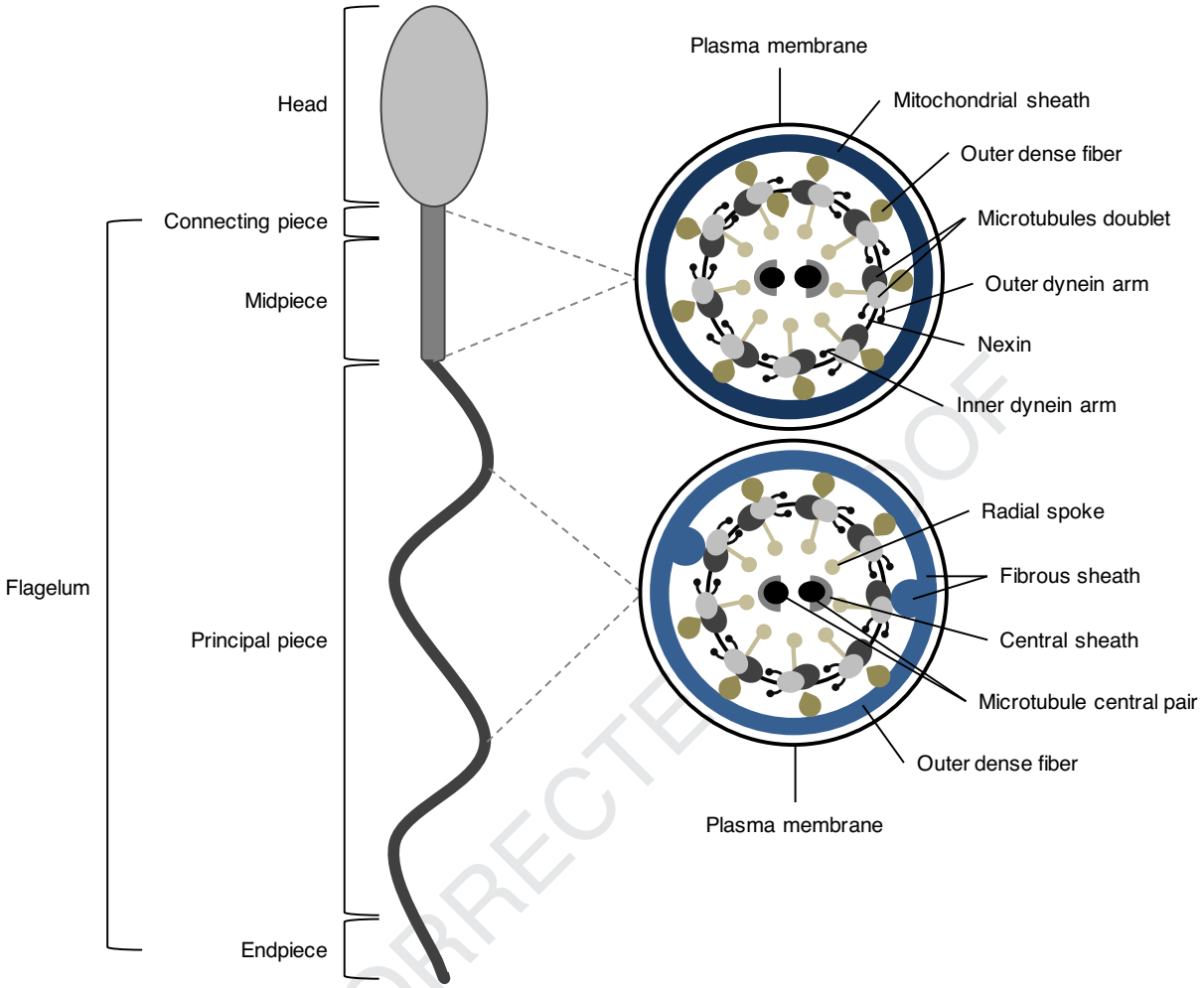

Figure 1. Schematic representation of human spermatozoon and flagellum structure. Human sperm is divided into two parts: head and flagellum. The flagellum is further divided into four structures: connecting piece, midpiece, principal piece, and endpiece. A cross-section shows that the flagellum structure differs between midpiece and principal piece. In midpiece, plasma membrane and mitochondrial sheath surround the outer dense fibers. Within outer dense fibers, the axoneme composed of the microtubule doublets associated with the dyneins arms (inner and outer), radial spoke, and microtubule central pair. Nexin connects adjacent microtubule doublet. In the principal piece, plasma membrane and fibrous sheath surround the outer dense fibers. In two opposing microtubule doublets, the outer dense fibers are replaced by longitudinal columns of fibrous sheath.

impulse that pushes the spermatozoon through the female genital tract [3].

Severe asthenozoospermia is one of the causes of male infertility, which arises from the inability of the sperm cell to reach the oocyte [4]. Primary or activated motility is acquired throughout the journey in the epididymis. Although the exact mechanism behind motility acquisition is still far from being fully understood, specific signaling events are described in the literature as essential for this process [2,5]. Low-amplitude symmetrical tail movements characterize sperm-activated motility and drive sperm in a straight line in a nonviscous media (seminal plasma) [6]. However, in fallopian tubes, sperm must acquire a specific type of motility, hyperactivated motility, which is characterized by high amplitude and asymmetric flagellar bends. Only this type of flagellar movement allows sperm to overcome dense mucus, detach from the oviductal epithelium, and penetrate the egg's protective vestments [7]. Curiously, in the viscous media hyperactivated sperm swim in a circular or figure-8 pattern [6,8]. Alterations in pH, specific molecules, and ion concentration changes are a few of the crucial events for the stimulation of hyperactivated motility [9,10]. However, the cellular mechanism and signaling pathways responsible for this type of motility are not fully described.

To be motile, human sperm need a morphologically complete flagellum, be able to produce energy to power flagellar movement and functional signaling pathways (to transduce external signals into internal signals). This review discusses these three topics, but mainly focuses on the signaling pathways involved in human sperm motility regulation. For an in-depth review on sperm bioenergetics, see du Plessis et al. [11]

Sperm flagellum—structure and function

The human sperm are composed of two main structures: head and flagellum (Figure 1). The head comprises the nucleus and the acrosome. The nucleus houses the genetic information to be delivered to the oocyte. Upon acrosome reaction, the acrosome integrity is disrupted and its content is released digesting the oocyte's zona pellucida [12]. The flagellum contains the motile apparatus necessary

for sperm motility [6,13] and is divided into four ultrastructures: connecting piece, midpiece, principal piece, and end piece [6]. The connecting piece attaches the flagellum to the sperm head, the midpiece contains the sperm mitochondria, the principal piece and the end piece generate the flagellar waveform pattern motility [6,13,14]. The main structure of the flagellum is the axoneme, which is the sperm motility motor. This structure is well conserved throughout evolution, present in flagella from protozoans to humans [1,13]. The axoneme originates in the connecting piece and terminates in the end piece. Typically, the axoneme is composed of nine microtubules doublets and a central pair, designated a 9+2 structure. The nine microtubules doublets connect to each other by nexin links and connect to the central pair by projections, the radial spokes. The latter are responsible for positioning and spacing the microtubules doublets in a perfect circle around the central pair microtubule. Projecting from the microtubules doublets are the inner and outer axonemal dynein arms (classified according to their position in relation to the doublet microtubule). These proteins are key for motility, by promoting sliding of a microtubule doublet in relation to the adjacent. The flagellar beating pattern begins with a dynein from one doublet transiently interacting with the following doublet. In the presence of ATP, axonemal dynein "walks" toward the base of the flagellum, forcing the adjacent microtubule doublet to slide down. Since microtubules are attached to the connecting piece, this movement encounters resistance, leading to the bending of the flagellum. At the end, the dynein detaches from the adjacent microtubule. To obtain a flagellum waveform movement and consequently motility, this process has to occur on one side of the axoneme and be inactive on the opposite site. Hence, the flagellar beat appears to be based on an "on-and-off" switch of the axonemal dynein arms, in specific points in the axoneme [1,6,13,14].

In mammalian sperm, between the axoneme and the plasma membrane, there are several accessory structures, such as the mitochondrial sheath, outer dense fibers, and fibrous sheath [1,6]. In the midpiece, the axoneme is surrounded by outer dense fibers and the mitochondrial sheath, while in the principal piece the axoneme is surrounded by outer dense fibers and fibrous sheath. The end piece has no accessory structures between the axoneme and the plasma membrane [13]. The mitochondrial sheath is composed of individual mitochondria coiled helically around the axoneme. In humans, the midpiece length is about a dozen mitochondrial turns [15]. The outer dense fibers have a petal-like shape, are directly above the axoneme microtubules doublets, and diminish in diameter from base to tip of the principal piece [16]. The outer dense fibers appear to be responsible for maintaining the passive elastic structure and recoil of the flagellum and to protect the axoneme against shearing forces [17]. In the principal piece, the fibrous sheath confers flexibility, shape and plane to the flagellar beat [18]. It also supports and ensures compartmentalization of signaling proteins that regulate motility, capacitation, and hyperactivation. In the principal piece, two opposing outer dense fibers are replaced by fibrous sheath projections [16] (Figure 1).

The regulation and propagation of this "on-and-off" signal and the conversion into flagellar bending appear to reside in the control of the ATPase activity of axonemal dynein arms. Although this process is not fully understood, alterations in pH, ATP availability, calcium concentration, and phosphorylation of key proteins appear to modulate axonemal dynein arms activity and consequently sperm motility. The process of ATP production and the signal pathways that control axonemal dynein activity will be discussed in the next topics [19].

Energy for motility—oxidative phosphorylation vs. glycolysis

One of the key requirements for sperm motility is energy availability. ATP is the fuel used by axonemal dynein ATPases within the flagellum [20], and active protein modifications, such as phosphorylation, also depend on ATP. Thus, it is not surprising that sperm requires exceptionally high amounts of ATP when compared with somatic cells [21]. Consequently, a constant and adequate supply of ATP is crucial [20]. In spite of the efforts [20,22,23], a long-standing debate exists on the metabolic pathway responsible for sperm motility bioenergetics: oxidative phosphorylation in mitochondria, glycolysis in the flagellum and head, or both.

In mammalian sperm, oxidative phosphorylation occurs in mitochondria, which are exclusively located in the midpiece. A mature mammalian spermatozoon contains approximately 72–80 mitochondria [24] and in theory can produce more than 30 ATP molecules per glucose molecule [25]. Since midpiece is localized at the anterior end of the flagellum, the transport of ATP to the all length of the flagellum must be efficient. Ford et al. believed that the model of flux transfer chains proposed by Dzeja and Terzic in 2003 is able to transport the ATP produced in mitochondria through the entire flagellum [23,26]. It was indeed shown that an increase in human sperm motility requires a parallel increase in mitochondrial activity [3,22,27]. Also, the use of specific inhibitors for the mitochondrial electron transport chain and ATP synthase decreases drastically human sperm motility [25]. Moreover, high mitochondrial activity levels increase the success of in vitro fertilization rate [20]. These studies suggest that human sperm motility correlates with mitochondrial functional status. Furthermore, mitochondrial activity is negatively correlated with morphological alterations in the midpiece, which appears to reinforce the role of mitochondrial ATP production in sperm motility [22].

In spite of the reports supporting the role of mitochondria in sperm motility, its contribution to flagellar beat can be questioned. Since mitochondria are localized in the midpiece, it has been argued if ATP diffusion and carrier systems are able to supply ATP throughout the entire length of the flagellum (about 50 μm in humans) [23,25]. Also, some authors argued that if ATP produced in the mitochondria fuels motility, the levels of reactive oxygen species produced during the electron transport chain would be harmful to DNA integrity [28]. However, both enzymatic (e.g., superoxide dismutase and glutathione peroxidases) and nonenzymatic antioxidants (e.g., glutathione and ascorbic acid) present in human sperm and seminal plasma appear to control the levels of ROS activity [28–30].

A growing hypothesis for the source of ATP (or at least part of the ATP) in sperm is the glycolytic pathway. Glycolysis is the process by which glucose is converted into pyruvate. During this process, energy is released in the form of ATP and NADH, with a rate of 2 ATP molecules per glucose. When human sperm are deprived of glucose (the starting unit of glycolysis) or when glycolysis is blocked, ATP content and protein tyrosine phosphorylation decreases. Consequently, sperm exhibits decreased motility [25,31–34]. Mukai and Okuno proved that even when mitochondria function is conserved, mouse sperm motility decreases when glycolysis is impaired. Moreover, a sperm-specific lactate dehydrogenase (LDHC) accounts for 80%–100% of the LDHC activity in human sperm and is anchored to the fibrous sheath along the length of the flagellum, representing a local ATP production closer to the site of ATP consumption [34]. Also, Odet et al. showed that a disruption in mouse sperm-specific *Ldhc* resulted in impaired fertility due to immotile sperm [35].

Furthermore, sperm-specific LDHC presents a low K_m for pyruvate and a high K_m for lactate, suggesting a higher affinity of LDHC for pyruvate and consequently a preference for the glycolytic energy pathway. It is noteworthy to mention that although most mammals rely, at least partially, on glycolysis for motility, the bull seems to be an exception. Oxidative phosphorylation in bull sperm appears to be the only source of ATP [36].

A third possibility for ATP availability in human sperm is a cooperation and dependence between oxidative phosphorylation in mitochondria and glycolysis in the flagellum. This hypothesis is supported by different energetic subtracts of the reproductive tract fluids [11,21]. It appears that mammalian sperm switch between metabolic pathways depending on oxygen availability and glucose, pyruvate, lactate, sorbitol, glycerol, and fructose concentration in the fluid [25,32,37–40]. For example, in the human female reproductive tract, glucose, pyruvate, and lactate are found in the range of 0.5–3.2, 0.1–0.2, and 4.9–10.5 mM, respectively. Sperm must adapt its bioenergetic metabolism according to the metabolites available from the epididymis until the fallopian tubes [41].

Signaling pathways in sperm motility

Sperm leaving the testes are immotile and acquire motility throughout the epididymis journey. Sperm is virtually devoid of transcription and translation due to highly condensed DNA and lack of endoplasmic reticulum [42]. Since gene expression cannot be accounted for functional alterations in sperm, activation or inhibition of specific signaling pathways and protein posttranslational modifications must be involved. The interaction between sperm and the environment created by the epididymis and the female reproductive tract are essential to trigger sperm motility.

Several signaling pathways have been described as having a role in mammalian sperm motility. In the next section, the most relevant signaling pathways and messengers involved in sperm motility acquisition in the epididymis and hyperactivated motility in the female reproductive tract will be described.

Sperm motility in the male reproductive system—a journey through the epididymis

After spermatogenesis, sperm is morphologically complete but functionally immature. When entering the epididymis, a long convoluted tubule that connects the testis to the vas deferens, human sperm is incapable of fertilizing an oocyte. The epididymis is roughly divided into three regions: caput, corpus, and cauda. The caput is adjacent to the testis and the caudal portion adjacent to the vas deferens [43]. Only during the journey through the epididymis, the sperm acquire fertilization ability. Epididymal maturation involves the interaction of sperm with proteins that are synthesized and secreted into the epididymis in a region-dependent manner [44]. The majority of studies concerning epididymis function are carried out in rodent models, due to the limited availability of human epididymal tissues at reproductive ages, the impossibility of mimicking the epididymis environment in vitro and the difficulty to manipulate the human epididymis experimentally. The exact mechanism behind sperm motility acquisition in the epididymis is still unknown [6].

One of the first described signaling events responsible for sperm motility acquisition within the epididymis is the control of phosphoprotein phosphatase 1 (PPP1, also known as PP1) activity in sperm (Figure 2). PPP1CC2 (also known as PP1γ2), a testis-enriched sperm-specific PPP1 isoform, is distributed throughout the flagellum, midpiece, and posterior region of the head [45], suggesting a role in motility and acrosome reaction [46,47]. In 1996, Smith et al. described, for the first time, the association between PPP1 activity and sperm motility. In caput sperm, PPP1 activity is high and sperm is immotile. Conversely, in caudal sperm PPP1 is inactive and sperm are motile [48,49]. In the following years, several studies attempted to unveil the signaling pathways responsible for PPP1 activity modulation in sperm, through the epididymis journey. PPP1 regulatory subunit 2, PPP1R2 (also known as Inhibitor-2), is a PPP1 inhibitor [50]. In sperm, PPP1R2 localizes throughout the principal piece, midpiece, and posterior and equatorial regions of the head. Former studies described PPP1R2 activity in human sperm and that some of the sperm PPP1 population is bound to PPP1R2 and is therefore inactive [51]. When phosphorylated at threonine 73, human PPP1R2 is unable to bind to PPP1, rendering it active [52]. Glycogen synthase kinase 3 (GSK3) is the kinase responsible for PPP1R2 phosphorylation. Interestingly, GSK3 activity has been extensible correlated with sperm motility regulation both in cauda and caput (bovine, mouse, and macaque models) [53–55]. GSK3 is six times more active in caput than in caudal sperm and its activity is correlated negatively with sperm motility [53,55]. Moreover, GSK3 appears to have an isoform-specific function on sperm motility. When GSK3alpha is knockout, there is a decrease in sperm motility and metabolism, while GSK3beta conditional knockout is fertile [56].

Recently, Koch et al. showed that the Wnt signaling can be partially responsible for GSK3 activity regulation in epididymal sperm. In corpus and caudal epididymis, Wnt signaling proteins are released in epididymosomes from epididymal principal cells. In the epididymis, lumen Wnt proteins bind to low-density lipoprotein receptor-related protein 6 receptor (LRP6), activating it. In turn, LRP6 induces GSK3 inhibition, which leads to decreased PPP1R2 phosphorylation [57]. Synergistically, GSK3 activity can be modulated by phosphoprotein phosphatase 2A (PPP2 also known as PP2A). Dudiki et al. demonstrated that in caput sperm demethylated and phosphorylated PPP2 isoform alpha (PPP2CA) is active. Consequently, it dephosphorylates GSK3 in serine residues rendering it active. Subsequently, PPP1R2 threonine 73 is phosphorylated and the inhibitor becomes inactive, resulting in active PPP1 and immotile sperm. In caput sperm, methylation of PPP2CA increases due to a decrease in protein phosphatase methylesterase 1 (PPME1) activity. In these conditions, PPP2CA becomes inactive resulting in increased GSK3 serine phosphorylation and thus its inactivation. Subsequently, PPP1R2 is active and inhibits PPP1, leading to motile sperm [58] (Figure 2).

Moreover, in 2013, Korrodi et al. identified a new PPP1R2 isoform in human sperm, PPP1R2P3 (also known as inhibitor 2-like) [51]. This isoform has the unique feature of threonine 73 being replaced by proline avoiding GSK3 phosphorylation. Korrodi et al. hypothesized that PPP1R2P3 is only present in caudal motile sperm, representing a constitutively inhibitor of PPP1, and therefore responsible for the process of sperm motility acquisition along the epididymis journey [51] (Figure 2).

Besides PPP1R2, PPP1 regulatory subunit 7 (PPP1R7) and PPP1 regulatory subunit 11 (PPP1R11), other two PPP1 inhibitors are present in sperm, suggesting a synergetic mechanism for PPP1 activity control [59]. PPP1R11 (also known as I3) is a human homolog of the mouse Tctex5, a protein associated with male infertility due to impaired sperm motility. On mouse sperm, PPP1R11 is localized in the head and principal piece of the flagellum, the same subcellular localization of PPP1 [60]. In rat liver cells, PPP1R7 (also known as sds22) inhibits PPP1, and in rat testis, it associates with PPP1CC2. In

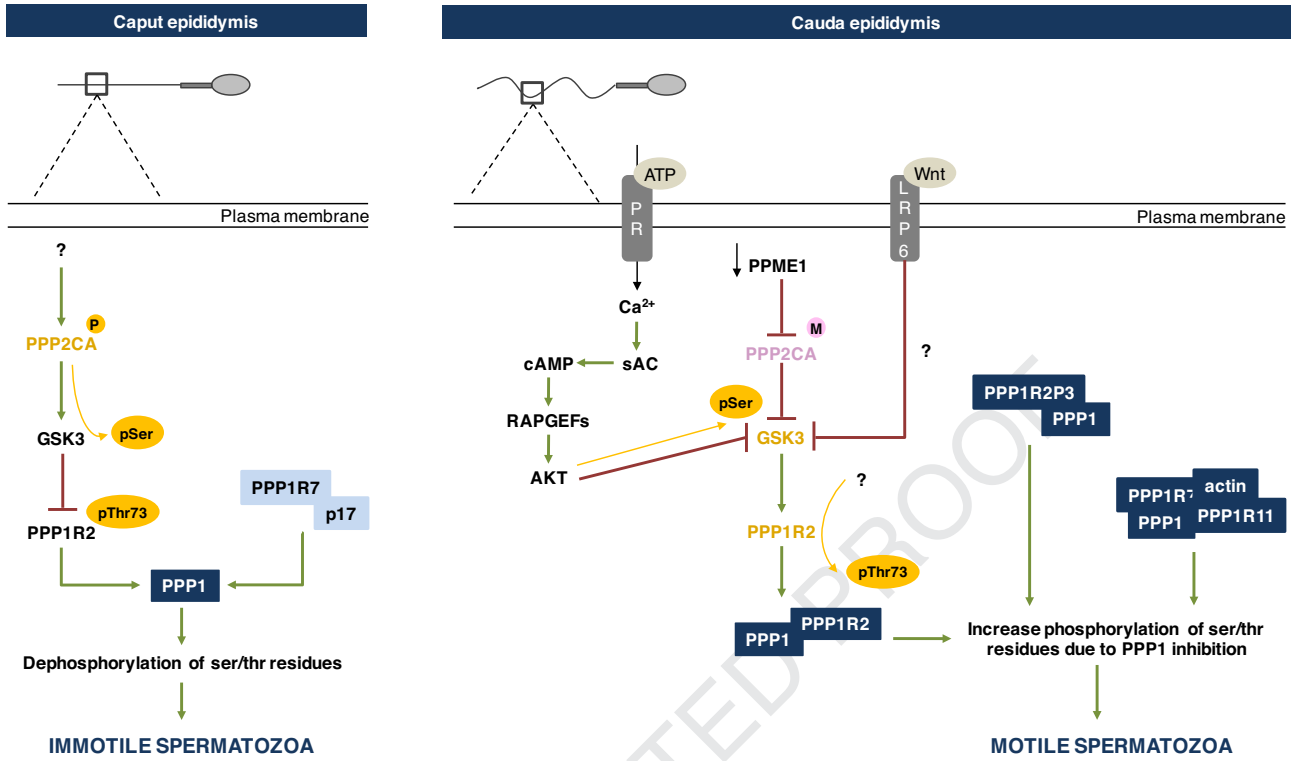

Figure 2. Schematic representation of the signaling events required for sperm motility acquisition in the epididymis. In caput epididymis, PPP2CA is phosphorylated and consequently active, which in turn dephosphorylates GSK3 at serine residues, rendering it active. GSK3 phosphorylates PPP1R2 at thr73 which inhibits the interaction between PPP1R2 and PPP1 resulting in active PPP1. PPP1R7 is bound to p17, which leads to free and active PPP1. Active PPP1 results in dephosphorylation of key residues and consequently immotile sperm. In cauda epididymis, PPME1 activity decreases increasing PPP2CA methylation, resulting in inhibition of PPP2CA. Consequently, GSK3 serine phosphorylation increases leading to GSK3 inhibition. Also, Wnt binds to LRP6 receptor which promotes GSK3 inhibition by an unknown mechanism. Moreover, ATP binds to purinergic receptors (PR), resulting in calcium influx. Calcium activates sAC, which produces cAMP activating Rap guanine nucleotide exchange factor (RAPGEFs). The latter activates AKT that phosphorylates GSK3 at serine residues inactivating it [65]. GSK3 is inhibited, which leads to decrease Thr73 PPP1R2 phosphorylation (the phosphatase responsible is unknown). Consequently, PPP1R2 binds PPP1. Also, PPP1 is bound to PPPP1R2P3 and in a complex with PPP1R7, actin, and PPP1R11. Thus, PPP1 activity is inhibited and ser/thr phosphorylation of key residues increases leading to motile sperm. P, phosphorylation; M, methylation; green arrows, activation; red arrows, inhibition; yellow arrows, phosphorylation; yellow, phosphorylated proteins; pink, methylated proteins.

caput, bovine sperm PPP1R7 and PPP1CC2 do not interact. Instead, PPP1R7 is associated with a 17 kDa protein (p17) [61], resulting in active PPP1. Conversely, in mouse caudal sperm, PPP1R7, PPP1R11, actin, and PPP1 form a complex that is catalytically inactive [59] (Figure 2). Although PPP1 plays a crucial role in keeping motility at check in caput sperm, its substrates are still unknown. Besides PPP1, a sperm-specific isoform of calcineurin (PPP3CC) appears to be involved in epididymal maturation. Upon ablation of PPP3 and regulatory subunit PPP3R2, male mice are infertile due to impaired hyperactivation and penetration of zona pellucida. Phenotypically, sperm without PPP3CC presents an inflexible midpiece. When sperm is hyperactivated, the bending capacity of the midpiece increases; however, PPP3CC null sperm are incapable of exhibiting this increase. Interestingly, inhibition of PPP3CC with specific inhibitors results in a quick phenotype (5 days) alteration from normal to inflexible midpieces. After 1 week of halting drug administration, the sperm are completely recovered and fertility is restored [62].

Since for sperm motility dephosphorylation must be shut down, it is not surprising that phosphorylation must increase. It is well known that the soluble adenylyl cyclase/cyclic adenosine monophosphate/cAMP-dependent protein kinase (sAC/cAMP/PRKA; cAC/cAMP/PKA) signaling pathway affects sperm motility positively. Although the sAC/cAMP/PRKA signaling is mostly associated with hyperactivated motility [9], its involvement in sperm motility acquisition is unquestionable (see below) [63,64]. In 2013, Vadnais et al. proposed a crosstalk between the GSK3/PPP1R2/PPP1 and sAC/cAMP/PRKA pathways during motility acquisition in the epididymis [65] (Figure 2). Figure 2 shows the main signaling pathways involved in motility acquisition in the epididymis.

Sperm motility in the female reproductive system

During unprotected intercourse, millions of sperm are deposited in the female reproductive tract, more specifically in the vagina. From there on, sperm must swim until they reach the oocyte in the fallopian tube. Although sperm is already motile when ejaculated, hyperactivated motility must be acquired to overcome all the filters and traps imposed by the female reproductive tract. Interestingly, it is the unique female environment that triggers the signaling pathways essential for sperm hyperactivated motility [66]. In the past years, many efforts have been made to unravel the role of key messengers and signaling pathways involved in hyperactivated motility.

First messengers—calcium, bicarbonate, and progesterone

In sperm, calcium (Ca^{2+}) plays a central role in events preceding fertilization, specifically, motility, chemotaxis, and acrosome reaction. The relevance of Ca^{2+} on eukaryotic cell physiology is reflected in several Ca^{2+}-dependent enzymes, intracellular Ca^{2+} stores, and Ca^{2+} channels [67]. Human sperm is no exception. The most described role of Ca^{2+} in human sperm motility is the activation of the sAC. Moreover, the inhibition of Ca^{2+} signaling is associated with male subfertility [68]

In human sperm, mean basal Ca^{2+} is kept around 100—200 nM, while in the extracellular medium it varies between 1 and 2 mM [69]. This gradient concentration is accomplished by a Ca^{2+}-ATPase pump, which promotes Ca^{2+} efflux with ATP consumption [70,71]. A low resting Ca^{2+} concentration is what keeps human sperm in a basal motility state in the caudal portion of the epididymis and vas deferens. However, in the female reproductive tract, Ca^{2+} concentration increases to induce hyperactivated motility. The female reproductive system controls the increase in Ca^{2+} concentration in the sperm through clues in specific places and menstrual cycle phase [72].

The influx of Ca^{2+} into human sperm is promoted by several mechanisms: increase in membrane permeability [73], depolarization [74], inhibition of the Ca^{2+}-ATPase pump, and activation of voltage-dependent calcium channel (VOCCs). Yet, the main known mechanism for Ca^{2+} influx into sperm is the CatSper (cation channel of sperm), identified in 2001 by Ren et al. [75]. This channel, located at the principal piece of the flagellum, is the only constitutively active Ca^{2+} conductance present in human sperm, responds weakly to voltage alterations, and is pH sensitive [75,76]. Moreover, null mice for *CatSper1* are infertile [75]. Human CatSper activation is triggered mainly by extracellular progesterone (see below), prostaglandins [77], and an alkaline environment (created by increasing HCO_3^- concentrations) [78]. Curiously, mouse CatSper is activated by neither progesterone nor prostaglandins. This suggests a species-specific Ca^{2+} influx process, possibly to avoid cross-species fertilization [77]. Although it is not located to the sperm's head, CatSper also appears to be involved in the acrosome reaction by increasing Ca^{2+} concentration [79]. Furthermore, Brenker et al. concluded that a range of small odorant molecules present in the female reproductive tract activate CatSper, resulting in chemotaxis of the sperm toward the oocyte [80].

Although the process of Ca^{2+} influx is essential for sperm motility, it is established that the human sperm has Ca^{2+} stores. The most promising candidates for Ca^{2+} stores in human sperm are the acrosome, the nuclear membrane, and the cytoplasmic droplet [81]. Interestingly, it appears that in the sperm flagellum there are no Ca^{2+} stores, suggesting that the stores are important on processes such as acrosome reaction, rather than in motility. Moreover, the presence of sarcoplasmic and/or endoplasmic reticulum calcium ATPases in human sperm, channels that transport Ca^{2+} from the intracellular medium to Ca^{2+} stores in somatic cells, further reinforces the presence and functional importance of Ca^{2+} stores [81,82].

Progesterone is probably the most potent activator of capacitation of human sperm [83]. It is produced by the *cumulus oophorus* cells that surround the oocyte. At nanomolar concentration range, progesterone induces Ca^{2+} influx and promotes extensive phosphorylation through the activation of several kinases, such as PRKA [84], protein kinase C (PRKC), mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 4,5-bisphosphate 3-kinase (PIK3C, PI3K) [85,86]. Phenotypically, progesterone increases the number of motile sperm, induces hyperactivated motility and acrosome reaction, and appears to be involved in sperm chemotaxis toward the oocyte [86–91].

In somatic cells, progesterone acts through classic nuclear progesterone receptor and regulates gene expression. Conversely, sperm is transcriptionally silent and the effect of progesterone on sperm physiology is far too quick to be explained by gene expression [92]. In 2011, Strünker et al. and Lishko and et al. concluded that progesterone activates the CatSper channel [77,78]. As the sperm leaves the epididymis and mixes with the prostatic seminal vesicle fluid, the bicarbonate (HCO_3^-) content increases [93]. Reaching the female reproductive system, sperm encounters an acidic environment, which should reduce motility. Yet, the basic pH of the seminal plasma neutralizes the acidic pH and allows sperm motility [94] and the semen is deposited closely to the uterus cervix so that sperm can quickly move out of the vagina [7,95]. Within the uterus, the rich HCO_3^- alkaline environment is essential for sperm hyperactivated motility [96]. Curiously, throughout the menstrual cycle, HCO_3^- concentrations vary from 35 nM at the follicular phase to at least 90 nM at ovulation, potentiating fecundation [95]. Sperm-specific Na^+/ HCO_3^- cotransporters mediate the influx of HCO_3^- and, as a result, there is an increase on sperm pH and hyperpolarization [97]. Though, to achieve complete hyperpolarization, there must be a Na^+ and K^+ influx, Na^+ is transported by the Na^+/ HCO_3^- cotransporters and K^+ influx is mediated by calcium-activated potassium channels and the ion transporter Na,K-ATPase. Calcium-activated potassium channels are regulated by intracellular alkalinization and cAMP, which hints a HCO_3^- indirect regulation of K^+ [94]. Within the sperm, HCO_3^- activates factors that exchange phospholipids within the bilayer plasma membrane. Consequently, cholesterol is vulnerable to albumin, which is the most abundant protein on the female reproductive system, and the main cholesterol acceptor. Albumin can decrease up to 40% of the sperm cholesterol content and this leads to an increase on membrane fluidity [98,99]. Hyperpolarization, intracellular alkalinization, and increased membrane fluidity promote influx of Ca^{2+}.

The Na,K-ATPase pump is a membrane protein found in all eukaryotes [100]. By using the energy released from ATP hydrolysis, the Na, K-ATPase pump promotes the efflux of three molecules of Na^+ and influx of two molecules of K^+ [101]. Two subunits compose the Na,K-ATPase protein: the alpha and beta subunits. In several species, including human, the alpha4 subunit presents the most restricted expression. It is present in sperm principal piece only in mature sperm of males in sexual maturity. Besides the Na,K-ATPase alpha4 subunit, only subunit alpha1 is present in sperm [100]. Knockout studies revealed that the Na,K-ATPase alpha4 subunit is crucial for sperm physiology, since alpha4 subunit KO is completely sterile (knockout sperm presents reduced primary and hyperactivated motility, bent flagellum, increased intracellular Na^+, and cell plasma membrane depolarization [102]). Mcdermott et al. reinforced the role of Na,K-ATPase alpha4 subunit on human male fertility by showing that an overexpression of this protein in mouse testis results in an increased total motility (among other parameters of sperm movement) [103]. Although the exact mechanism underlying the role of Na,K-ATPase in sperm physiology is not fully characterized, Na,K-ATPase alpha4 isoform appears to regulate intracellular H^+. Since it is unlikely that the Na,K-ATPase transports H^+, its ability to regulate intracellular H^+ arises from its effect on the activity of a Na^+/H^+ exchanger (NHE). NHE uses the Na^+ gradient established by the Na,K-ATPase to extrude H^+ in exchange for the influx of Na^+ [104] and, consequently, there is an increase in the intracellular pH [104] (Figure 3). In

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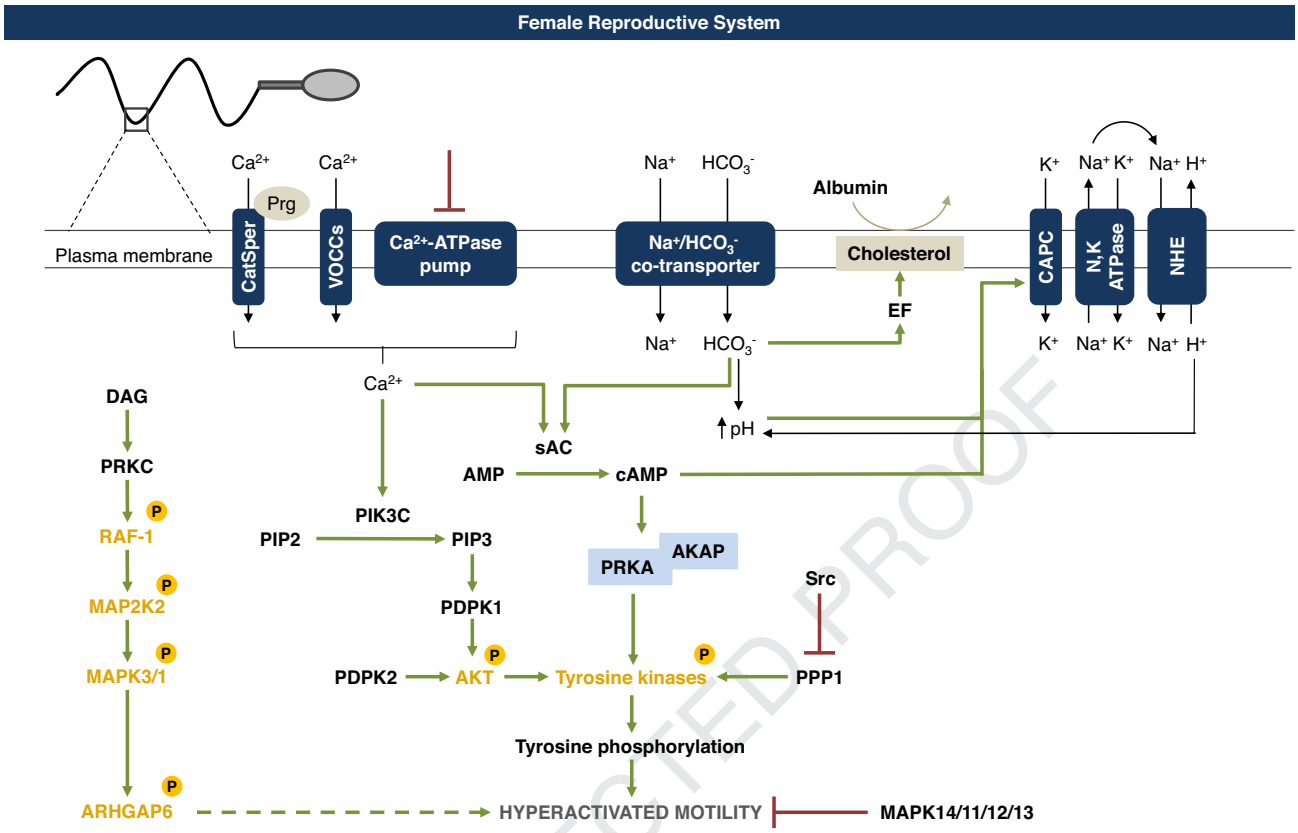

Figure 3. Schematic representation of the signaling events required for sperm hyperactivated motility in the female reproductive system. Several mechanisms are responsible for intracellular Ca^{2+} increase in sperm. Progesterone binding to CatSper activates VOCCs and inhibition of Ca^{2+}-ATPase pump promotes Ca^{2+} influx. In sperm, HCO_3^- and Na^+ increase due to activation of Na^+/HCO_3^- cotransporter. Potassium enters through a calcium-activated potassium channels (CAPC) and Na,K-ATPase. The Na+ gradient created by the Na,K-ATPase activates the Na^+/H^+ exchanger that promotes the influx of Na^+ and de efflux of H^+. HCO_3^- activates exchange factors (EF) that promote cholesterol externalization, becoming vulnerable to albumin. HCO_3^- and Ca^{2+} activate sAC, which converts AMP to cAMP and activates PRKA. PRKA activates tyrosine kinases, by phosphorylation. Also, Ca^{2+} activates PIK3C, which forms PIP3, that in turn activates PDPK1 and PDPK1 and PDPK2 activate AKT by phosphorylation. AKT activates tyrosine kinases by phosphorylation. At the same time, DAG activates PRKC which phosphorylates RAF-1, RAF-1 activates MAP2K2, and MAP2K2 activates MAPK3/1. Again, MAPK3/1 activates tyrosine kinases by phosphorylation. MAPK3/1 phosphorylates ARHGAP6 which may be involved in hyperactivated motility. MAPK14/11/12/13 inhibits hyperactivated motility. Src inhibits PPP1, which allows an increase of tyrosine kinases phosphorylation. Tyrosines kinases phosphorylate key proteins inducing hyperactivated motility. P, phosphorylation; Prg, progesterone; green arrows, activation; red arrows, inhibition; dashed arrow, predicted function; yellow, phosphorylated proteins.

bovine sperm, Jimenez et al. demonstrated that Na,K-ATPase activity is upregulated during capacitation. Also, when Na,K-ATPase activity is impaired, the intracellular decrease in Na^+ and plasma membrane hyperpolarization that typically accompany sperm capacitation are inhibited [105]. Ouabain, a cardiac glycoside produced in adrenal glands, is a Na,K-ATPase inhibitor which may have a physiological role in fertilization. [106–108].

Signaling pathways in hyperactivated motility

It appears that all the processes that occur in the female reproductive system increase the Ca^{2+} and HCO_3^- concentrations in the sperm. This raises the question: Within the sperm, what signaling pathways Ca^{2+} and HCO_3^- modulate to promote hyperactivated motility?

The most well-known pathway that controls hyperactivity and is highly dependent on Ca^{2+} and HCO_3^- concentrations is the sAC/cAMP/PRKA pathway. The sAC is specific to sperm, does not interact with guanosine-5′-triphosphate, and, binds to HCO_3^- and Ca^{2+} for its activation [97]. Upon activation, sAC converts adenosine monophosphate into 3′5′-cAMP. The increase of cAMP activates PRKA, a serine/threonine kinase that is dependent on cAMP [5,109]. The fact that when sAC and Calpha2 sperm-specific PRKA subunit are knockout, sperm does not acquire motility reinforcing the necessity of such signaling pathway in sperm motility [110,111]. PRKA appears to target and activate tyrosine kinases since inhibition of PRKA is correlated with a decrease in tyrosine phosphorylation [112].

PRKA activity control and its subcellular localization are crucial for compartmentalization of its effect. In sperm, PRKA is typically tethered to A-kinase anchor proteins (AKAPs), which in turn targets PRKA to specific subcellular sites and provides a mechanism for defining its substrates [84]. Besides anchoring PRKA, it has been showed that AKAPs can also scaffold phosphatases and other kinases from macromolecular complexes essential for signaling cascades within the sperm [84]. In flagellum sperm, AKAPs have a prominent role, and AKAP4 is the main component of the fibrous sheath. Moreover, when AKAP4 is knockout, sperm numbers are normal but sperm is incapable of progressive motility and proteins usually associated with the fibrous sheath, such as PRKA, are absent or significantly reduced [70].

Besides increasing PRKA activity, Battistone et al. proved that serine/threonine phosphatases must be inactivated to allow the increase in serine/threonine phosphorylation. Members of the Src

family kinase (SFK) inactivate members of the serine/threonine phosphatases family possibly by tyrosine phosphorylation. In human sperm, PPP1CC2 is the most promising candidate since it exhibits predicted tyrosine phosphorylation sites and only high okadaic acid concentrations overcome the SKI606 effect (an SFK inhibitor) [113]. Nevertheless, the involvement of other serine/threonine phosphatases, such PPP2CA or PPP4C, cannot be ruled out [113].

Although sAC/cAMP/PRKA plays a central role in hyperactivated motility, it is not the only signaling pathway involved in this process. The involvement of the PIK3C-AKT pathway in sperm hyperactivated motility was described by Sagare-Patil et al. Progesterone promotes the influx of Ca^{2+} through the CatSper channel. Within the sperm, Ca^{2+} activates PIK3C (unknown mechanism) converting PIP2 into PIP3. The latter binds and activates 3-phosphoinositide-dependent protein kinase 1 (PDPK1), which phosphorylates RAC-alpha serine/threonine-protein kinase (AKT or PKB) in threonine 308. Consequently, AKT serine 473 becomes exposed and vulnerable to phosphorylation by PDPK2. Both phosphorylations render an active AKT, which phosphorylates serine residues on key proteins in sperm motility [86].

The MAPK signaling is also involved in human sperm hyperactivated motility, although its role is controversial. In 2005, a study in human sperm stated that tryptase, a product of mast cells in the female reproductive system, activates MAPK3/1 (also known as ERK1/2), which in turn inhibits motility [114]. However, in 2008, Almog et al. demonstrated the existence of the MAPK cascade elements, more specifically, MAPK3/1, SOS, RAF-1, MAP2K1, and MAPK14/11/12/13 (also known as p38 proteins) in the tail of mature ejaculated human sperm and revealed a positive correlation between MAPK3/1 and motility. Upon activation by diacylglycerol (DAG), protein kinase C (PRKC, PKC) becomes active and phosphorylates RAF-1, which in turn phosphorylates and activates MAP2K1. MAP2K1 activates MAPK3/1 by phosphorylation. One of the identified substrates of MAPK3/1 was Rho GTPase-activating protein 6 (ARHGAP6). This protein may control the active slide of microtubules in sperm flagellum since it has already been described as being involved in cell motility and actin remodeling [115]. On the other hand, active MAPK14/11/12/13 inhibits sperm motility [116]. Again in 2015, Silva et al. also showed a negative correlation between MAPK14/11/12/13 activation and human sperm motility [117]. Figure 3 shows the signaling pathways involved in sperm hyperactivated motility in the female reproductive system.

Correlation between sperm motility and tyrosine phosphorylation

In 1989, Leyton and Saling described for the first time the presence of tyrosine phosphorylation in mammalian sperm (mouse) [118]. Twenty-six years later, the importance of tyrosine phosphorylation in capacitation and motility is unquestionable. The increase of proteins tyrosine phosphorylated in the human sperm is a hallmark of capacitation and has been positively associated with acquired and hyperactivated motility [112,119,120]. Most of the signaling pathways involved in human motility, including the ones described previously, culminate in activation of tyrosine kinases. The identity of most tyrosine kinases is unknown, still the tyrosine kinases Scr [121], FGFR1 [122], and ABL1 [123] are already associated with tyrosine phosphorylation in mammalian sperm.

Several studies proved that dozens of proteins undergo tyrosine phosphorylation during capacitation in the sperm, mainly proteins localized in the flagellum [55,124–127]. In fact, human AKAP4 (see above) was one of the first proteins to be identified as a substrate for tyrosine phosphorylation [128]. Fibrous sheath protein of 95 kDa [129], CABYR [130], and HSP90 [131] are other targets of tyrosine kinases [97]. Also, it has been hypothesized that dyneins are tyrosine phosphorylated and that this posttranslation modification controls the sliding of microtubules and therefore motility. The challenge for the next years is to identify new tyrosine phosphorylation targets and their relationship with sperm motility [112].

Concluding remarks

This review attempts to summarize the current knowledge on the signaling pathways involved in sperm motility regulation. Since the first observation of the sperm by Anton van Leeuwenhoek in 1677, the knowledge concerning the sperm cell grew exponentially. The sperm structure, energy metabolism, epididymal maturation, and capacitation are indispensable for fertilization. Nevertheless, the road to unraveling the molecular players involved in the regulation of this processes is still long. Specifically, the molecular basis of sperm motility is not fully understood. Nowadays, we believe that the major setback to fully comprehend the molecular basis of human sperm motility is a technical one. Animal models and *in vitro* experiments are the only options to study epididymal maturation and capacitation. Understanding the mechanism responsible for human sperm motility is of great value. Sperm motility is the perfect target for male contraception since it does not disturb spermatogenesis and hormone production. Also, decreased sperm motility is increasing in developed countries, resulting in an escalation in male infertility rates. Understanding the signaling pathways behind sperm motility can help pinpoint the cause of male infertility and contribute to the development of new therapies.

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