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Sperm Capacitation associated increase in Tyrosine Phosphorylation: kinetic of the increase and novel members involved in the process.

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SPERM CAPACITATION ASSOCIATED INCREASE IN TYROSINE PHOSPHORYLATION: KINETIC OF THE INCREASE AND NOVEL CANDIDATES INVOLVED IN THE PROCESS

A Dissertation presented

By

ANTONIO ALVAU

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DEDICATION

To my parents Clara and Giovanni, because this is what happened when you use the Odyssey as bedtime story, thank you.

To Franco, because he is not just an uncle, he's a lifetime friend.

EPIGRAPH

‘‘A Negroni, Signor Barone?” asked Mr. Cipriani.
But the good lion had flown all the way from
Africa and Africa had changed him.
“Do you have any Hindu trader sandwiches?”
he asked Cipriani. “No, but I can get some.”
“While you are sending for them make me a
very dry martini.” He added, “With Gordon’s gin.”
“Very good,” said Cipriani. “Very good indeed.”
Now the lion looked about him at the faces
of all the nice people and he knew that he was at
home but that he had also traveled. He was very happy.

Ernest Hemingway, *The good Lion.*

“Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza

Call to mind from whence ye sprang:
Ye were not form’d to live the life of brutes,
But virtue to pursue and knowledge high.”

Dante Alighieri*, The Comedy, Hell*
Canto XXVI 118-120.

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Pablo, you realized that with me, a good old argument works better than thousand words. Thank you but whom I'm going to discuss with now?

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David unfortunately we worked only few months together, very intense though. Thanks for your support, hope we can work together again in the future.

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My long life friend Gian Battista, always on my side, although thousands miles apart.

Ilaria and Lorenzo for this great last year of trips, movies, spritz, parties and fun, I wouldn’t have made it without you.

ABSTRACT

SPERM CAPACITATION ASSOCIATED INCREASE IN TYROSINE PHOSPHORYLATION: KINETIC OF THE INCREASE AND NOVEL CANDIDATES INVOLVED IN THE PROCESS.

SEPTEMBER 2016

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Sperm capacitation is a post-ejaculatory maturational event required for successful fertilization. Specific molecular events are induced during capacitation: a cAMP-dependent activation of protein kinase A (PKA) leading downstream to the increase of tyrosine phosphorylation. Activation of PKA and tyrosine phosphorylation was shown to occur on different time scale. Here we showed that phosphorylation events during capacitation are tightly regulated over time, with fast activation of PKA and a late and continuous increase in the level of tyrosine phosphorylation to guarantee a pool of capacitated sperm at the right time/site of fertilization.

Despite several studies on tyrosine phosphorylation, the identity of the tyrosine kinase(s) that mediate these increases has not been conclusively demonstrated. Recently a role for *focal adhesion kinases* (PYK2 and FAK) was proposed in stallion, based on the use of PF431396, a small molecule inhibitor directed against this kinase family, but critical loss of function experiment have not been reported. We used both pharmacological tools and genetically modified mice models to investigate the identity of the tyrosine

kinase(s) mediating the increase of tyrosine phosphorylation in human and mouse sperm. PF431396 blocks the capacitation-associated increase in tyrosine phosphorylation in both human and murine. On the other hand, *Pyk2*$^{-/-}$ mice showed a physiological capacitation-associated tyrosine phosphorylation and the specific inhibition of FAK by PF573228 showed no decrease in the levels of tyrosine phosphorylation, indicating that *focal adhesion kinases* are not responsible for this phosphorylation process. Here we show that PF431396 can also inhibit the tyrosine kinase FER and that sperm from mice targeted with a kinase inactivating mutation in *FER* failed to undergo capacitation-associated increases in tyrosine phosphorylation. While these mice are fertile, their sperm displayed normal levels hyperactivation but have reduced ability to fertilize metaphase-II arrested eggs *in vitro*.

Keywords: Sperm Capacitation, Tyrosine phosphorylation, Tyrosine Kinases, FER.

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CHAPTER 1

INTRODUCTION: SIGNALING PATHWAYS REGULATING SPERM CAPACITATION

1.1 Spermatogenesis

Spermatozoa are highly specialized and motile germ cells, and represent the final product of male gametogenesis, known as spermatogenesis, which occurs in the testis. Testis has both endocrine and reproductive functions, it responds to stimuli from anterior pituitary producing androgens, finally stimulating male gametogenesis (Weinbauer et al., 2001). Spermatogenesis occurs in the functional unit of the testis, the *seminiferous tubule*s. Seminiferous tubules are composed by a germinal epithelium, mostly formed by the *Sertoli cells* which function not only as a barrier to protect haploid sperm from the attack of the immune system but also to provide essential paracrine factors (cytokines, proteins, growth factors and steroids) selectively up-taken by germ cells to produce mature spermatozoa (Weinbauer et al., 2001). When sperm reach their structural maturation, they are released from the germinal epithelium into the *rete testis*, where spermatozoa are concentrated before moving toward the epididymis. Although morphologically mature, these spermatozoa have not reached full fertilizing competence and need to transit through *epididymis* and *vas deferens* in order to acquire motility and later fertilizing ability.

1.2 Epididymal Maturation

Mammalian spermatozoa produced in the testis leave the rete testis and slowly transit through the *efferens ducts*, ultimately reaching the epididymis. The epididymis is a long, convoluted tube that covers a fundamental role in post gonadal maturation of male gametes and is divided in three portions: *caput* (proximal), *corpus* (medial) and *cauda* (distal) (Hinton and Palladino, 1995; Jones and Murdoch, 1996; Robaire and Viger, 1995; Yeung et al., 1993). Spermatozoa slowly transit across the epididymis in order to acquire motility, ability to respond to the female environment, and fertilizing ability. The prominent feature of epididymal lumen is a highly specialized epithelium (Da Silva et al., 2007). Primary function of epithelial cells is to secrete a luminal fluid to create the optimal environment for sperm maturation during epididymal maturation. Low levels of bicarbonate ions (HCO_3^-) and pH typical of the luminal fluid (Kirichok et al., 2006; Levine and Kelly, 1978; Levine and Marsh, 1971) ensure the maintenance of spermatozoa in a quiescent state (Acott and Carr, 1984; Carr et al., 1985; Kirichok et al., 2006). Furthermore, interactions between epididymal factors and gametes are further facilitated due to high levels of water reabsorbtion. At the end of their transit through the epididymis, immotile spermatozoa finally reach the *cauda*, where they are stored in a quiescent state before ejaculation.

1.3 Sperm Capacitation

1.3.1 Definition and General Description

Ejaculated spermatozoa are motile but, before becoming fully competent for fertilization, they must reside in the female reproductive tract for a certain amount of time: the combination of physiological changes that occur to spermatozoa during the transit across the female reproductive tract and that prepare spermatozoa to interact with the egg is defined as Capacitation.

Dr. Austin and Dr. Chang independently introduced the term *capacitation* in the early fifties (Austin, 1951; Chang, 1951). Since then, several studies have focused their interest on understanding features and regulatory mechanisms of sperm capacitation.

Physiologically, capacitation occurs after ejaculation, during the transit toward the oocyte, and is characterized by a series of maturational events that render spermatozoa able to appropriately swim through the *fallopian tubes*, to bind to the oocyte's extracellular matrix, to penetrate egg vestments and to fuse with the egg (Aitken and Nixon, 2013). Both functional and molecular modifications are typical of sperm capacitation and both are necessary to render spermatozoa fully capacitated (Yanagimachi, 1994a).

Two major functional changes characterize capacitated spermatozoa: the acquisition of hyperactive motility (Suarez, 1996, 2008) and the ability to undergo the *acrosomal reaction* (Breitbart, 2003; Florman et al., 2008). When spermatozoa transit through the female tract, it responds to the various environmental signals by modifying their motility pattern: ejaculated spermatozoa show progressive motility, which is characterized by a high

flagellar beat frequency and low bend angle, which helps mature sperm to swim out from the seminal plasma and to rapidly move through the vaginal and uterine environments, toward the oviducts. By the time sperm reach the utero-tubal junction (UTJ), they must acquire hyperactive motility; reduced flagellar beat frequency, increased bending angle and higher propulsive force distinguish hyperactive motility (Suarez and Ho, 2003). Hyperactive sperm are therefore slower but able to pass the UTJ and swim through the high viscosity medium typical of the fallopian tubes, detaching from the tubes epithelium to finally reach the oocyte.

Another functional change characterizing capacitated sperm and that is necessary for fertilization is the ability to undergo to the *acrosomal reaction*. The acrosome is a large secretory vesicle located on the tip of the sperm head and its lumen is filled with both soluble and particulate components called acrosomal matrix (Buffone et al., 2014a). Mostly proteases, glycosidases and several binding proteins form the acrosomal matrix (Buffone et al., 2008) and when capacitated spermatozoa reach the oviducts, release their acrosomal content in response to signals produced by the *zona pellucida*, in a process called Acrosomal Reaction (AR). This exocytotic event allows spermatozoa to digest and penetrate egg vestments and, at the same time to expose molecules present on the sperm membrane that mediate sperm-egg binding and fusion. In order to be fully competent for fertilization, sperm must develop a functional acrosome and to be able to release its contents with the appropriate timing (Buffone et al., 2008; Florman et al., 2008).

Besides the functional changes described above, molecular and biochemical events must also take place during capacitation. These changes must not be considered as single events but as a series of somehow connected and sequential phenomena that ultimately render spermatozoa fully competent for fertilization (Salicioni et al., 2007). Biochemical events include: loss of cholesterol from sperm plasma membrane, fluxes of Ca^{2+}, K^{+}, HCO_3^{-} and other extracellular ions, changes in the cAMP levels, increased sperm plasma membrane potential and increased levels of Tyrosine Phosphorylation (PY). Modifications associated with capacitation occur both in the sperm flagellum and in the sperm head rendering spermatozoa competent for fertilization (Ickowicz et al., 2012; Salicioni et al., 2007; Visconti et al., 2011).

Although investigating mechanism of sperm capacitation *in vivo* is challenging, sperm capacitation can be mimicked *in vitro* by exposing epididymal or ejaculated spermatozoa to a standard culture media with a limited number of ions (Na^{+}, K^{+}, Cl^{-}, HCO_3^{-}, Mg^{2+}, Ca^{2+} and PO_4^{3-}), energy metabolites (pyruvate, lactate and glucose) and cholesterol acceptors (usually Serum Albumin) (Bavister, 1973; Edwards et al., 1969; Lee and Storey, 1986).

Historically, the ability to capacitate spermatozoa *in vitro* has represented a milestone not only to pinpoint the relevance of female factors during fertilization (Yanagimachi, 1994a) but to finally develop reproducible methods to fertilize metaphase-II arrested oocytes *in vitro* in humans (Steptoe and Edwards, 1978) and other mammalian species (Chang, 1959).

Furthermore, eliciting sperm capacitation *in vitro*, easily has allowed researchers to study behavior and mechanisms of regulation of sperm during capacitation.

1.3.2 Importance of the cAMP/PKA cell signaling pathway

Due to the relatively easy conditions of stimulation *in vitro*, different molecular mechanisms associated with sperm capacitation have been characterized. Studies over the past 20 years have clearly shown that capacitation is associated with the activation of the cAMP/PKA pathway in several species (Esposito et al., 2004; Vijayaraghavan et al., 1997; Xie et al., 2006).
In order to induce capacitation *in vitro*, the presence of a cholesterol acceptor (usually Bovine Serum Albumin [BSA]), Ca^{2+} and HCO_3^- are required. BSA induces loss of cholesterol (Visconti, 2009) and an increased membrane fluidity, producing elevation of the intracellular pH (pH_i), activation of the Na^+/HCO_3^- co-transporter (Demarco et al., 2003) and a rapid collapse of the asymmetry of the sperm plasma membrane (Gadella and Harrison, 2000; Signorelli et al., 2012). Increased levels of intracellular HCO_3^- and Ca^{2+}` promotes the activation of the unique soluble Adenyl Cyclase expressed in sperm, ADCY10 (sAC) (Buck et al., 1999). The presence of ADCY10 has been shown to be necessary for fertility. In fact, sAC null mice are infertile and show defects associated with capacitation and with the acquisition of inducing a hyperactive motility pattern (Esposito et al., 2004; Xie et al., 2006). In turn, sAC activation induces an elevation of the intracellular levels of cAMP. The role of cAMP in the pathway conducive to sperm capacitation has been elucidated over the years by both biochemical and pharmacological approaches: increased cAMP levels promote the release of the CαII catalytic subunit of the Protein Kinase A (PKA) from the regulatory subunits stimulating the activity of the kinase (Nolan et al., 2004). Therefore, also PKA was shown to be a necessary element for sperm capacitation *in vivo*: animals lacking the

PKA catalytic subunit Cαll are indeed not fertile, despites a normal mating behavior (Nolan et al., 2004).

The increase of cAMP levels and activation of PKA are considered early events of sperm capacitation: cAMP levels increases within 60 seconds with a full activation of the kinase in less than 90 seconds (Salicioni et al., 2007; Visconti et al., 1995a; Visconti et al., 1995b). Upon activation, PKA induces the phosphorylation of target proteins on Serine and Threonine residues, modulating their activity, thus initiating several signaling pathways downstream. The early activation of cAMP/PKA is also a necessary step to stimulate the phosphorylation of a number of protein substrates on tyrosine residues, a late event also considered a hallmark of sperm capacitation in several species (Baldi et al., 2002; Ficarro et al., 2003; Harrison, 2004; Jagan Mohanarao and Atreja, 2011; Roy and Atreja, 2008; Visconti et al., 1995a). Aforementioned studies using sAC and PKA null animals revealed the necessity of this pathway for sperm capacitation: spermatozoa with a defective cAMP/PKA pathway are infertile and their ability to undergo capacitation is lost (Esposito et al., 2004; Hess et al., 2005; Nolan et al., 2004). Therefore the early activation of cAMP/PKA is necessary not only to induce early events as the acquisition of progressive motility, membrane depolarization, activation of cAMP/PKA pathway and intracellular alkalinization but to also coordinate, in some yet unknown way, later events such as increased tyrosine phosphorylation levels, acquisition of hyperactive motility (Suarez, 2008) and chemotactic behavior, ability to undergo to AR (in response to *zona pellucida*, progesterone or other biological agonists *in vitro*)

(Breitbart, 2003; Florman et al., 2008), and ability to fertilize metaphase II-arrested oocytes *in vitro*.

1.4 Capacitation-associated Tyrosine Phosphorylation (PY)

1.4.1 Post-translational modifications and Phosphorylation in sperm

Spermatozoa are transcriptionally and translationally silent cells, therefore sperm functions mostly relies on different post-translational modifications. Ubiquitination was detected in different regions of human spermatozoa, where appears to negatively correlate with semen quality (Sutovsky et al., 2001), in contrast different results suggested ubiquitination being important for physiological sperm function (Haraguchi et al., 2007; Muratori et al., 2005; Sutovsky, 2003). Small ubiquitin-like modifiers (SUMO), another type of post-translational modification, are also present in human and murine sperm, where it has been localized to different subcellular sperm compartments (Rogers et al., 2004; Vigodner et al., 2006; Vigodner and Morris, 2005). Marchiani *et al* have shown a negative correlation between sumoylation and progressive motility in human spermatozoa (Marchiani et al., 2011). Several proteins were also shown to be the target of nitrosylation in human sperm (Lefievre et al., 2007). Finally histones acetylation and methylation are known to regulate histone-protamines replacement process, therefore promoting chromatin remodeling and compaction in sperm (Baker, 2016).
Although several types of post-translational modifications regulate different events associated with sperm function, most detailed studies have been focused on regulation of phosphorylation/dephosphorylation of pre-existing

proteins during capacitation (Naresh and Atreja, 2015). Earlier studies by Visconti *et al* have shown that the presence of HCO_3^-, Ca^{2+} and BSA in the capacitating media not only promote the PKA-mediated phosphorylation of proteins on Ser/Thr residues and the acquisition of a hyperactive motility and AR, but that they also stimulate tyrosine phosphorylation (PY) of several protein substrates in mouse sperm (Visconti et al., 1995a; Visconti et al., 1995b). Subsequent studies have confirmed occurrence of PY during capacitation in several species like stallion (Pommer et al., 2003), boar (Flesch et al., 1999), bull (Galantino-Homer et al., 1997) and human (Aitken et al., 1996). Recent investigations have shown that also Na^+, Cl^- and glucose are required to stimulate PY and ultimately capacitation (Hernandez-Gonzalez et al., 2006; Urner et al., 2001; Wertheimer et al., 2008).

Elevation of tyrosine phosphorylation levels during sperm capacitation was shown to be a downstream event respect to the activation of cAMP/PKA. cAMP agonists in fact are able to induce PY even when elements necessary for capacitation (Na^+, Cl^-, glucose, HCO_3^-, Ca^{2+} and BSA) are lacking (Wertheimer et al., 2008). In addition, pharmacological inhibitors of PKA as H89 and PKI, besides affecting cAMP/PKA activation, are also effective in inhibiting the increase of tyrosine phosphorylation associated with capacitation (Visconti et al., 1995b). Even though increased tyrosine phosphorylation levels are known to be necessary for capacitation, a precise relationship between tyrosine phosphorylation status of mammalian spermatozoa and their ability to fertilize has not been ultimately established yet (Naresh and Atreja, 2015).

Besides the fundamental role covered by tyrosine phosphorylation during sperm capacitation, other types of phosphorylation/dephosphorylation events were shown to be associated with sperm capacitation. Phosphorylation on serine/threonine (ser/thr) was also reported during capacitation. Besides the well known role of PKA-mediated phosphorylation was shown for human (Moseley et al., 2005; O'Flaherty et al., 2004) and boar (Harrison, 2004), also ser/thr kinases different from PKA were show to be present in sperm: Protein Kinase C (PKC) (Breitbart et al., 1992; Kalina et al., 1995), Protein Kinase B/Akt (PKB/Akt) (Nauc et al., 2004), glycogen synthase kinase 3β (GSK3β) (Vijayaraghavan et al., 1996), mitogen-activated kinases (MAPKs) (O'Flaherty et al., 2005; Thundathil et al., 2002) and extracellular signal-regulated kinase 1/2 (ERK1/2) (de Lamirande and Gagnon, 2002; Luconi et al., 1998a). Other studies also reported proline-directed ser/thr phosphorylation during phosphorylation of murine spermatozoa. This type of phosphorylation, known to be important for cell homeostasis, proliferation and differentiation it depends in sperm on the presence of BSA (or other cholesterol acceptors as β-cyclodextrins) (Jha et al., 2006). The onset of proline-directed phosphorylation occurs earlier (after ~15 min in capacitating conditions) compared to PY increase, but does not depend on the presence of HCO_3^- (Jha et al., 2006). It is therefore essential to understand how these different post-translational mechanisms are regulated and whether they are inter-connected in order to finally promote sperm capacitation.

1.4.2 Substrates phosphorylated on Tyrosine residues during Capacitation

Sperm flagellum appears to be the major site of tyrosine phosphorylation in most of mammalian species (Leclerc et al., 1997; Naz et al., 1991; Urner et al., 2001) except for boar (Petrunkina et al., 2001; Tardif et al., 2001). Mammalian spermatozoa modify their motility pattern during capacitation to vigorously swim through the oviduct (Luconi et al., 2006), therefore requiring high levels of ATP, mostly produced by glycolysis occurring in the principal piece of the sperm tail (Fraser and Quinn, 1981; Miki et al., 2004).

Different studies using 2-dimensional (2D) gel electrophoresis, together with tandem mass spectrometry, (MS/MS) have unveiled the identity of various substrates phosphorylated on tyrosine during capacitation in different species. The first identified proteins being phosphorylated on tyrosine belong to the family of A-kinase anchor protein (AKAPs). AKAP3 and AKAP4 were found being phosphorylated in murine (Moss et al., 1999), human (Ficarro et al., 2003) and hamster sperm (Jha and Shivaji, 2002). In addition, AKAPs members are activated by direct interaction with PKA (Carr et al., 1992) and localize within organized super-molecular structures in different cells (Luconi et al., 2011). AKAPs family members are also important during spermatogenesis, where some members regulate the assembly and stability of the sperm flagellum (Lin et al., 1995). The sperm flagellum, like other types of cilia, is organized in a typical 9+2 microtubular arrangement (axoneme) and is surrounded by 9 Outer Dense Fibers (ODFs); two opposite ODFs fuse with two surrounding longitudinal columns, which are connected throughout the tail by semilunar ribs forming the *fibrous sheet*, a cytoskeletal structure unique to the sperm flagella (Li et al., 2010; Luconi et al., 2011). Miki *et al.* showed that

spermatozoa lacking AKAP4 have a normal number of spermatozoa but fail to show progressive/hyperactive motility, they are infertile and they display an underdeveloped fibrous sheet and a short flagellum (Miki et al., 2002). Additional studies also showed that reduced tyrosine phosphorylation of ODF-2 and tektin-2 results in circular motility in hamster sperm (Mariappa et al., 2010), supporting the idea that PY and other changes in sperm motility are indeed associated events.

Studies from Naaby-Hansen *et al.* (Naaby-Hansen et al., 2002) also showed that CABYR, a calcium binding protein present in sperm, known to be associated with the fibrous sheet, is regulated by phosphorylation during capacitation. Interestingly, CABYR, a highly polymorphic protein shown to be phosphorylated both on tyrosine (Naaby-Hansen et al., 2002) and on serine/threonine during capacitation (Ficarro et al., 2003), shares high similarity with the human PKA regulatory domain (RαII), and appears to directly interact with AKAP3 in human spermatozoa (Li et al., 2011). The presence of CatSper 1-4 in the membrane of the principal piece, where also CABYR localize, further supports the hypothesis that CABYR plays a role in the cross-talk between calcium signaling and tyrosine phosphorylation (Li et al., 2010). CatSper proteins are in fact calcium-selective channels unique to spermatozoa that were recently shown to regulate the spatio-temporal occurrence of PY during capacitation (Chung et al., 2014).

Besides the abovementioned proteins associated with tail structure and motility, other substrates fundamental for cell metabolism are regulated by phosphorylation during capacitation. ATP produced by spermatozoa to support motility changes and capacitation is mostly produced by Glycolysis

(Miki et al., 2004), leading to the idea that PY also regulates the energetic state of spermatozoa during capacitation: pyruvate dehydrogenase (Arcelay et al., 2008; Jagan Mohanarao and Atreja, 2011), dihydrolipoamide dehydrogenase (Mitra et al., 2005a; Mitra and Shivaji, 2004) and two testis-specific Aldolase A isoforms, present in mature sperm (Vemuganti et al., 2007), are indeed phosphorylated on tyrosine during capacitation.

Although sperm flagellum appears to be the major site of tyrosine phosphorylation, tyrosine-phosphorylated substrates were also known to be present in the sperm head: various studies revealed that heat shock protein 60, 70 and 90 (HSP-60, HSP-70 and HSP-90) are localized on the sperm head in humans and are phosphorylated during capacitation (Asquith et al., 2004; Mitchell et al., 2007). Molecular chaperones have an important role in basic cellular processes as protein folding and degradation, intracellular transport or membrane translocation and are usually activated under conditions of stress like elevated temperatures or osmotic and oxidative stress (Lund, 1995). In mature spermatozoa, heat shock proteins are somehow involved in the sperm-oocyte recognition, binding to the *zona pellucida* and the phosphorylation of these chaperones on tyrosine during capacitation regulates conformational changes on the sperm head facilitating sperm-egg recognition (Cole and Meyers, 2011; Dun et al., 2012; Ecroyd et al., 2003; Huszar et al., 2000; Mitchell et al., 2007; Naresh and Atreja, 2015). Valosin-containing protein (VCP/p97) is another member of the chaperone family known being phosphorylated on tyrosine during sperm capacitation. This protein was shown to translocate from the sperm neck to the head during

capacitation of human sperm, highlighting a possible role as a connecting player between capacitation and AR (Ficarro et al., 2003).

Recently, Mohanarao & Atreja have shown that actin is another important cytoskeletal protein phosphorylated during sperm capacitation in buffalo sperm (Jagan Mohanarao and Atreja, 2011). Various studies showed that during capacitation actin cytoskeleton remodeling occurs in the sperm head of different species (Brener et al., 2003), facilitating the occurrence of acrosomal reaction. Finally, inhibition of actin polymerization in human and guinea pig prevent sperm penetration of zona-free hamster oocytes (Rogers et al., 1989), highlighting the role of this structural protein as important player during sperm-egg interaction and fertilization. Other important cytoskeletal proteins like different tubulins (Kadam et al., 2007), spectrin (Dvorakova et al., 2005) and dynein (Travis et al., 2001) are also tyrosine phosphorylated during capacitation. Proacrosin binding protein/p32 is likely to be involved in the regulation of AR and capacitation; it is localized both on the acrosomal cap and in the mid piece and its phosphorylation was reported in boar (Dube et al., 2005), mouse (Arcelay et al., 2008), human (Kumar et al., 2006; Schumacher et al., 2013) and buffalo (Jagan Mohanarao and Atreja, 2011).

Similarly, membrane channels appear to be post-translationally regulated during sperm capacitation. Recent studies in fact show that Voltage-Dependent Anion Channels (VDAC) are tyrosine-phosphorylated in different species (Arcelay et al., 2008; Ficarro et al., 2003; Jagan Mohanarao and Atreja, 2011). VDACs in sperm are largely localized in proximity of ODFs on the flagellum of bovine sperm (Hinsch et al., 2004) and murine models lacking VDAC gene are infertile and show reduced motility (Sampson et al., 2001).

These results further support the importance of proteins present in the principal piece for the regulation of sperm motility.

Protein phosphatases are another essential group of proteins regulated by phosphorylation on tyrosine during capacitation. Changes in the phosphorylation status during sperm capacitation (as in any other system) are tightly regulated by the balanced activity of kinases and phosphatases, therefore accounting for phosphatases activity during capacitation. Phosphatases were known to have a significant role during sperm maturation (Chakrabarti et al., 2007a; Mishra et al., 2003; Vijayaraghavan et al., 1996), with data revealing an important function in regulating sperm motility (Fardilha et al., 2011; Hoskins et al., 1983; Smith et al., 1996). Four isoforms of PP1 (PP1α, PP1β, PP1γ1 and PP1γ2) were shown to be present in mammalian sperm (Chakrabarti et al., 2007b), with PP1γ2 being the most abundant. This isoform has been detected in hamster, mouse, bull, primates and human (Smith et al., 1996) and has a major role in regulating sperm motility (Mishra et al., 2003). A different phosphatase found in mammalian sperm is PP2, which has been described in human, bovine and fowl spermatozoa; and PP2B (calcium/calmodulin-dependent phosphatase, calcineurin) found in dog, goat, pig, bovine, mouse and human sperm (Ashizawa et al., 2006; Vijayaraghavan et al., 1996). Importantly, recent data highlight a role for phosphatases in regulating the activity of PKA during capacitation: Goto and Harayama in 2009 first showed that the PP1/PP2 inhibitor cyclosporine-A (CL-A) increases the phosphorylation pattern of PKA during capacitation, with a decrease in the progressive motility and an increase in hyperactive population (Goto and Harayama, 2009). Later studies by Krapf *et al.*, 2010 have proposed that

PP2A could be the phosphatase inhibiting the activity of PKA, and that its activity would be regulated by Src family kinases (SFK) (Krapf et al., 2010). Although these studies helped elucidating the role for certain phosphatases during capacitation, the precise relationship between phosphatases activity, tyrosine phosphorylation and capacitation has not been elucidated yet.

Proteins involved in the regulation of oxidative stress are also phosphorylated during capacitation. Glutathione-S-transferase (GST) is located in the principal- and end piece of the flagellum, where it is involved in maintaining the oxidative balance and promoting detoxification (Naresh and Atreja, 2015). GSTmu5 isoform was found in murine (Arcelay et al., 2008) and hamster sperm (Ashrafzadeh et al., 2013), whereas GSTmu3 was found in buffalo (Jagan Mohanarao and Atreja, 2011, 2012) and human (Kumar et al., 2006) with both isoforms phosphorylated on tyrosine during capacitation.

Finally, other phospho-proteins have been described in sperm during capacitation including tyrosine kinase c-yes (Leclerc and Goupil, 2002), alpha enolase, succinate dehydrogenase and glutamate synthase (Jagan Mohanarao and Atreja, 2011, 2012).

Although several studies revealed the identity of different proteins phosphorylated on tyrosine, the relationship between phosphorylation, time and specific functions of each of these substrates during capacitation requires further investigation.

1.4.3 Kinases mediating capacitation-associated Tyrosine Phosphorylation

It is well established that in mammals, elevated levels of phosphorylated tyrosine observed during sperm capacitation are stimulated *in* vitro by the

same components promoting the activation of the cAMP/PKA pathway [HCO_3^- and a cholesterol acceptor, usually BSA (Hernandez-Gonzalez et al., 2006; Urner et al., 2001; Visconti et al., 1995a; Wertheimer et al., 2008)]. In addition, calcium is known to be necessary for capacitation (Visconti et al., 2002; Yanagimachi, 1994b) acquisition of hyperactive motility and AR (Santi et al., 2010), with recent data showing that extracellular Ca^{2+} negatively regulates PY, possibly controlling its time of occurrence (Navarrete et al., 2015). Since PKA is a serine/threonine kinase and does not promote phosphorylation on tyrosine residues directly, it is reasonable to think that PKA induces the increase of tyrosine phosphorylation through a protein kinase cascade [reviewed by (Salicioni et al., 2007)]. Furthermore, the cAMP-dependent increase of tyrosine phosphorylation has not been reported in cell types other than spermatozoa (Leclerc et al., 1996). Therefore, another fundamental and long-standing question in the field of male reproduction, particularly in studies on sperm capacitation, is to identify the (one or more) Tyrosine Kinase(s) that participate(s) in this process, and to ultimately comprehend how these kinase(s) are being regulated.

Protein tyrosine kinases (TKs) are divided in two major groups: non-receptor tyrosine kinases (PTK) and receptor tyrosine kinases (RTK), and both were shown to be present in mammalian spermatozoa (Gangwar and Atreja, 2015). Recent studies have reported that besides PKA-dependent tyrosine phosphorylation, a different subset of phosphorylated proteins is present on the membrane of capacitated spermatozoa (Nixon et al., 2010). Early studies on tyrosine phosphorylation were focused mostly on receptor tyrosine kinases expressed on cell surface. EGF receptor was found to be present on the

membrane of human, murine, rabbit (Naz and Ahmad, 1992) and bovine spermatozoa, where somehow promote the occurrence of acrosome reaction (Lax et al., 1994); p190 c-met tyrosine kinase receptor was also found to be localized in both human sperm tail and head, and to be phosphorylated on tyrosine upon activation (Herness and Naz, 1999); the Insulin growth factor receptor I (IGF-IR) has been identified in human (Naz and Padman, 1999) and bovine sperm. In addition, bull seminal plasma carry a high concentration of Insulin growth factor (IGF), (mostly produced in the testis and epididymis) (Henricks et al., 1998). IGFR, upon activation phosphorylates itself (auto-phosphorylation) and other adaptor proteins downstream: one of these adaptor protein is c-ras, shown to be present in human spermatozoa (Naz et al., 1992). The role of c-ras in different cell systems is well established: this adaptor protein promotes the activation of the mitogen-activated protein kinase (MAPK) cascade. Besides c-ras, other MAP kinases were found in sperm: ERK2 (p42ERK), which localizes in the head of human (Luconi et al., 1998b) and fowl (Ashizawa et al., 1997) spermatozoa. Nixon *et al.* showed that inhibitors of ERK1/2 kinases promote a decrease of tyrosine phosphorylation of membrane proteins in murine (Nixon et al., 2010) and boar sperm (Piehler et al., 2006) but not in human, hypothesizing different mechanisms of regulation between different species (Mitchell et al., 2007). It has been shown that ERK1/2 inhibitors are not able to completely suppress increased membrane tyrosine phosphorylation levels during capacitation, opening the possibility of a cross-talk between cAMP/PKA and MAP kinase cascades during capacitation of murine spermatozoa.

Although tyrosine phosphorylation could be mediated by receptor tyrosine kinase(s) in the sperm head, the major cascade of signaling events inducing the increase of tyrosine capacitation in the sperm tail during capacitation is dependent on the cAMP/PKA pathway. sAC and the CαII catalytic subunit of PKA are both essential for fertilization, as the loss of each of these genes produce a sterile phenotype with defects associated with capacitation: knock-out (KO) mice do not show any increase of PY and do not acquire hyperactive motility (Burton and McKnight, 2007; Xie et al., 2006). In this regard, it is of great importance to identify the protein tyrosine kinase(s) activated downstream of cAMP/PKA and that stimulate the increase of tyrosine phosphorylation. Since Visconti *et al* report in 1995 (Visconti et al., 1995a; Visconti et al., 1995b), several studies tried to reveal the identity of the kinase(s) activated during capacitation and different candidates were proposed over the years.

1.4.4 The case of c-Abl tyrosine kinase

In somatic cells, c-Abl is a proto-oncogenic non-receptor tyrosine kinase that localizes both in the nucleus and in the cytoplasm, is ubiquitously expressed and plays a central role in different cellular processes as differentiation, proliferation and apoptosis. Since its presence was showed in human spermatozoa, tyrosine kinase c-Abl was considered as a possible candidate playing a role during sperm capacitation (Naz, 1998). Recent studies from Baker *et al* suggested a role for this kinase during the onset of PY associated with capacitation. c-Abl was found to be present in both the tail and the head

of murine spermatozoa and the CαII catalytic subunit of PKA *in vitro* was shown to induce c-Abl phosphorylation (Baker et al., 2009). c-Abl inhibition during *in vitro* capacitation, on the other hand, slightly affects PY increase (Baker et al., 2009), suggesting that c-Abl probably plays a marginal role in PY-dependent signaling during sperm capacitation.

1.4.5 The role of Src Family Kinases

Several studies point out to different Src Family Kinases (SFKs) to be responsible for the capacitation-associated increase in tyrosine phosphorylation. Leclerc and Goupil in 2002 (Leclerc and Goupil, 2002) first proposed c-yes: this tyrosine kinase was found in both sperm membrane and cytosol and its activity was up regulated by cAMP and down regulated by calcium. Although up regulation of c-yes by cAMP was in accordance with the paradigm of tyrosine phosphorylation, the fact that this kinase is negatively regulated by increased Ca^{2+}, (which is known to augment during capacitation) suggested that a different kinase was involved in regulating tyrosine phosphorylation (Leclerc and Goupil, 2002). Another proposed candidate belonging to Src family was Fyn, but analysis of *fyn*-null spermatozoa showed defects associated with morphology and fertilizing ability but no difference in the pattern of tyrosine capacitation was observed compared to wild type, suggesting a role during sperm development in the testis (Luo et al., 2012). The major body of investigation on SFKs during capacitation was done on the role of c-Src. Baker *et al* in 2006 first proposed c-Src as the key tyrosine kinase regulating PY during capacitation: c-Src was found to be present in

murine sperm and to physically interact with the catalytic subunit of PKA (Baker et al., 2006). The phosphorylated active form of c-Src (c-Src PY^{416}) was detected only after capacitation, and found to be sensitive to H89 (specific inhibitor of PKA). In addition, the presence of SFKs inhibitor SU6656 in the capacitating media inhibited tyrosine phosphorylation (Baker et al., 2006). Studies conduced in human spermatozoa confirmed that tyrosine phosphorylation was sensitive to SU6656 but, different from previous data in mouse, c-Src failed to immunoprecipitate with the $C\alpha 2$ catalytic subunit of PKA and no changes in hyperactive motility were observed (Varano et al., 2008). This set of results supported a possible role for c-Src during the increase of tyrosine phosphorylation but raised a question about the PKA direct activation of SFKs during capacitation (Varano et al., 2008). Later, studies from our lab (Krapf et al., 2010) provided further evidence on the role of SFKs during murine sperm capacitation indicating that, although SFKs inhibitors such as SU6656 do not affect the activity of PKA *in vitro*, the same concentrations of the inhibitor *in vivo* cause a decrease in the level of phosphorylation of the PKA substrates (Krapf et al., 2010), suggesting that the inhibition of tyrosine phosphorylation is due to a down regulation of the cAMP/PKA pathway upstream and not to a direct effect on the activity of c-Src or other tyrosine kinases. To further confirm that c-Src is not the unique tyrosine kinase involved in the pathway, levels of tyrosine phosphorylation were measured in a murine model lacking the *c-src* gene (Src null mice). Both sperm from c-Src null and their respective wild type littermates showed no differences in tyrosine phosphorylation levels after capacitation (Krapf et al., 2010). Based on the fact that the inhibitory effect of SFKs inhibitors on the

phosphorylation of PKA substrates and tyrosine during capacitation was abolished when inhibitors of serine/threonine phosphatases were added during capacitation, Kraft et al. proposed an alternative mechanism of regulation: SFKs are part of a parallel pathway that promote the full activation of PKA through the inhibition of serine/threonine phosphatases, which are known to be present in murine sperm, as mentioned above. More recently, our group has provided evidence for a similar mechanism of regulation of PKA activity mediated by SFKs through the down regulation of the serine/threonine phosphatases in human spermatozoa (Battistone et al., 2013). Although these later studies have represented a great advance in studies on sperm capacitation revealing the presence of another pathway parallel to cAMP - the involvement of SFKs and phosphatases in the activation of the PKA-, the original question of which tyrosine kinase(s) promote tyrosine phosphorylation during capacitation remains still open.

1.4.6 Role of Focal Adhesion Kinases

Recently, Gonzalez-Fernandez *et al* have proposed a different mechanism of PY stimulation mediated by calcium and pH in stallion (Gonzalez-Fernandez et al., 2013): they showed that in stallion sperm the capacitation-associated increase of tyrosine phosphorylation is lost in the presence of Ca^{2+} at physiological pH ~7.4 (Gonzalez-Fernandez et al., 2012), leading them to hypothesize that, beside cAMP/PKA cascade, calcium may also regulate tyrosine phosphorylation in order to prevent a premature occurrence of PY (and capacitation) (Gonzalez-Fernandez et al., 2012). . In this study, the

authors open the possibility that a different calcium-dependent tyrosine kinase(s) is activated during capacitation in stallion sperm: Focal Adhesion Kinases. This kinase subfamily includes two members, FAK (PTK2) and PYK2 (PTK2B): both are non-receptor tyrosine kinases that, although sharing high similarity in structure, exhibit clear differences in their functions and mechanisms of regulation in different cells types such as fibroblasts, osteoblasts, osteoclasts and others in the immunological compartment. Both FAK members mediate phosphorylation of targets downstream, but also act as scaffolding proteins (Hall et al., 2011). FAK localizes to focal adhesion contacts where it responds to integrin clustering, growth factors and mechanical stimuli, ultimately regulating survival (Gilmore et al., 2009), proliferation (Assoian and Klein, 2008) and motility through the regulation of the actin cytoskeleton (Schaller, 2010). PYK2 shares a high degree of structure similarity and localization with FAK, yet it is regulated by integrin clustering, environmental stress and growth factors. Different from FAK, PYK2 responds to extracellular stimuli to regulate Rho activity (Okigaki et al., 2003), thus controlling actin cytoskeleton, finally promoting cell motility (Owen et al., 2007a; Sun et al., 2011), cell processes formation (Gil-Henn et al., 2007) and phagocytosis (Owen et al., 2007b). PYK2 also regulates mitogenic and hypertrophic response upstream the functionality of various ion channels (Lakkakorpi et al., 1999; Lev et al., 1995), and its full activity depends on the presence of Calcium (Wu et al., 2006).

Gonzalez-Fernandez *et al* showed that both members of FAK family are present in stallion spermatozoa. In addition, the use of the Focal Adhesion Kinase specific inhibitor PF431396 reduced both activity of the active

phosphorylated forms of FAK and PYK2 and the occurrence of tyrosine phosphorylation associated to sperm capacitation in stallion (Gonzalez-Fernandez et al., 2013).

Recent studies also investigate the role of both FAK members in oocytes from different species: Sharma and Kinsey showed that in *zebra-fish* oocytes, fertilization is associated with a rapid (~2 minutes) accumulation of PYK2, with an increase of the active phosphorylated form of PYK2 (PYK2-PY^{579}) (Sharma and Kinsey, 2013). PYK2-PY^{579} increases at the site of sperm-egg binding. A similar role for PYK2 during sperm-egg interaction was also confirmed in mammals: both FAK and PYK2 are found in murine oocytes. After fertilization FAK retain its punctate localization but PYK2 appear to concentrate in proximity to the region where sperm head bound to the oocyte (McGinnis et al., 2013). Furthermore PYK2 plays a role either during sperm-egg fusion or sperm engulfment into the cytoplasm (Luo et al., 2014).

These results altogether highlight the role of Focal Adhesion Kinases as players during fertilization, which has lead us to hypothesize for a role for PYK2 / FAK in regulating the timing and occurrence of Capacitation-associated Tyrosine Phosphorylation in human and murine sperm.

1.5 Spatio-temporal considerations and Kinetics of Sperm Capacitation Signaling Events

Different from the antigen mediated T-cell activation, or cell signaling that takes place upon hormone-receptor binding, sperm capacitation signaling is not initiated by the binding of a ligand to a specific receptor. Moreover, unlike most of fast signaling cascades, also timing of sperm capacitation appear to be tightly regulated: when incubated in capacitating conditions, sperm reach their full fertilizing ability only after several minutes or even hours, depending on the species (Salicioni et al., 2007). It is important to note that, when studying the process of sperm capacitation from a kinetics point of view, an immediate paradox stands out: although the so-called 'triggers' of capacitation – the sAC-mediated increase of cAMP levels and the activation of PKA – are described as fast events, spermatozoa show macroscopic features of capacitation (hyperactive motility and acrosomal reaction) only minutes or even hours later. Furthermore, cAMP may regulate, either directly or indirectly, different biochemical events associated with capacitation: intracellular alkalinization (Zeng et al., 1996), membrane lipid remodeling (Gadella and Harrison, 2000) and hyperpolarization of the sperm plasma membrane (De La Vega-Beltran et al., 2012; Escoffier et al., 2012; Zeng et al., 1995). Although in the last years several advances have been made in understanding mechanisms mediating capacitation, little is known about how the early triggers of capacitation may coordinate events occurring later on in order to ensure the presence of a pool of capacitated spermatozoa able to reach the oocyte at an appropriate time. As previously stated, the increase of tyrosine phosphorylation (PY) associated with capacitation is also activated by

the initial rise in cAMP but it was shown to occur on a different time in different models. In murine sperm, increased levels of PY are observed only 45 minutes after the beginning of capacitation in vitro, reaching their maximum at 60 minutes, and remaining steady thereafter (Visconti et al., 1995a). In bovines, PY levels start to rise after 3 hours with a full increase at 5 hours (Galantino-Homer et al., 1997); a similar time course is also observed for humans (Liu et al., 2006). Using a chemical-genetic approach that allows to specifically inhibit sperm PKA without affecting off-target kinases, Morgan *et al* 2008 showed that, although the activation of PKA during capacitation is a fast event, the activity of this ser/thr kinase must be sustained for at least 30 minutes in order to promote the increase of PY and finally capacitation (Morgan et al., 2008). Recent data also showed that the activity of c-Src, a tyrosine kinase that promotes activation of PKA through down regulation of ser/thr phosphatases (Krapf et al., 2010), is tightly regulated over time. Krapf further showed that auto-phosphorylation (and activation) of cSrc is dependent on the activation of PKA and that begins only 15 minutes after capacitation (and PKA activation) has started, finally reaching its maximum after 60 minutes (Stival et al., 2015). Therefore c-Src activation is delayed compared to the activation of PKA but precede the onset of tyrosine phosphorylation. Altogether, these results suggest that the activities of the different kinases involved in capacitation are tightly regulated over time. Therefore it is critical to investigate the spatio-temporal occurrence of the different hallmarks of capacitation for a full comprehension of the process. Due to the importance of different phosphorylation events during capacitation, it is of great importance to investigate the possible role of protein

phosphatases in regulating the temporal occurrence of the events linked to sperm capacitation. Our efforts in the fourth chapter of this dissertation will be focused on investigating and understanding the kinetics of phosphorylation events during capacitation in murine spermatozoa.

1.6 Hypothesis and specific aims

The purpose of this project is to contribute elucidating molecular events that participate in the process of sperm capacitation in murine spermatozoa. Our working hypothesis is that a protein tyrosine kinase downstream of PKA is responsible for the onset of tyrosine capacitation.

This hypothesis gives rise to some basic questions: 1. What is the tyrosine kinase that promotes the increase of tyrosine phosphorylation? 2. Does the increase in tyrosine phosphorylation depend on the presence of a single specific tyrosine kinase? 3. What is the temporal relationship between tyrosine phosphorylation and the upstream activation of PKA?

In order to elucidate the questions mentioned above, we propose two specific aims.

1.6.1 Specific Aim 1: Investigate the role of focal adhesion kinase during capacitation in human and murine sperm.

The focal adhesion kinase specific inhibitor PF431396 was shown to inhibit capacitation-associated PY in stallion sperm (Gonzalez-Fernandez et al., 2013). No evidence so fare were given about a possible role of this family in regulating tyrosine phosphorylation during capacitation.

1.6.2 Specific Aim 1: Investigate the role of FER tyrosine kinase during capacitation in human and murine sperm.

Recently phospho-proteome analysis of capacitated mouse sperm showed that the tyrosine kinase FER is present and phosphorylated (Chung et al., 2014). However no studies were focus on investigating the role of FER kinase during sperm capacitation.

1.6.3 Specific Aim 2: Determine the kinetic of phosphorylation events associated with capacitation.

A study in murine showed that the activity of PKA have to be sustained to induce tyrosine phosphorylation during capacitation (Morgan et al., 2008). Beside this publication, no studies so far focused on the temporal requirement for phosphorylation events in order to guarantee a pool of capacitated sperm.

CHAPTER 2

INVESTIGATING THE ROLE OF FOCAL ADHESION KINASES DURING SPERM CAPACITATION

2.1 Analyzing the activity of Focal Adhesion Kinases during sperm capacitation in human spermatozoa.

Focal adhesion kinase family represents a class of protein tyrosine kinases involved in the control and regulation of cell migration. This coordinated event requires both a fast regulation of the dynamics of actin cytoskeleton and the continuous assembly and disassembly of adhesion sites (Brakebusch and Fassler, 2003) to promote the generation of membrane processes and traction forces that allow cells to migrate (DeMali et al., 2003). Extracellular stimuli that control cell migration are transduced to intracellular signals through the activation of integrins. Several extracellular signals induce the formation of integrins heterodimers and the assembly of intracellular dynamic protein complexes named *focal contacts*, which promote the activation of biochemical signaling downstream (Mitra et al., 2005b). *Focal adhesion kinases* (*fak*) are present at focal contacts, where rapidly respond to different stimuli activating their tyrosine kinase activity. FAK family includes two members: FAK (*PTK2*) and PYK2 (*PTK2B*). The structures of both members of the family show high similarity. FAK and PYK2 indeed share the FERM, FAT and kinase domains as well as the presence of three proline-rich regions (PRRs). FERM domain mediates interaction with receptor tyrosine kinases (EGFR or PDGF) as well as with G-protein couple receptors (GPCRs), promoting the activation of FAK and other non-receptor tyrosine kinase; FAT on the other hand is the domain that regulate the interaction

with other integrin-associated proteins of the focal contact including paxilin and talin (Schlaepfer et al., 2004); the PRR domains function as binding sites for Src Homology domains 3 (SH3) containing proteins, promoting FAK's function as adaptor protein (Chodniewicz and Klemke, 2004; Hanks et al., 2003). The kinase domain both promotes the phosphorylation of specific substrates and auto-phosphorylation: auto-phosphorylation of Tyr397 on FAK and of Tyr402 on PYK2 induces the formation of a recognition motif for protein with SH2 domains, finely regulating the activity of the kinase (Toutant et al., 2002; Wu et al., 2006). Although both FAK and PYK2 shares high similarity and can bind to Src Family Kinases (SFKs) (Calalb et al., 1995), these kinases cover different cellular roles due to different binding affinity to the FERM and FAT domains. Furthermore PYK2, different from FAK, which always localize at focal contact, have a perinuclear distribution and its activity is dependent on intracellular Ca^{2+} (Klingbeil et al., 2001; Wu et al., 2006). Finally these two kinases are differentially expressed with FAK mostly widely express compared to PYK2, which is mostly restricted to cell of the endothelium, the central nervous system and hematopoietic lineage (Mitra et al., 2005b). Recent data showed that both members of focal adhesion family are present in stallion spermatozoa, where have been suggested to regulate the onset of tyrosine phosphorylation associated with capacitation (Gonzalez-Fernandez et al., 2013).

2.1.1 Presence of Focal Adhesion Kinase members in human spermatozoa

Focal Adhesion Kinases were shown to be present in stallion sperm where appear to regulate the increase of PY associated with capacitation (Gonzalez-

Fernandez et al., 2013). These results led us to investigate whether FAK members were present in human sperm and if these kinases cover a similar role in regulating mechanisms conducive to sperm capacitation. The presence of both FAK members was tested by Western blotting together with the use of specific antibodies directed against either total levels of FAK or PYK2. We showed that in total cell lysates from human sperm, only one member of the Focal Adhesion Kinase family was present, PYK2 (Fig 2.1A). In fact, antibodies directed against total levels of FAK did not detect any band correspondent to the molecular weight of FAK in human spermatozoa (Fig 2.1B).

2.1.2 Effect of PF431396 on capacitation-associated phosphorylation events in human spermatozoa

To further investigate whether PYK2 play a role in regulating PY during sperm capacitation in human sperm, we tested the effect of a small molecule inhibitor of FAK kinase members, PF431396 (Buckbinder et al., 2007; Han et al., 2009). This inhibitor in stallion was shown to negatively affect PY increase associated with capacitation (Gonzalez-Fernandez et al., 2013). Ejaculated spermatozoa were incubated for 6 hours in a complete BWW media alone (in presence of HCO_3^-) or in presence of increasing concentrations of PF431396 (0–30μM): the inhibitor cause a slight increase in the levels of phosphorylation of PKA substrates (pPKAs) (Fig 2.2, upper panel) but induce a significant decrease in the level of tyrosine phosphorylation (PY) associated with capacitation (Fig 2.2, lower panel) with a clear decrease in PY levels observed at 3μM. The result suggests a role for

the FAK member PYK2 during capacitation of human spermatozoa, most likely being involved in the induction of tyrosine phosphorylation.

2.1.3 PYK2 phoshorylation/activation and effect of PF431396 during capacitation

In order to gain further insights on the possible role of PYK2 during capacitation in human, the activation status of this kinase was tested by western blot: data in the literature showed that in different cellular systems, upon activation, PYK2 is trans/auto phosphorylated on tyrosine 402 (PYK2-PY^{402}) (Avraham et al., 2000). We used antibodies directed against the phosphorylated form of PYK2 (anti-PYK2-PY^{402}) to investigate the kinetic of activation of PYK2 during capacitation. The phosphorylated form of PYK2 is not detected in fresh ejaculated sperm, but the signal appear only after two hours incubation in complete BWW media, and PYK2-PY^{402} phosphorylation reach its maximum after 6 hours incubation in capacitating conditions (Fig 2.3A, upper panel). On the other hand, levels of total PYK2 remained constant during the same time of incubation (Fig 2.3A, lower panel). The similarity between the kinetics of PY and PYK2 during capacitation further supports a role for the FAK member as regulator of PY during capacitation (Liu et al., 2006). To test a possible relationship between PYK2 activity and tyrosine phosphorylation during capacitation, we examined whether the activity of PYK2 is affected by the presence of PF431396. Sperm were incubated in BWW media, in absence or presence of PF431396, and after 6 hours the phosphorylation level of PYK2 (PYK2-PY^{402}) was analyzed. Phosphorylated PYK2 appear only in presence of bicarbonate (complete BWW media), and the signal is

lost when the sperm population is incubated with 5μM PF431396 (Fig 2.3B, higher panel), no change in the levels of total PYK2 were observed (Fig 2.3B, lower panel). Additionally, the kinetics of activation of the enzyme and PY are very similar, with a slow increase over time when sperm are incubated in capacitating conditions. Altogether, these results suggest that PYK2 is activated during capacitation and that its activity correlate with the occurrence of PY.

2.1.5 Effects of PF431396 on capacitation-associated events in human sperm

In order to investigate whether PYK2 have a function in regulating the occurrence of late capacitation-associated events, we next tested the effect of PF431396 on two well-known functional events associated with sperm capacitation: motility and acrosomal reaction (AR). When sperm were incubated in presence of PF431396, increasing concentrations of the inhibitor negatively affect several parameters associated with sperm motility (all except linearity [LIN] and straightness [STR]), indicating that PYK2 is somehow involved in the regulation of sperm motility (Fig 2.4). Furthermore, the presence of PF431396 negatively affect both progressive motility and hyperactive motility in capacitating conditions: in presence of the inhibitor in fact, the percentage of hyperactive sperm is reduced from ~12% in the capacitated sperm to ~2% in sperm treated with PF431396 (both 10 and 30μM), similar decrease was also shown for progressive motility (from 86% to 25%) (Fig 2. 4) No effect on total motility was observed (data not shown). The effect of PF431396 on the *in vitro* progesterone-induced AR was also tested and was shown to negatively affect the occurrence of acrosome reaction: 10 and 30μM PF431396 added during capacitation cause a significant decrease in the number

of progesterone-reacted spermatozoa without affecting the rate of spontaneous AR (Fig 2.5). Observations discussed in this paragraph strongly suggest that the focal adhesion kinase PYK2 in human sperm, play a role downstream to cAMP/PKA during capacitation and appear to regulate the onset of slow events of capacitation as onset of PY, hyperactive motility and AR.

2.2 Analyzing the activity of Focal Adhesion Kinases during sperm capacitation in murine CD1 sperm.

Results showed in the above section highlight a role for the *focal adhesion kinase* PYK2 in the regulation of PY during sperm capacitation in human spermatozoa. In this second section of the chapter, we used both pharmacological and genetic approaches to investigate the presence of *focal adhesion kinases* and whether any of these proteins play a role in the regulation of PY during murine capacitation, similar to what we suggested in human.

2.2.1 Effect of PF431396 on murine sperm capacitation associated protein phosphorylation

In the case of murine spermatozoa, we decided to use a slightly different strategy and to test the effect of the *fak* members-specific inhibitor PF431396 on the phosphorylation of both PKA substrates and tyrosine before examining the presence of focal adhesion members. When murine sperm are stimulated with HCO_3^- and BSA *in vitro*, events associated with sperm capacitation as cAMP-mediated activation of PKA and a late increase of PY levels are induced (Visconti

et al., 1995a; Visconti et al., 1995b). Murine spermatozoa are incubated for 60 minutes in modified TYH medium containing 15mM HCO_3^- and 5mg/mL BSA together with different concentrations of PF431396 (1–30μM). After 60 minutes of incubation, protein extracts were produced and phosphorylation of PKA substrates (pPKAs) and of tyrosine (PY) were analyzed using anti-pPKAs and anti-PY antibodies, respectively. PF431396 induces a concentration-dependent decrease in the levels of PY with an EC_{50} ~1μM with maximum effect observed at 10μM (Fig 2.6, upper panels). On the other hand when the capacitation-associated levels of pPKAs in presence of the same concentrations were examined, no significant decrease was observed compared to non-treated capacitated control (Fig 2.6, lower panels). Therefore PF431396 inhibits the capacitation-associated onset of tyrosine phosphorylation (PY) in a concentration-dependent manner, without affecting early events as increase of cAMP levels and PKA activation in murine (pPKAs), confirming previous results in human spermatozoa (Fig 2.2). In order to confirm PF431396 was not affecting early events associated with capacitation, we additionally examined the effect of the inhibitor when the signaling was also stimulated by exogenous triggers of pPKAs and PY in different species: dibutyryladenosine cyclic monophosphate (dbcAMP) and isobuthilmethylxanthine (IBMX) (Esposito et al., 2004). Sperm were co-incubated with modified TYH media (containing HCO_3^- and BSA) together with 1mM dbcAMP/100μM IBMX, both in presence and absence of 10μM PF431396. The presence of exogenous triggers did not modify the pattern of inhibition of PY mediated by PF431396, with a clear decrease in the levels of PY (Fig 2.7, upper panels) and no effect on the phosphorylation of PKA substrates (Fig 2.7, lower panels).

Src Family Kinases (SFKs) were also showed to have a positive role in the full activation of Protein Kinase A during capacitation both in human (Battistone et al., 2013) and murine (Krapf et al., 2010), by down regulating the inhibitory effect of Serine/Threonine phosphatases on PKA, in a parallel pathway to the one stimulated by sAC/cAMP. The specificity of the pharmacological approach in use was also assessed by examining the effect of PF431396 on PY, in presence of different concentrations of the phosphatases inhibitor okadaic acid (OA). The SFKs inhibitor SKI606 (50μM) induces a decrease in the capacitation-associated pPKAs and PY levels and this decrease is rescued in presence of OA (Krapf et al., 2010). Different from what was shown for SFKs inhibitors SKI606 and SU6656, increasing concentrations of OA (1–100nM) are not able to rescue the inhibition of PY mediated by 10μM PF431396 (Fig 2.8) consistent with previous results in human (Battistone et al., 2014). These data indicate that PF431396 inhibits the late increase of PY associated with capacitation without affecting the parallel pathway mediated by SFKs and phosphatases, which regulates the full activation of PKA during capacitation. This result suggests that, similar to what we shown in human, *focal adhesion kinases* could be the target of PF431396 during capacitation therefore possibly playing a role in the increase of PY.

2.2.2 Effect of PF431396 on in vitro fertilizing ability of CD1 spermatozoa

The focal adhesion kinase inhibitor PF431396 was shown to abolish the increase of PY observed during capacitation (Fig 2.6). Since the well established importance of PY increase during capacitation in different species (Baldi et al., 2002; Ficarro et al., 2003; Liu et al., 2006; Roy and Atreja, 2008; Visconti et al.,

1995b), we decided to test the effect of the same inhibitor also on the ability of CD1 spermatozoa to fertilize metaphase-II arrested oocytes *in vitro*. We used 10μM PF431396, a concentration showed to abolish tyrosine phosphorylation increase in sperm from CD1 mice (Fig 2.6). In presence of PF431396, the ability of CD1 epididymal spermatozoa to fertilize metaphase II arrested oocytes *in vitro* is reduced of about 40% compared to untreated sperm (Fig 2.9A). Furthermore when the same concentration of the inhibitor was present both during capacitation and co-incubation the inhibitor displayed a more pronounced effect with a stronger decrease in fertilizing ability (Fig 2.9A). Recent studies showed that the calcium-dependent activation of PYK2 is required in Zebra-fish oocyte to guarantee successful fertilization (Sharma and Kinsey, 2013) and that in murine, PYK2 somehow mediates sperm-egg fusion and anaphase resumption (McGinnis et al., 2013). Due to a possible effect of PF431396 on the activation of PYK2 during meiotic resumption of metaphase II arrested oocytes (McGinnis et al., 2013) during our experimental procedure, we therefore decided to test the effect of different concentrations of PF431396 (0.01–1μM, carryover concentrations used during IVF experiments) only when incubated with metaphase II arrested oocyte: after collection, eggs were incubated in presence of PF431396 for 30 minutes and then co-incubated with capacitated spermatozoa (not treated with the FAK inhibitor) for the following 4 hours in presence of PF431396. We showed that concentrations up to 1μM have a negligible effect on PYK2 activation in metaphase arrested II oocytes (Fig 2.9B), confirming that the decrease of fertilization rate observed in Fig 2.9A is the results of the inhibitory effect of PF431396 throughout capacitation and not on the meiotic resumption of murine oocytes during *in vitro* fertilization.

The reduced ability of PF431396 incubated during capacitation, to strongly inhibit fertilization rate in compared to other treatments, is due to a time-dependent increase of PY phosphorylation during sperm-oocyte co-incubation the carryover concentrations of PF431396 drop to 10nM) (see Fig 4.13).
Although this observation confirmed that PF431396 negatively affect fertilizing ability *in vitro*, suggesting PYK2 play a role in coordinating later events of capacitation, the data does not indicates which functional feature was negatively affected by the inhibitor during capacitation.

2.2.3 Effect of PF431396 on sperm motility of CD1 spermatozoa

In order to investigate which functional parameter of capacitation was affected by PF431396, we decided to analyze the effect of the inhibitor on motility of murine sperm. Mammalian spermatozoa modify their motility behavior during capacitation: while ejaculated spermatozoa mostly show a progressive motility that allows them to rapidly swim-out from the seminal plasma, capacitated spermatozoa in the utero-tubal junction (UTJ) acquire hyperactive motility, which render this cells slower but able to swim through the viscous tubal mucus of Fallopian tubes (Fraser, 1977, 2010; Suarez, 1996, 2008; Suarez and Osman, 1987). Studies also showed that most of the tyrosine phosphorylated substrates are located in the sperm tail, suggesting a possible role in regulating motility behavior (Carrera et al., 1996; Leclerc et al., 1997; Lewis and Aitken, 2001; Si and Okuno, 1999; Urner et al., 2001).
After collection from epididymis, sperm were incubated in capacitating condition for 60 minutes, in absence or presence of 10μM PF431396, and motility was then

measured using Hamilton-Thorne CASA system (Fraser, 1977, 2010; Suarez, 1996, 2008; Suarez and Osman, 1987) together with the software CASAnova (Goodson et al., 2011). Incubation of murine spermatozoa with 10μM PF431396 during capacitation does not affect motility behavior; the percentage of motile spermatozoa in fact is similar between treated and untreated capacitated control (Fig 2.10A). Similarly, the acquisition of hyperactive motility during capacitation was also not affected by the presence of PF431396, with comparable percentage of sperm with hyperactive motility (Fig 2.10B).

The analysis of other motile subpopulations, also based on the use of CASAnova (Goodson et al., 2011) showed no difference between treated sperm and controls (data not shown). Different from human spermatozoa (Fig 2.4), here we showed that the *fak* inhibitor PF431396 has no effect on the motility behavior of capacitated spermatozoa in mouse. The different sensitivities to PF431396 between human and murine spermatozoa potentially highlight some difference in the regulation of sperm motility between human and mouse.

2.2.4 Presence of Focal Adhesion Kinase members in murine spermatozoa

Because capacitation-associated phosphorylation events in murine spermatozoa were shown to be sensitive to the FAK members inhibitor PF431396 in both stallion (Gonzalez-Fernandez et al., 2013) and human (Battistone et al., 2014), we therefore decided to investigate which of the focal adhesion kinases members were present in murine spermatozoa. Detection of *fak* was performed by western blot: epididymal sperm were lysed and both soluble and insoluble protein fractions were tested using antibodies directed against total levels of FAK (anti-FAK) and

PYK2 (anti-PYK2). Similarly to human, total cell lysates from murine spermatozoa probed with anti-PYK2, showed a band at ~116kDa, corresponding to the molecular weight of murine PYK2 (Fig 2.11A). Different from human, when total cell lysates were tested for the presence of the other member of focal adhesion kinase family FAK in murine, we also detected a band at ~123kDa, corresponding to the expected size of FAK (Fig 2.11B). Although both anti-PYK2 and anti-FAK recognized additional bands at different molecular weights, no relationship between those bands and our proteins of interest was established. Although the presence of both members was in contrast with results previously obtained in human (Battistone et al., 2014), the data were consistent with the presence of both members in stallion (Gonzalez-Fernandez et al., 2013). We therefore decided to test the solubility of both members to the non-ionic detergent Triton X-100 after 60 minutes incubation in capacitating conditions. Different from the analysis of PYK2 solubility in human, both PYK2 (Fig 2.11C, left panel) and FAK (Fig 2.11C, right panel) were soluble to Triton X-100. This result again was in argument with was shown in human (Battistone et al., 2014), highlighting possible differences between species in mechanisms regulating phosphorylation associated with sperm capacitation.

2.2.5 Effect of PF573228 on pPKAs and PY in CD1 sperm and Effect of PF431396 and PF573228 on the In vitro activities of recombinant FAK and PYK2

The presence of both members of the FAK family was an encouraging result to support our hypothesis. Although PYK2 and FAK present differential expression

and roles, they share high similarity both in structure and function (Mitra et al., 2005b), PF431396 was shown being able to block both their activities with a similar IC_{50} (Bhattacharya et al., 2012); PF431396 therefore, is not the ideal tool to investigate whether PYK2 or FAK is the protein that plays a role in regulating the increase of PY associated with capacitation. In order to circumvent this limitation, we adopted an alternative pharmacological approach: PF573228 is another inhibitor of FAK kinases, but different from PF431396, have higher specificity for FAK than PYK2 (IC_{50} for FAK is one order of magnitude higher than for PYK2) (Bhattacharya et al., 2012). We therefore examined the sensitivity of phosphorylation events to PF573228 (Fig. 2.12). Epididymal Murine sperm were incubated in modified TYH medium in capacitating condition (15mM HCO_3^- and 5mg/mL BSA) and in presence of different concentrations of PF573228. Different from what showed for PF431396, PF573228 is not able to completely inhibit the capacitation-associated increase of PY to non-capacitated control levels, but only cause a small decrease in the level of PY (Fig 2.12B). Levels of pPKAs were not affected by the presence of the FAK specific inhibitor PF573228, consistent with previous results using PF431396 (Fig 2.12A). The significantly reduced inhibition observed in presence of PF573228 suggested that, even if FAK is present in murine spermatozoa, is not the kinase responsible for PY increase during capacitation. Although these data were consistent with our hypothesis of PYK2 being the tyrosine kinase responsible for PY increase in murine sperm, further investigation were required to elucidate the function of PYK2 in murine sperm during capacitation. In the previous section we showed that during *in vitro* capacitation PF573228 has a negligible effect on both early (pPKAs) and late (PY) events associated with capacitation. We therefore examine the specificity of both

inhibitors on the *in vitro* activity of His-tagged recombinant forms of FAK and PYK2. Recombinant kinases activity was monitored *in vitro* following radiolabeled $\gamma[^{32}P]$-phosphate from ATP to the peptide substrate Poly (Glu:Tyr). Increased concentrations of PF431396 negatively affect the activity of both recombinant FAK and PYK2 *in vitro* (Bhattacharya et al., 2012), with similar patterns of inhibition and IC_{50} for both kinases (Fig 2.13A). FAK and PYK2 on the other hand showed different sensitivity to PF573228, with FAK being affected at lower concentrations (Fig 2.13B). This observation, together with results showed in the previous paragraph, suggests that FAK is not involved in the regulation of PY during capacitation.

2.2.7 Analysis of the capacitation-associated phosphorylation events in PYK2$^{-/-}$ spermatozoa

PF431396 pointed out to PYK2 as the member responsible for PY increase during mouse sperm capacitation. However, pharmacological inhibitors are known to also affect off-targets kinase, therefore results above were not conclusive. Loss of function experiments using knock out animals gives the advantage of investigating the role of a particular protein in a given process, when the protein under investigation is lacking. We therefore decided to investigate whether the lack of PYK2 (*Pyk2*$^{-/-}$) affect the fertility profile of this murine model. *Pyk2*$^{-/-}$ animals we have been using for our experiments were characterized (Guinamard et al., 2000; Okigaki et al., 2003), and kindly donated by Dr. Assoian (Dept. of System Pharmacology and Translational Therapeutics, University of Pennsylvania). We first validated the model in our hand testing the presence of *fak* members in total

sperm lysate from both *Pyk2*$^{+/+}$ and *Pyk2*$^{-/-}$ animals. Sperm form *Pyk2*$^{+/+}$ exhibit a band correspondent to the molecular weight of PYK2 (~116kDa), but this band was not detected in sperm from *Pyk2*$^{-/-}$ animals (Fig 2.14A, higher panel). This observation validates the use of the knockout model for our studies and confirms the specificity of anti-PYK2 antibody in use. The analysis of cell lysates from *Pyk2*$^{-/-}$ animals showed no defect in the expression of the other focal adhesion kinase member FAK (Fig 2.14A, lower panel). Since this model appear to be suitable for studies on sperm capacitation, we next evaluated events associated with capacitation in this model: first we compared the phosphorylation of both PKA substrates and Tyrosine in epididymal spermatozoa from *Pyk2*$^{-/-}$ and *Pyk2*$^{+/+}$ animals incubated in capacitating conditions (15mM HCO_3^- and 5mg/mL BSA) for 60 minutes. Spermatozoa from *Pyk2*$^{-/-}$ animals surprisingly showed no differences in the levels of tyrosine phosphorylation (Fig 2.14B, left panel) compared to their wild type littermates (Fig 2.14B, right panel). No defects in the phosphorylation of the PKA substrates after 1 hour capacitation, was observed for *Pyk2*$^{+/+}$ animals (Fig 2.15, upper panel). These results were in sharp contrast with our hypothesis and showed that, at least in the case of mouse sperm, PYK2 is not the kinase responsible for the increase of PY associated with capacitation and is not necessary for capacitation and fertilization.

2.2.8 Effect of PF431396 and PF573228 on capacitation associated protein phosphorylation in spermatozoa from Pyk2$^{-/-}$ animals

We reasoned that, in animals that lacks *Pyk2*, a different kinase, is involved in the regulation of PY. We hypothesized that this unknown kinase belong to FAK family

and therefore is sensitive to PF431396. Consistent with this hypothesis, the increase of tyrosine phosphorylation in *Pyk2*$^{-/-}$ sperm was inhibited by PF431396 in a concentration dependent manner as showed for wild type littermates (Fig 2.15B) and CD1 sperm (Fig 2.6). The effect of PF431396 on PKA substrates phosphorylation upstream was negligible (Fig 2.15A). These observations opened the possibility that, when PYK2 is lacking, a compensatory mechanism regulates the capacitation-associated increase of PY and due to the ability of PF431396 to inhibit both *fak* members, this result highlight the other member FAK as the most likely candidate in compensating the lack of PYK2. If this is the case, *Pyk2*$^{-/-}$ animals should be also sensitive to the FAK specific inhibitor PF573228, as shown for PF431396. Caudal epididymal spermatozoa from both *Pyk2*$^{-/-}$ and *Pyk2*$^{+/+}$ animals were exposed to different concentrations of PF573228 and sperm lysates collected after 1 hour. Analysis of the phosphorylation profile associated with capacitation showed that PF573228 have no effects on pPKAs phosphorylation (Fig 2.16A). Surprisingly, the FAK specific inhibitor only induce a slight decrease in the levels of PY associated with capacitation also in *Pyk2*$^{-/-}$ animals (Fig 2.16B) but is not able to abolish the PY signals to non-capacitated levels, as shown for PF431396 (Fig 2.15B). Moreover, FAK protein levels are not increased in *Pyk2*$^{-/-}$ sperm compared to wild type (Fig 2.14A, lower panel). The results indicate that FAK, in absence of PYK2, is not the kinase that mediates the increase of PY and therefore is not compensating for the lack of PYK2. These observations were in conflict with our original hypothesis, and different from what we proposed in human, we showed that *fak* members were not involved in the regulation of PY during capacitation in mouse sperm. Although we ruled out the involvement of *fak* members on regulation of PY during capacitation, the

conserved effect of PF431396 on tyrosine phosphorylation in *Pyk2*$^{-/-}$ spermatozoa raised some concerns about the specificity of PF431396. The catalytic subunit of protein kinases is a conserved domain even in kinases belonging to different families, therefore although specificity claimers, potentially any pharmacological inhibitors of kinases could affect the activity of different off-target kinases (Bain et al., 2003; Bain et al., 2007; Davies et al., 2000). Results obtained in *Pyk2*$^{-/-}$ animals with PF431396, suggests that in murine spermatozoa, another kinases sensitive to PF431396 and that not belong to the *fak* family regulate increase of PY during capacitation

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CHAPTER 3

INVESTIGATING THE ROLE OF TYROSINE KINASE FER DURING SPERM CAPACITATION

3.1 FER tyrosine kinase: a novel candidate for studies on capacitation-associated PY in murine sperm.

Results in the previous chapter demonstrated that *focal adhesion kinases (fak)* do not play a unique role in regulating phosphorylation events associated with capacitation in murine sperm. Therefore, we concentrated our efforts in the identification of the tyrosine kinase(s) affected by the pharmacological tool in our hand during mouse sperm capacitation. We assume that PF431396 is affecting different kinase(s) not belonging to *fak* family. Recently using Tandem Mass Spectrometry (MS/MS), Chung *et* al revealed the identity of several proteins phosphorylated on tyrosine during capacitation in murine (proteins of the sperm head, signaling, structural and mithocondrial proteins as well as uncharacterized) (Chung et al., 2014). Of all the signaling proteins identified in the study, only few kinases were found to be phosphorylated on tyrosine and only one member was an appealing candidate for our research: the tyrosine kinase FER. Chung showed the auto-phosphorylation activation loop of FER is phosphorylated in murine capacitated sperm (Chung et al., 2014). FER is a non-receptor tyrosine kinase that belongs to the Fps/Fes family (*fps*, Fujinami poultry sarcoma; *fes*, Feline sarcoma)(Shibuya et al., 1980; Snyder and Theilen, 1969). First described as proto-oncogene, the activated version of this kinase is shown to induce cellular transformation (Roebroek et al., 1985; Wilks and Kurban, 1988). Fps/Fes and FER shared close structural similarity indicating they might cover similar, if not

redundant biological functions (Shibuya et al., 1980). Growth factors, cytokines and immunoglobulin induce the activation of FER and Fes, which have been implicated in the regulation of cell-cell and cell-matrix interactions (Greer, 2002). Somatic FER has a molecular weight of 94kDa (p94-FER) and its structure includes 4 different domains: 1. The carboxy-terminal **Kinase** domain mediates the phosphorylation of SH2-containing substrates on tyrosine (Maru et al., 1995); 2. **SH2** (Src-homology 2 domain) is a non-catalytic domain that both modulates the activity of the kinase and regulates protein-protein interactions (SH2 domains are known to mediate interactions with cortactin, EGF/PDGF receptors as well as insulin receptor 1) (Hjermstad et al., 1993; Iwanishi et al., 2000; Kim and Wong, 1995, 1998); 3. The presence of three **coiled-coiled** domains is also typical of FER, these domains regulate FER oligomerization (trimerization) and appear to also mediate the interaction of FER with adherent junctions (through p120catenin) (Craig et al., 1999); 4. The amino-terminal **FCH** domain was shown being implicated in the regulation of cytoskeletal re-arrangements, vesicular transport and endocytosis (Modregger et al., 2000; Qualmann and Kelly, 2000; Tian et al., 2000; Yeung et al., 1998).

The genomic locus of FER express a different splicing variant during the first meiotic prophase in pachytene spermatocytes, called FERT (Fischman et al., 1990). Different from somatic FER, FERT has a lower molecular weight of 51kDa (p51-FER) and this isoform looses both the FCH and coiled-coiled domains but retains the SH2 domain and kinase activity. Studies in rat spermatids revealed this protein appear to be important during spermatogenesis where is involved in the formation of acrosome-acroplaxome-manchette complex (Chen et al., 2003; Kierszenbaum et al., 2008).

The aim of our study was to investigate whether any of the isoforms of FER was present in murine spermatozoa and if this tyrosine kinase takes part in phosphorylation pathways conducive to sperm capacitation.

3.1.1 Expression of the Tyrosine Kinase FER in murine spermatozoa

In order to elucidate a possible role of FER in regulating phosphorylation events (pPKAs and PY) during sperm capacitation in mouse sperm, we decided to first examine the presence of the kinase in mouse sperm. Total protein extracts were collected from epididymal spermatozoa and proteins were separated by 1D gel electrophoresis, transferred to PVDF membranes and tested using antibodies directed against total levels of FER protein (anti- FER). As shown in Fig 3.1A, antibody against FER detected a band at ~50kDa, correspondent to the molecular weigh described for the testis specific isoform FERT (p51-FER) (Hazan et al., 1993). We therefore analyzed the solubility of FER to the non-ionic detergent Triton X-100 in capacitated spermatozoa and compare it with the solubility of the substrates phosphorylated on tyrosine. Results depicted in Fig 3.1B, show that the testis specific isoform of FER is present in both Triton X-100 soluble and insoluble fractions and it appear that during solubilization FERT is post-translationally modified. A detailed analysis of the same blot showed a faint band around 100kDa, suggesting that both testis-specific (p51-FER) and somatic (p94-FER) isoforms of FER are present in mouse sperm. Interestingly, different from what we shown for PYK2 and FAK, FER is present in both soluble and insoluble fraction, in some way correlating with PY signal in sperm, which is present only in the insoluble fraction of capacitated spermatozoa (Fig 3.1C).

This preliminary result confirmed the presence of the sperm specific variant FERT in murine sperm and showed that somatic FER is also present in mouse sperm, even if at lower concentration compared to the testis specific isoform. These observations represented a promising result and support our new working hypothesis of FERT playing a role in the regulation of PY during capacitation.

3.1.2 Effect of PF431396 on the activity of recombinant FER in vitro

We therefore decided to further investigate the function of FERT during capacitation in murine spermatozoa. In order to further support our hypothesis, we next examined the *in vitro* sensitivity of the commercially available recombinant form of FER to the *fak* inhibitor we have been using throughout this study, PF431396. Similar to the analysis of recombinant PYK2 and FAK in the previous chapter, the activity of FER was monitored following the transfer of γ^{32}P phosphate from ATP to the peptide substrate of FER Poly(Glu:Tyr). The activity of the kinase was tested in absence and in presence of different concentrations of PF431396 (0.1–30μM). Results in Fig 3.2 indicate that the activity of tyrosine kinase FER *in vitro* is sensitive to increasing concentration of PF431396, with an IC_{50} of ~1μM, and a complete abolition of FER activity at 10μM. Worth to notice, the pattern of inhibition of FER activity was comparable to the one observed for recombinant PYK2 *in vitro*. Our concerns about the specificity of PF431396 toward *focal adhesion members* were therefore confirmed. In addition, we showed that the activity of recombinant FER *in vitro* and the levels of PY during *in vitro* capacitation are sensitive to PF431396 with a similar IC_{50}/EC_{50} (~1μM) (Compare Fig 2.1 and Fig 3.2), supporting a possible role for tyrosine kinase FER in the

regulation of capacitation-associated PY. Despite this result support our hypothesis, does not conclusively demonstrate the function of FER during capacitation.

3.1.3 Analysis of capacitation-associated phosphorylation events in $Fer^{DR/DR}$ spermatozoa

In order to further investigate the role for FER during capacitation, we decided to analyze the capacitation events in a murine model that express non functional copies of *Fer* gene. Since murine models in which the *fer* gene is knockout are note viable, due to the importance of this kinase in regulating cell-cell and cell-matrix interactions, Dr. Greer at Queen's University developed a murine model that include an inactivating point mutation (D743R) in the catalytic loop of the kinase, $Fer^{DR/DR}$ (Craig et al., 2001). FER expression is not affected but in this model the kinase activity of the enzyme is abolished. The kinase inactivating mutation abolishes not only the activity of somatic FER but also affect the testis-specific isoform FERT (Craig et al., 2001). Therefore $Fer^{DR/DR}$ represents a suitable tool to investigate the function of FER during capacitation. We therefore decided to examine phosphorylation events associated with capacitation in animals lacking FER activity. Spermatozoa from $Fer^{DR/DR}$ animals and their wild type littermates ($Fer^{+/+}$) were incubated in modified TYH medium in capacitating conditions (15mM HCO_3^- and 5mg/mL BSA) and level of phosphorylation of PKA (pPKAs) substrates and tyrosine (PY) were checked after 60 minutes. When $Fer^{+/+}$ spermatozoa were capacitated *in vitro*, pPKAs levels were physiologically increased compared to the non-capacitated sperm population (Fig 3.3A, higher

panel), and similarly a normal increase in the level of tyrosine phosphorylation (PY) was observed (Fig 3.3A, lower panel). The increase of tyrosine phosphorylation levels is observed at 60 minutes and remains elevated for the following hour (Fig 3.3A, lower panel). On the other hand, when *Fer*$^{DR/DR}$ spermatozoa are incubated in capacitating conditions, although no defects in the levels of phosphorylation of PKA substrates (pPKAs) were detected (Fig 3.3B, higher panel), no increase in PY levels was showed compared to non-capacitated controls (Fig 3.3B, lower panel). In order to rule out the possibility that the lack of FER was inducing only a delay in the onset of capacitation-associated PY, we decide to extend the time of incubation in capacitating condition to 120 minutes. At all the time points examined (between 60 and 120 minutes) we did not observed any increase in the levels of PY compared to non-capacitated control (Fig 3.3B, lower panel). This observation represent a groundbreaking result toward the investigation of phosphorylation events associated with capacitation. We demonstrated for the first time that there is a unique kinase that regulates the onset of tyrosine phosphorylation associated with capacitation in murine spermatozoa and that this kinase is the testis-specific isoform of the tyrosine kinase FER (FERT). The absence of the tyrosine kinase does not have any effect on the activation of elements that are known being activated upstream, the cAMP-dependent phosphorylation of PKA substrates is indeed not affected in *Fer*$^{DR/DR}$. This result assign for the first time a role to a specific kinase (FERT) a unique role in inducing the onset of PY associated with capacitation.

3.1.4 Analysis of FER localization in $Fer^{DR/DR}$ animals

Results above confirmed that a band corresponding to the testis-specific isoform FERT, similar to tyrosine phosphorylated substrates, is found in the fraction insoluble to Triton X-100, suggesting a possible co-localization of FER with PY signal (Fig 3.1B, C). In order to gain further insight about the localization of FER in murine sperm, we performed an immunocytochemical analysis (ICC) of FER in *Fer*$^{DR/DR}$ animals. According to Craig *et al* the D743R mutation only affect the activity of the kinase without affecting its expression (Craig et al., 2001; Khajah et al., 2013; Kierszenbaum et al., 2008). Before analyzing FER localization by ICC, we decided to test the use of FER antibody by western blot on total sperm lysate from both *Fer*$^{DR/DR}$ and *Fer*$^{+/+}$. Surprisingly, the band correspondent to the testis-specific isoform of FER at 50kDa (FERT) was not detected in lysate collected from *Fer*$^{DR/DR}$ sperm (Fig 3.4A). On the other end the band correspondent to FERT is present in sperm from wild type animals of the same strain (*Fer*$^{+/+}$). We therefore reasoned that due to the difference in length and therefore structure between somatic and testis-specific FER, in the latter the D743R mutation not only affect the kinase activity but could also affect protein folding, somehow disrupting the FERT epitope recognized by the antibody in our hand. This result shows on one hand that in sperm from *Fer*$^{DR/DR}$ animals, not only the activity but also the presence of FERT is lacking and, on the other hand represent an unexpected validation of the antibody in use to further examine the localization of FER in murine sperm. Analysis of FER localization by ICC showed that tyrosine kinase FERT is localized in the tail of murine spermatozoa. The antibody both labels the mid- and the principal piece (Fig 3.4B). Semi quantitative analysis of FERT signal confirmed the difference showed by ICC, with a strong reduction in

signal corresponding to FER in *Fer*$^{DR/DR}$ compared *to* activity (Fig 3.4C). The result suggests co-localization of PY and FERT and further supporting a role for tyrosine kinase FER in regulating capacitation-associated tyrosine phosphorylation.

3.1.5 Analysis of FER and PY signaling through STORM microscopy

Immunocytochemistry results showed that FER signal is localized to the tail of murine spermatozoa (Fig 3.4B), a previously shown for proteins phosphorylated on tyrosine (Carrera et al., 1996; Leclerc et al., 1997; Lewis and Aitken, 2001; Si and Okuno, 1999; Urner et al., 2001). The use of light or confocal microscopy is restricted to the diffraction limit of ~200nm (which depend on wavelength and on the numerical aperture of the objective), therefore limiting the accuracy of spatial observations on a smaller scale. We therefore take advantage of Stochastic Optical Resolution Microscopy (STORM) to investigate the localization of FER and PY signals in murine spermatozoa. This technique allows the detection of single fluorescent molecules that are closer than the resolution limit, with higher resolution than confocal microscopy both in the *x-y* and the *z* planes. Moreover the analysis of tyrosine phosphorylation signal during murine sperm capacitation through STORM showed that PY signal is restricted to the tail and that in the principal piece of sperm flagellum is restricted to the axoneme (Chung et al., 2014). Results in Fig 3.5D, G and H were consistent previous studies showing the distribution of PY signal within the sperm flagellum (Chung et al., 2014), confirming the validity of STORM technique to investigate the localization of FER in sperm. When the 3D spatial distribution of FER was examined, the signal

corresponding to FER was detected throughout both the mid- and principal piece (Fig 3.5A) and the analysis of cross-sections of mouse sperm tail showed that FER signal, similar to PY was localized within 250nm from the center in the mid-piece (Fig 3.5A, B, D, E and G). Observations on the spatial distribution of FER in the principal piece showed that the signal is restricted to the axoneme and overlaps with signal corresponding to PY (Fig 3.5A, C, D, F and H), as shown in the mid-piece. These last observation, further confirmed previous ICC observations in $FER^{DR/DR}$ animals (Fig 3.4B), supporting the role of tyrosine kinase FER in regulating tyrosine phosphorylation during murine sperm capacitation.

3.1.6 Analysis of in vitro fertilization capability of $Fer^{DR/DR}$ animals

Once the localization of FER and its role in inducing tyrosine phosphorylation were established, we next decided to evaluate whether the absence of FERT affect the ability of spermatozoa to fertilize metaphase II arrested oocytes *in vitro*. Epididymal spermatozoa from *Fer*$^{DR/DR}$ and wild type animals were capacitated *in vitro* for 60 minutes and then co-incubated with metaphase II arrested oocytes collected from 8 weeks old CD1 females. After 4 hours of co-incubation, the sperm in excess was washed out and the oocytes that reached two polar bodies/two cells stages were counted as fertilized after 24 hour. Different from what was shown *in vivo* (Craig et al., 2001), spermatozoa from *Fer*$^{DR/DR}$ animals have a limited capability to fertilize oocytes *in vitro*: fertilization rate for wild type animal was ~35%, which is in accordance with data showed for the 129/SVJ murine strain (Kawai et al., 2006). On the other hand, *Fer*$^{DR/DR}$ spermatozoa

showed a very limited capability to fertilize metaphase-II arrested eggs *in vitro* (Fig 3.6). This observation suggests that FER activity and PY are required for fertilizing capability *in vitro*. The fact that *in vivo* these mice are fertile, suggest that during natural mating, either PY increase is not required for fertilization and therefore sperm without increased levels of PY are fertile or that during the transit through the female reproductive tract certain unknown mechanism compensate the lack of FER, inducing the increase of PY.

3.1.7 Analysis of functional parameters associated with capacitation in $Fer^{DR/DR}$ animals

The results above determined the role of FER in inducing the increase of PY and in regulating the fertilizing ability of murine sperm *in vitro*. We therefore decided to examine whether functional parameters associated with capacitation were affected in absence of FER and tyrosine phosphorylation. We therefore tested the motility profile of *Fer*$^{DR/DR}$ spermatozoa compared to wild type. Ejaculated mammalian spermatozoa once deposited into the vagina, acquire progressive motility in order to quickly swim-out from the seminal plasma (that contains several de-capacitating factors) and to finally begin their journey toward the oviduct. Once capacitated spermatozoa reach the utero-tubal junction, sperm must acquire a different patter of motility characterized by slow speed and higher propulsive force, the hyperactive motility: this motility pattern allow them to swim through the thick viscous mucus of Fallopian Tubes (Fraser, 1977, 2010; Suarez, 1996, 2008; Suarez and Osman, 1987). Epididymal sperm from *Fer*$^{DR/DR}$ and *Fer*$^{+/+}$ animals were incubated with 15mM HCO_3^- and 5mg/mL BSA for 60

minutes. After capacitation, an aliquot of sperm was diluted and motility was assessed using Hamilton Thorne Computer Assisted Semen Analysis (CASA), the analysis of different motile populations was measured through the software CASAnova (Goodson et al., 2011). Results in Fig 3.7A showed that, the percentage of *Fer*$^{DR/DR}$ motile spermatozoa is comparable to the motile population of *Fer*$^{+/+}$. This observation was expected as several studies showed that the onset of motility in ejaculated/epididymal sperm is dependent on sAC activation and cAMP pathway (Esposito et al., 2004). Surprisingly, when populations of spermatozoa with hyperactive motility were compared between *Fer*$^{DR/DR}$ and *Fer*$^{+/+}$, no difference between the two strains was observed (Fig 3.7B). This observation, although in argument with previous studies showing a relationship between PY increase and hyperactive motility (Mahony and Gwathmey, 1999; Si and Okuno, 1999), clearly showed that *Fer*$^{DR/DR}$ sperm although lacking tyrosine phosphorylation acquire hyperactive motility.

Studies on sperm capacitation in different species suggested a relationship between tyrosine phosphorylation and later functional events of capacitation as the ability to undergo to AR in response to extracellular agonists (Asquith et al., 2004). Results showed in the previous section, showed that the absence of FERT kinase does not have any impact on neither total nor hyperactive motility. We therefore decided to examine the ability of these cells to undergo to acrosome reaction *in vitro*. Epididymal sperm collected from *Fer*$^{DR/DR}$ and *Fer*$^{+/+}$ animals, as previously showed for other experimental methods, were capacitated *in vitro* for 60 minutes and then incubated with the Ca^{2+} ionophore A23187 [shown to induce Acrosomal Reaction *in vitro* (Balao da Silva et al., 2013)] or DMSO (as control) for the following 30 minutes. The percentage of acrosome reacted spermatozoa was

then assessed by PNA-Alexa488 staining. Although significant differences were observed between control and sperm treated with A23187, results in Fig 3.7C revealed no difference between *Fer*$^{DR/DR}$ and their wild type littermates in the level of reacted sperm in response to the A23187. As shown for motility, this observation is in sharp contrast with previous data in the literature showing a relationship between the occurrence of PY and Acrosomal Reaction (Asquith et al., 2004). Lack of positive correlation between tyrosine phosphorylation and the regulation of functional parameters in *Fer*$^{DR/DR}$ model highlight the possibility that different pathways present in murine sperm, bypass through certain unknown mechanisms the lack of PY in order to guarantee, the occurrence of capacitation. To summarize the evidences presented in this section, we demonstrated that tyrosine kinse FERT is present in mouse sperm, where regulates the occurrence of PY during capacitation. Although capacitated *Fer*$^{DR/DR}$ sperm *in vitro* showed hyperactive motility and acrosome reaction, their ability to fertilize metaphase arrested II eggs in vitro is reduced compared to Fer$^{+/+}$ sperm. On the other hand *Fer*$^{DR/DR}$ animal are fertile at mating, delivering viable and healthy offspring.

CHAPTER 4

INVESTIGATING THE KINETIC OF PHOSPHORYLATION EVENTS ASSOCIATED WITH CAPACITATION

4.1 Analysis of the Kinetics of phosphorylation events associated with Capacitation in murine sperm: the chemical-genetic approach.

Different from other signaling pathways, capacitation is not a rapid signaling event triggered by ligand-receptor binding. Mammalian spermatozoa need to reside in the female tract for a certain amount of time and to respond to different modulators promoting the occurrence of capacitation in a time-dependent fashion (Ickowicz et al., 2012). It is therefore fundamental to examine the time relationship between phosphorylation events and the occurrence of capacitation in murine spermatozoa.

In this third section, our effort is focused on understanding the kinetic of occurrence phosphorylation of PKA substrates and PY during capacitation in order to gain further insights about the time dependent mechanisms of regulation during capacitation. We have used both pharmacological and chemical-genetic strategies to elucidate the temporal requirements of these signals during sperm capacitation.

4.1.1 Time of occurrence of pPKAs and PY during capacitation in CD1 mice

The presence of ADCY10 and of the CαII catalytic subunit of PKA are known to be necessary requirements to guarantee the occurrence of capacitation in murine sperm (Esposito et al., 2004; Nolan et al., 2004), moreover the presence and

activation of PKA are also necessary to induce one of the hallmark of sperm capacitation, the increase of tyrosine phosphorylation (PY) levels (Visconti et al., 1995a; Visconti et al., 1995b). Further studies revealed that, although regulated by cAMP/PKA pathway, PY occurs on a different time scale compared to PKA activation (Arcelay et al., 2008; Visconti et al., 1995b). In order to set the ground for studies on kinetic of capacitation, we decided to first examine the time dependent activation of PKA and PY phosphorylation during capacitation. Epididymal spermatozoa, after collection were challenged with 15mM HCO_3^- and 5mg/mL BSA in order to stimulate capacitation. Spermatozoa were then collected at different time points (1 through 60 minutes) during capacitation and the level of phosphorylation of PKA substrates and PY was tested using anti-pPKAs and anti-PY antibodies. Activation of PKA and the increase of PY levels occur at different time: phosphorylation of PKA substrates already reach its maximum after 1 minute and it is shown to last for the following hour (Fig 4.1A); on the other hand, onset of tyrosine phosphorylation is delayed compared to pPKAs: a subtle rise in the level of PY is first observed after 30 minutes of incubation with a full increase observed at 45 minutes (Fig 4.1B), confirming previous observation in murine sperm (Arcelay et al., 2008). The delay in the rise of PY levels compared to pPKAs could be explained by either a slow accumulation of second messengers or by a down regulation of the activities of protein phosphatases, showed to be present in murine sperm (Signorelli et al., 2012). We therefore try to gain further insights on the possible mechanisms regulating the times of occurrence of these phosphorylation events using different approaches.

4.1.2 Effect of the broad inhibitor H89 on pPKAs and PY during capacitation

Before the advent of knockout genetic models, studies on sperm capacitation strongly relied on pharmacological approaches. Together with cAMP analogues and phosphodiesterases (PDE) inhibitors, pharmacological inhibitors of PKA were broadly used to demonstrate the requirement of cAMP/PKA pathway to induce sperm capacitation in different species. H89, is a small inhibitor that bind to the catalytic subunit of Protein Kinase A (PKA) (including the sperm-specific subunit Cα II) and studies on sperm showed its ability to negatively affect the activity of PKA with a good degree of specificity (Vijayaraghavan et al., 1997; Visconti et al., 1995b). We therefore decided to first examine the concentration-dependent effect of this inhibitor on pPKAs and PY phosphorylation: Epididymal spermatozoa, were challenged with different concentrations of H89 for 5 minutes in absence of HCO_3^- and BSA and then incubated in capacitating conditions in presence of the same concentrations of the inhibitor for the following hour. Phosphorylation of pPKAs and PY were tested as previously shown. As expected, H89 is effective in inhibiting capacitation-associated pPKAs (Fig 4.2A) and PY (Fig 4.2B) as previously showed, with an EC_{50} in murine spermatozoa of ~10μM. Consistent with previous data in the literature (Visconti et al., 1995b), when sperm are capacitated in presence of H89, the phosphorylation of PKA substrate is inhibited in a concentration-dependent manner and similarly, downstream events regulated by PKA activation as tyrosine phosphorylation are as well hampered. Our results were consistent with previous published data showing that during sperm capacitation, the late onset of PY is dependent on the early activation of PKA. Similar conclusions were also drawn by loss of function experiment using knockout models of sAC (Esposito et al., 2004) and Cα II (Nolan et al., 2004) as

well as gain of function experiments using exogenous cAMP agonists (Visconti et al., 1995b).

4.1.3 Limits of competitive kinase inhibitors and the chemical-genetic strategy

As described in the previous section, H89 is a broad competitive kinase inhibitor with high specificity against Protein Kinase A (PKA) and was used in different studies to elucidate the role of PKA in different systems (Krapf et al., 2010; Visconti et al., 1995b). Although several efforts over the years were made to improve the specificity of competitive kinase inhibitors, the conserved nature of the ATP binding site between different classes of kinases represent a limiting factor to develop potent inhibitors with a high degree of specificity (Dar and Shokat, 2011). In order to circumvent this issue Dr. Shokat developed a chemical-genetic strategy that allow to inhibit specific classes of kinases with minimum off-target effect: the principle is to render the kinase of interest sensitive to a specific inhibitor rather than create inhibitors that are specific for the kinase of interest. The method include the introduction of a point mutation within the gatekeeper amino-acid within the kinase catalytic domain of interest, the mutation causes a conformational change of the catalytic subunit that render the kinase sensitive to specific bulky inhibitors, without affecting the activity of the kinase (Bishop et al., 2000). Based on this strategy, Niswender identified a point mutation (M120A) that render the CαII catalytic subunit of Protein Kinase A sensitive to a series of C-3 derivatized pyrazolo[3,4-d]pyrimidine-based inhibitors (Niswender et al., 2002) and a knock-in murine model that express the CαII-M120A mutation only after Cre-mediated recombination *in vivo* was created by Morgan *et al* (Morgan et al.,

2008). The mutation did not affect the activity of PKA, therefore CαII-M120A animals were viable and fertile. Using a bulky pyrimidine based inhibitor (1NM-PP1) the activity of PKA can be specifically down regulated during capacitation without affecting the activity of other kinases present in sperm (therefore avoiding off-target effects). We therefore decided to test whether this tool is suitable to examine the kinetic of phosphorylation events during capacitation, together with the broad inhibitor H89.

4.1.4 Effect of 1NM-PP1 on pPKAs and PY in sperm from CαII-M120A animals

The first step towards the investigation of sperm capacitation kinetic was to test whether CαII-M120A model was suitable for our studies on sperm signaling. Spermatozoa from both CαII-M120A and their wild type littermates (CαII-WT) were collected by swim out from epididymis and then incubated in capacitating conditions (15mM HCO_3^- and 5mg/mL) for the following 60 minutes. Levels of pPKAs and PY phosphorylation were then tested using anti-pPKAs and anti-PY antibodies, as previously showed. After 60 minutes in capacitating condition both sperm from M120A and WT animals, in absence of 1NM-PP1 showed physiological increased level of both pPKAs and PY phosphorylation similar to CD1 sperm (Fig 4.3). This preliminary result confirmed that in sperm the activity of PKA is not affected by the presence of the mutation and that phosphorylation events associated with capacitation are not affected. We also tested the effect of different concentrations of 1NM-PP1 (3 – 30μM) on pPKAS in both CαII-WT and CαII-M120A animals. 1NM-PP1 negatively affect the phosphorylation of pPKAs in a concentration dependent manner only in CαII-M120A animals (Fig 4.3A, left

side), on the other hand CαII-WT are not sensitive to the same concentrations of 1NM-PP1 (Fig 4.3A, right side). The same blot was stripped and probed again with anti-PY antibodies (Fig 4.3B). Similar to what we previously shown for pPKAs, the increase of PY levels was also sensitive to increasing concentrations of 1NM-PP1, displaying similar EC_{50} (~3μM) (Fig 4.3B, left side), with no effect observed on PY signals in CαII-WT animals (Fig 4.3B, right side). These data confirmed that sperm from CαII-M120A animals are sensitive to 1NM-PP1, and that in presence of the inhibitor, phosphorylation events associated with sperm capacitation are depleted. We therefore use this engineered knock-in murine model to investigate the kinetic of phosphorylation events associated with sperm capacitation.

4.1.5 Effect of H89 and 1NM-PP1 on the in vitro activity of PKA from CD1 mice

Preliminary results in the previous section showed that when CD1 sperm were incubated with increasing concentrations of H89, pPKAs phosphorylation is reduced in a concentration dependent manner. Although the use of anti-pPKAs antibody is an established technique to monitor the activity of PKA during capacitation and in other signaling, it still remains an indirect method of analysis of PKA activity that only mirrors the kinase behavior. We therefore decided to test the effect of the pharmacological tools in our hand to (H89 and 1NM-PP1) on the activity of Protein Kinase A in homogenates collected from CD1 murine spermatozoa. Sperm Homogenates containing PKA were challenged with different concentration (1–100μM) of both H89 and 1NM-PP1. The transfer of radiolabeled phosphate from γ^{32}P-ATP to the peptide substrate of PKA *kemptide*

(Leu-Arg-Arg-Ala-Ser-Leu-Gly) was used to monitor the activity of PKA *in* vitro (Visconti et al., 1997). Results depicted in Fig 4.4 showed a concentration dependent effect of H89 on the *in vitro* activity of PKA from CD1 mouse sperm, with an IC_{50} of 3μM (Fig 4.4, black line).

To test whether 1NM-PP1 has any effect on the activity of CD1 PKA activity, we also examined the *in vitro* activity of PKA from CD1 sperm homogenates, in presence of increasing concentration of the other PKA inhibitor, 1NM-PP1. Different from H89, 1NM-PP1 has a negligible effect on the activity of PKA at the lower concentrations tested, with a slight decrease of PKA activity in presence of very high concentrations of 1NM-PP1 (Fig 4.4, gray line). These results are consistent with previous observations, where H89 was affecting the phosphorylation of PKA substrates during *in vivo* capacitation (Fig 4.2A). The effect of 1NM-PP1 is specific to CαII-M120A animals and does not affect the activity of PKA in sperm from CD1 mice.

4.1.6 Effect of 1NM-PP1 on in vitro activity of PKA in sperm from CαII-M120A animals

Results in Fig 4.3 showed that the pPKAs and PY phosphorylation levels associated with capacitation are negatively affected by increasing concentration of 1NM-PP1 in sperm from CαII-M120A but no effect is observed in sperm from CαII-WT littermates. To further confirm that the effect on pPKAs and PY is caused by a reduction in the activity of PKA, we decided to directly examine the activity of PKA in homogenates from CαII-M120A and CαII-WT in response to increasing concentrations of 1NM-PP1 (1–30μM). As shown in the previous section, the *in*

vitro activity of the PKA was monitored following the transfer of radiolabeled phosphate from γ^{32}P-ATP to the peptide substrate of PKA *kemptide.* Consistent with data obtained during *in* vitro capacitation (Fig 4.3), PKA activity in homogenates from CαII-M120A animals was sensitive to increasing concentrations of 1NM-PP1 with an IC_{50} of ~3μM, and is abolished at 10μM (Fig 4.5, gray line). On the other hand, PKA activity in homogenates from CαII-WT animals (not including CαII-M120A mutation) was not sensitive to 1NM-PP1, with a slight reduction of the activity observed only at 30μM (Fig 4.5, black line). This observation confirmed that CαII-M120A mutation render PKA sensitive to increasing concentration of 1NM-PP1, with a with IC_{50} ~2μM and 30μM are shown to reduce completely the activity of the kinase. As previously shown during *in vitro* capacitation the effect is specific to M120A animals, with no effect on sperm WT animals. We therefore confirmed that the chemical-genetic tool we have been using is suitable for studies on phosphorylation events associated with capacitation.

4.1.7 Effect of 1NM-PP1 on the kinetic of pPKAs and PY during capacitation

In order to elucidate the kinetic of phosphorylation events during capacitation, we used the CαII-M120A mouse model to investigate the possible relationship between the kinetic of activation of PKA and the onset of PY during *in vitro* capacitation. We previously showed that pPKAs and PY phosphorylation occur on a different time scale during capacitation (Fig 4.1), we were therefore interested in understanding whether the time-dependent activation of PKA affects the later onset of PY during capacitation. Epididymal spermatozoa from both CαII-M120A

and CαII-WT animals, after swim-out were incubated in capacitating conditions for 90 minutes, 30μM 1NM-PP1 was added to the incubation media at different time points (1 through 89 minutes) and at 90 minute, sperm lysates were produced and the levels of phosphorylation of pPKAs and PY were tested by western blot. The inhibitory effect of 1NM-PP1 on pPKAs in CαII-M120A is maintained even after the onset of capacitation and not only when sperm are treated with 1NM-PP1 prior to the incubation in capacitating conditions, indicating a good degree of specificity of the inhibitor (Fig 4.6A). Down regulation of pPKAs levels mediated by 1NM-PP1 occurs rapidly, as shown when the inhibitor is added at later time points (minute 89) (Fig 4.6A).

Consistent with previous results (Fig 4.3), the effect of 1NM-PP1 is specific to CαII-M120A and did not affect pPKAs phosphorylation in sperm from WT littermates (see Fig 4.7A). Interestingly, when levels of PY phosphorylation were examined in the same conditions, we observed a time dependent effect of 1NM-PP1: if 1NM-PP1 was added at early time points during capacitation (between 1 and 45 minutes), the onset of PY is inhibited. On the other hand, if 1NM-PP1 inhibitor was added at later time points (after 45 minutes), the inhibitor have a negligible effect on tyrosine phosphorylation and we still observed physiological increase in the levels of PY associated with capacitation (Fig 4.6B). As shown for pPKAs, the effect of 1NM-PP1 is specific to sperm collected from CαII-M120A animals with no inhibitory effect observed on PY for 1NM-PP1 at any of the time points examined in sperm from WT animals (see Fig 4.7B). These results indicate that in order to guarantee the onset of PY during capacitation, the activity of PKA upstream have to be sustained for at least 30/45 minutes to induce the onset of PY associated with sperm capacitation. Sustained activation of PKA is therefore a

key element in the regulation of the late increase in tyrosine phosphorylation during capacitation.

4.1.8 Effect of SKI606 on the kinetic of pPKAs and PY during capacitation in CD1 mice

As described in previous sections of this dissertation, besides the prominent role of sAC and cAMP, the full activation of PKA during capacitation also require Src-mediated down regulation of phosphatase activity and this mechanism of regulation was suggested being present both in murine and human sperm (Battistone et al., 2013; Krapf et al., 2010). These studies revealed that phosphorylation of PKA substrates and PY were inhibited in presence of the Src Family Kinase (SFK) inhibitor SKI606. We therefore decided to examine what was the impact of a time-dependent down regulation of the parallel pathway mediated by the SFK/phosphatases on pPKAs and PY phosphorylation during capacitation. Because the inhibition mediated by SKI606 is directed against SFKs and not directly on PKA, we analyze the effect of SKI606 on sperm collected from CD1. After swim-out, epididymal spermatozoa were incubated in capacitating conditions and the SKI6060 was added at different time points during incubation in capacitating condition, sperm were then harvested after 60 minutes and pPKAs and PY levels were tested by western blot, as previously described. Analysis of the data in Fig 4.8A showed that SKI606 abolish the phosphorylation of PKA substrates when sperm are challenged with the inhibitor prior to capacitating conditions or at early time points (0 to 10 minutes). On the other hand, when

SKI606 was added at later time points during capacitation (15 to 59 minutes), phosphorylation of PKA substrates was not affected and showed level similar to capacitated control. Consistent with results on pPKAs, the analysis of tyrosine phosphorylation in similar conditions confirmed a clear inhibition of PY when sperm were incubated with the inhibitor at early time points (1 to 15 minutes), and the effect is lost when sperm were incubated with SKI606 at later time points (Fig 4.8B). These observations, together with previous results showing time dependent effect of 1NM-PP1 in CαII-M120A animals further support the importance of the time dependent activation of PKA during capacitation: an early and sustained activation of PKA is a fundamental event of capacitation required for the late onset of PY. Furthermore, the parallel pathway regulated by SFKs and phosphatases and that control PKA full activation, also appear to be time dependent.

4.1.9 Effect of PKA inhibitors (H89 and 1NM-PP1) on pPKAs and PY on capacitated sperm

Besides the importance of an early activation of PKA during capacitation, our results also showed that the level of tyrosine phosphorylation (PY) are affected by the presence of PKA inhibitors (H89 and 1nM-PP1) only during early time points. We therefore hypothesized that when sperm reach full capacitation (at late time points) the upstream activity of PKA is no more required to induce PY phosphorylation downstream. In order to support our hypothesis, we took advantage of both pharmacological and chemical-genetic strategies to analyzed phosphorylation kinetics after full capacitation occurred: caudal epididymal spermatozoa are incubated in capacitating conditions for 1 hour. At 60 minutes,

30μM H89 were added to the capacitated sperm populations, protein extraction was then performed at different time points between 60 and 120 minutes. Sperm were harvested after 2 Hours, sperm lysates were produced and examined with anti-pPKAs and anti-PY antibodies western blotting. 30μM H89 even when incubated with capacitated spermatozoa, retain its ability to effectively inhibit pPKAs signal. Analysis of the kinetic of pPKAs inhibition show a fast inhibition mediated by H89 with a significant decrease in the level of pPKAs substrates already after 10 minutes and a full decreased observed after 30 minutes (Fig 4.9A). When the same blot was re-probed with anti-PY antibodies, any significant change in the level of tyrosine phosphorylation was observed: PY levels in fact remains elevated throughout the 2 hours incubation with no effect observed for H89 (Fig 4.9B). To further confirm these observations, a similar experiment was performed using sperm from CαII-M120A and CαII-WT animals, together with 10μM 1NM-PP1. Consistent with its effect during early time of capacitation, 1NM-PP1 affects the pPKAs in CαII-M120A sperm also when added after 1 hour. Furthermore 1NM-PP1 showed a faster kinetic of inhibition compared to H89 (Fig 4.10A). We reasoned that the more rapid effect of 1NM-PP1 compared to H89 could be due to the M120A mutation in CαII subunit of PKA. Similar to what observed for H89, 1NM-PP1 only affects pPKAs phosphorylation without having any impact of PY signal (Fig 4.10B). As previously showed, 1NM-PP1 does not affect neither pPKAs (Fig 4.11A) nor PY phosphorylation in CαII-WT animals (Fig 4.11B). Similar experiments were also performed extending the time of incubation (to 180 minutes) of capacitated sperm with both inhibitors, and similar results were obtained, with a rapid inhibition of pPKAs signal with no effect on PY (data not shown). Altogether these two sets of results suggest that, once the

capacitation process is fully accomplished, the inhibition of the cAMP/PKA signaling pathway has a negligible effect on PY phosphorylation and possibly on other end points of capacitation.

4.1.10 Time dependent effect of H89 during capacitation on fertilization rates in vitro

Results in the above section suggest that not only the presence of CαII catalytic subunit is required to promote phosphorylation events associated with capacitation (Nolan et al., 2004) but also that its time dependent activity appear to be a key element. Although the onset of PY during capacitation is considered a fundamental requirement for capacitation, we wanted to investigate whether time-dependent PKA activation affect the ability of spermatozoa to fertilize metaphase II arrested oocytes *in vitro*.

Epididymal spermatozoa, after swim out were incubated in capacitating conditions (TYH- 25mM HCO_3^- and 4mg/mL BSA) for 60 minutes. PKA inhibitor H89 was added either before incubation in capacitating conditions or during the last 10 minutes of incubation. Aliquots containing 10^5 sperm were then co-incubated with metaphase II arrested oocytes and 24 hours after, the fertilization rate was calculated. Similar to what observed for PY signal, when H89 is present throughout the whole capacitation, the ability of sperm to fertilize metaphase II arrested was strongly reduced compared to untreated control. On the other hand, when the PKA inhibitor was added only at late time points of capacitation, although a significant decrease in the fertilization rate was observed, the effect was much less pronounced and several spermatozoa were able to fertilize the

oocytes *in vitro* (Fig 4.12) despite the fact that the inhibitor affected phosphorylation of PKA substrates (Fig 4.9) This observation is consistent with previous results, showing that PY is not affected during late PKA inhibition and supports the hypothesis that PKA activity covers a fundamental role during early time points of capacitation but that later on is less necessary.

4.1.11 Reversibility of the effect of PF431396 on PY during sperm capacitation

As a next step toward the investigation of kinetic of phosphorylation signaling associated with capacitation, we decided to use the pharmacological inhibitor of PY (PF431396), described in previous chapters of this thesis, to further examine the relationship between PY and pPKAs phosphorylation. We previously showed that an early activation of PKA is necessary to induce capacitation-associated PY. We therefore wanted to investigate whether PKA is able to induce PY even after tyrosine phosphorylation was inhibited during the first hour of capacitation: epididymal spermatozoa were incubated in capacitated conditions in the presence of 10μM PF431396, concentration shown to affect PY with no effect on pPKAs. After 1 hour, PF431396 was washed out and spermatozoa were incubated in capacitated media for another 60 minutes. Sperm were collected at different time points, and pPKAs and PY phosphorylation levels were then examined by Western blot. The presence of PF431396 during the first our in CAP conditions has no effect on pPKAs, as shown in the second chapter of this dissertation (Fig 2.9). PY levels, after 60 minutes incubation in presence of PF431396 are depleted. When the inhibitor is removed from the incubation media, increased level of PY appear only if sperm are incubated in capacitating conditions for at

least 30 additional minutes or longer (Fig 4.13B). This observation was consistent with the kinetic observed for PY during *in vitro* capacitation in absence of any inhibitor (Fig 4.1B), with a later increase in PY levels that are then maintained throughout the whole incubation. Results showed above confirmed once again the different kinetic of onset of PY during capacitation, even when cAMP/PKA is fully active and suggest that although PKA induce the onset of PY, the activation is not direct but is mediated by a different player downstream of PKA and that might also regulate the time of occurrence of PY. Overall these data confirmed the key importance of time during capacitation, underscoring again difference compared to classical agonist-receptor signaling in other systems.

4.1.12 Kinetic of inhibition of PF431396 on pPKAs and PY phosphorylation in capacitated sperm

The recent data confirmed that the onset of PY during capacitation is a slow event that require long incubation (30/45 minutes) in presence of HCO_3^- and BSA, next we wanted to investigate the kinetic of inhibition of PY when both pPKAs and PY levels had reached their maximum levels. We took advantage of the specificity of PF431396 in inhibiting only the tyrosine phosphorylation without affecting PKA activity. Sperm, after swim out were incubated with 15mM HCO_3^- and 5mg/mL BSA for 60 minutes, in order to induce full increase of PY levels. At minute 60, 10μM PF431396 was added to capacitation media. Sperm were then harvested at different time points during the second hour of incubation and pPKAs and PY were tested by western blot. As expected PF431396 has no effect on the phosphorylation of PKA substrates at any of the time point examined (Fig 4.14A).

When added after 1 Hour capacitation, PF431396 is still able to induce a decrease in the levels of PY, but with a slow kinetic of inhibition (Fig 4.14B). Sperm indeed have to be incubated in presence of the inhibitor for at least further 30 minutes to induce a significant decrease in the level of PY, with a depletion of PY signal observed only after 60 minutes. Similar to PY increase, this result showed that also the kinetic of inhibition of PYF431396 on PY is a slow event that required a certain amount of time. This observation suggests that level of PY during capacitation does not only depend only on the activity of tyrosine kinase downstream of PKA, but could also require the down regulation of phospho-tyrosine phosphatases that maintain PY levels in a quiescent state in the early phase of capacitation. All these elements could cooperate together to guarantee the occurrence of capacitation at the appropriate time during mating.

CHAPTER 5

DISCUSSION

Capacitation is the ensemble of physiological changes that occurs to spermatozoa during their transit through the female tract and that render spermatozoa fully competent for fertilization (Austin, 1951). The independent discovery of capacitation by Dr. Austin (Austin, 1951) and Dr. Chang (Chang, 1951) represented a revolutionary step not only for studies on fertilization but also for setting the ground for the advent of Artificial Reproductive Techniques. The birth of Louis Joy Brown (Steptoe and Edwards, 1978) almost three decades later would not have been possible without thez works of Dr. Austin and Dr. Chang on sperm capacitation.

Capacitated sperm shows hyperactive motility to swim through and interact with the oviduct (Suarez and Ho, 2003) and has the ability to release their acrosomal content (Buffone et al., 2008; Buffone et al., 2014a). *In vitro* incubation in a normal culture media supplemented with HCO_3^- and BSA induce sperm capacitation (Buffone et al., 2014b) and molecular events associated with it: increase of membrane fluidity (Ickowicz et al., 2012) and of intracellular pH (pH_i) (Parrish et al., 1989; Zeng et al., 1996); hyperpolarization of the plasma membrane (Escoffier et al., 2012; Zeng et al., 1995); and increase in intracellular Ca^{2+} levels (Baldi et al., 1991; Carlson et al., 2007; Marin-Briggiler et al., 2003).

Sperm capacitation is associated with the activation of ADCY10 (Buck et al., 1999), an atypical soluble Adenyl Cyclase (sAC) stimulated by intracellular HCO_3^-. Increased cAMP levels induce the activation of PKA. The presence of

sAC and PKA is required for the onset of sperm capacitation (Esposito et al., 2004; Nolan et al., 2004). Also the increase of PY levels, another hallmark of capacitation, also depend on the activation of cAMP/PKA (Visconti et al., 1995a; Visconti et al., 1995b). Although correlated these events occur at different times during capacitation. Following studies confirmed the importance of PY in other species (Baldi et al., 2002; Ficarro et al., 2003; Harrison, 2004; Jagan Mohanarao and Atreja, 2011; Roy and Atreja, 2008; Signorelli et al., 2012). Although the identity of several substrates of phosphorylation were revealed (Ickowicz et al., 2012; Naresh and Atreja, 2015; Visconti et al., 2011), still there is a limited knowledge about roles and mechanisms regulating each of these substrates (Visconti et al., 2011).

Different studies focus on unveiling the identity of the tyrosine kinase(s) that induce PY increase during capacitation. Baker proposed a role for Src tyrosine kinase, based on tandem mass-spectrometry data showing its presence in murine sperm, on the interaction of Src with the CαII PKA subunit and on the sensitivity of PY to Src inhibitors (Baker et al., 2006). Similar results were shown also in human sperm (Varano et al., 2008). ON the other hand, later studies were in contrast with Baker's model and an alternative role for the kinase was proposed (Krapf et al., 2010): Src induces full activation of PKA down regulating phosphatases activity during capacitation (Krapf et al., 2010).

Recently a different candidate tyrosine kinase family was proposed to regulate PY in stallion: *focal adhesion kinases* (Gonzalez-Fernandez et al., 2013). We therefore used a pharmacological approach to investigate role of FAK members during capacitation in human. The *focal adhesion kinase* (*fak*)

members' inhibitor PF431396 induced a concentration-dependent decrease in the level of PY, without affecting the phosphorylation of PKA substrates and, only one member of the FAK family was detected in human: PYK2 (Battistone et al., 2014). PYK2 auto-phosphorylation/activation occurs after 2 hours incubation in capacitating conditions, and is sensitive to increasing concentration of PF431396 (Battistone et al., 2014). We also showed the effect of PF431396 is specific to events downstream of PKA (Battistone et al., 2014). As part of this manuscript as well as the one in horses, our group suggested that *fak* members have a role in regulating PY during capacitation. However, these results were based on pharmacological loss of function experiments. To be more conclusive, our hypothesis was tested in mouse spermatozoa, due to the possibility of using knockout mice models to test the function of our protein of interest.

We showed that in murine, the *fak* kinase PYK2 is not the responsible for PY increase during capacitation. *Pyk2*$^{-/-}$ animals showed no defect in the capacitation-associated levels of PY and a possible compensatory role played by the other *fak* member FAK, which we found to be present in murine sperm was also rule out due to a lack of inhibition of PF573228 (specific inhibitor of FAK) on PY levels.

Therefore, we search for other tyrosine kinase candidates. One of them was the tyrosine kinase FER, which is present in murine sperm as a testis-specific isoform (FERT). First, PF431396, which block the increase of tyrosine phosphorylation without affecting phosphorylation of PKA substrates during capacitation, was shown to block FER activity in vitro. Second, sperm from animals lacking FER activity showed no increase in the levels of PY during *in*

vitro capacitation. This genetic loss of function approach strongly suggested that FER was indeed the tyrosine kinase involved in the capacitation-associated increase of tyrosine phosphorylation. This is an important step-forward in understanding molecular events associated with capacitation. Although assigning an unambiguous role to FERT only represents a starting point to further investigate events that downstream of PKA induce PY increase. A direct mechanism of activation of FERT mediated by PKA is unlikely. PKA only induces phosphorylation on serine and threonine and FER activity depends on phosphorylation on the activation loop at Tyr715 (Greer, 2002). FERT retain the Src homology 2 (SH2) and the tyrosine kinase domains (Greer, 2002); SH2 domain mediates interactions between several classes of proteins, promoting both interaction with activators upstream and substrates downstream (Songyang et al., 1995). Phosphorylation/activation of FER was shown in response to different signals as EFG or PDGF (Craig et al., 2001; Kim and Wong, 1995). Although the identity of the kinases promoting FER phosphorylation was not clear, Src family kinases (SFKs) were candidates. In murine Src appears to be a connecting player between PKA and membrane hyperpolarization (Stival et al., 2015).

While *Fer*$^{DR/DR}$ animals lacking the increase of PY associated with capacitation showed no functional defects, physiological levels of total and hyperactive motility were similar to controls.

Given the high correlation between PY and capacitation, the lack of a functional phenotype associated with capacitation was surprising.

An interesting study in rat showed that during spermatogenesis FERT co-localize with cortactin (Kierszenbaum et al., 2008), a protein known to be

activated by tyrosine phosphorylation and to regulate actin dynamics (Head et al., 2003). Our results suggest that FERT cover no function during spermatogenesis in mouse, but future works should focus on a possible FERT-mediated phosphorylation of cortactin during capacitation and whether this has a possible outcome in controlling actin dynamics.

Similarly to PYK2, FAK tyrosine kinase includes a recognition motif for SH2 domain (Mitra et al., 2005b). Whilst we showed FAK has not a unique role in regulating PY is present in mouse sperm, a recent study in suspended hepatocytes showed that FER kinase promote the phosphorylation of FAK on different tyrosine kinase residues (Oh et al., 2009). It is therefore possible that FAK in sperm could function as substrate of FERT. Further investigations should be done to test this possibility.

The observation that *Fer*$^{DR/DR}$ showed no defects associated with AR, also argue with a previous study in rat, where FERT has been suggested to play a role during acrosome-acroplaxome complex development, ultimately contributing to the formation of a functional acrosome and of head shape (Kierszenbaum et al., 2008). Sperm collected from *Fer*$^{DR/DR}$ animals showed no defect in sperm morphology compared to WT littermates, arguing against a role of FERT during acrosome formation in mouse sperm. A plausible explanation could be due to different mechanism of regulation of acrosome development between different species.

We also examined the ability of *Fer*$^{DR/DR}$ sperm to fertilize metaphase II arrested oocytes *in vitro*. Our results indicate that sperm lacking FER kinase activity shows a strong decrease in their fertilizing ability compared to wild type. Few oocytes reach the 2-cell developmental stage when incubated with

Fer$^{DR/DR}$ sperm. On the other hand *Fer*$^{DR/DR}$ animals are fertile and produce viable and healthy offspring following natural mating (Craig et al., 2001). Analysis of our results *in vitro* is in accordance with the current paradigm of sperm capacitation, tyrosine phosphorylation *in vitro* is required for fertilizing ability (Naresh and Atreja, 2015; Visconti et al., 2011). *In vivo* results show that the fertilizing ability of these mice is not affected by the absence of FER activity despite the clear lack of PY. These data challenge the current paradigm of sperm capacitation.

This discrepancy between could be explained by separate mechanisms that control PY occurrence between *in vitro* fertilization and natural mating. To further elucidate the mechanisms underlying this difference, PY analysis of both WT and *Fer*$^{DR/DR}$ sperm recovered from the oviduct after copula will therefore be necessary, opening to possible new scenarios in the field of sperm capacitation. If *Fer*$^{DR/DR}$ sperm recovered from the oviduct does not show any increase of PY, the current paradigm of sperm capacitation will be changed, somehow downsizing the necessity of PY for full capacitation after mating. In this case, it will be necessary to investigate what are the different mechanisms that regulate capacitation *in vivo* and *in vitro*. Although unlikely, this is still an open possibility.

On the other hand if the recovered sperm showed a physiologic increase in the level of PY, it will be then fundamental to understand what are the mechanisms that *in vivo*, in absence of FERT cause the onset of PY. One possibility is that different kinases are activated between *in vivo* and *in vitro* capacitation. Different tyrosine kinases are present in sperm (Signorelli et al., 2012). Although the cAMP/PKA/FER pathway *in vitro* is the pathway of

election that induces PY, we cannot rule out the possibility that during *in vivo* capacitation other unknown pathways are activated. It is fundamental to remember that the occurrence of sperm capacitation *in vivo* is a time dependent event most likely regulated by several different factors encountered during the transit through the different female environments (vaginal, uterine and tubal). Finally, an alternative possibility is that PY gives a competitive advantage to certain spermatozoa during capacitation.

Although some of our results raise some concerns about the absolute requirement of tyrosine phosphorylation increase during sperm capacitation *in vivo*, we decided to investigate the *in vitro* kinetic of phosphorylation events during capacitation. We confirmed that the increase of PY depends on the cAMP/PKA signaling pathway. However, both phosphorylation types occur on a different time scale. Phosphorylation of PKA substrates is in fact a rapid event immediately induced when sperm are incubated in capacitating conditions (in presence oh HCO_3^- and BSA), PY increase on the other hand requires longer time of incubation in capacitating conditions. As part of this thesis, we showed that the continuous activation of PKA at the beginning of capacitation is required to induce the slow increase of PY. This result was consistent with previous publication (Morgan et al., 2008).

These observations suggest that the time of different phosphorylation events associated to capacitation is tightly coordinated.

We also showed the time of inhibition of cAMP/PKA is a key element to regulate *in vitro* fertilizing ability. Only the early inhibition of cAMP/PKA strongly affects the *in vitro* fertilization rate of this sperm.

The effect of different inhibitors added at later times of capacitation was also examined. We showed that, at these later time periods, inhibitors of PKA rapidly inhibit pPKAs signal but have a negligible effect on PY levels, even if incubated for longer periods. Furthermore when sperm are incubated with PY inhibitor after capacitation has occurred, we observed a slow kinetic of inhibition, with a full reduction only showed after 60 minutes of incubation. These results support our idea of tyrosine phosphorylation having a high correlation with occurrence of sperm capacitation. Other functional events that require longer times of incubation in capacitation media are the preparation for the acrosome reaction and the onset of hyperactive motility (Salicioni et al., 2007).

To explain our data, one possibility is that the late occurrence of PY during capacitation is due because of a slow (30/45 minutes) down-regulation of protein phosphatases activity.

In order to test this hypothesis, future works will include the use of phosphatases inhibitors to examine any changes in the kinetic of PY signals. As far of our knowledge, a limited body of work was done in investigating the time dependent PY phosphorylation, it will be therefore also beneficial to study the time course of phosphorylation of some of the substrates known being tyrosine phosphorylated as AKAP family kinases, CABYR or heat shock proteins. To fully understand the mechanism underlying late PY occurrence, it would also be fundamental to examine the time dependent activity of FERT during capacitation.

To conclude, we showed that in mouse sperm the testis specific isoform of tyrosine kinase FER, induce the increase of PY associated with capacitation.

Despite our finding showed that the increase of tyrosine phosphorylation levels associated with sperm capacitation is not an absolute requirement for capacitation and fertilizing ability, it is fundamental to underline difference between *in vitro* and *in vivo* capacitation. In vivo capacitation in fact include the transit of spermatozoa through different environment and, although not its occurrence is absolutely required, we cannot rule out the possibility that tyrosine phosphorylation give a competitive advantage to certain sperm subpopulations. If this is the case, it is reasonable that these competitive differences will only be evident when sperm capacitation is stimulated in competitive conditions.

CHAPTER 6

MATERIALS & METHODS

6.1 Materials

Reagents used for our project were obtained from various sources. Sodium bicarbonate ($NaHCO_3$), bovine serum albumin (BSA, fatty acid free), dibutyryl cAMP (Bt_2cAMP), 3-isobutyl-1-methylxanthine (IBMX), adenosine triphosphate (ATP), magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$), aprotinin, leupeptin, sodium orthovanadate ($NaVO_4$), *p*-nitrophenyl phosphate (NPP), β-glycero phosphate (βGP), β-mercaptoethanol, Poly (Glu:Tyr)(1:4), *Pisum sativum* agglutinin coupled to fluorescein isothiocyanate (PSA-FITC) (cat# L0770), gonadotropin from pregnant mare serum (PMSG) (cat# G4877), human chorionic gonadotropin (hCG) (cat# C1063), and PF431396 were purchased from Sigma-Aldrich (St Louis, MO). SU6656, H89 and Triton X-100 were obtained from Cayman Chemical (Ann Harbor, MI), okadaic acid (OA) and SKI606 were bought from LC Laboratories (Woburn, MA). PF573228 was obtained from Selleck Biochem (Houston, TX). HEPES was purchased from Roche (Basel, Switzerland) and paraformaldehyde (PFA) from Electron Microscopy Science (Hatfield, PA). Phosphorous 32 Radiolabeled adenosine triphosphate (γ-^{32}P-ATP) was obtained from Perkin Elmer (Waltham, MA). Mouse monoclonal anti-total PYK2 (clone 5E2) (Tang et al., 2002), rabbit polyclonal anti-PYK2PY^{402} (cat#3291), rabbit polyclonal anti-FAK (cat# 3285) (Battistone et al., 2014), rabbit monoclonal anti-phospho-PKA substrates (clone 100G7E) (anti-pPKAs) (Navarrete et al.,

2015) and mouse monoclonal anti-FER (cat#4268) (Greer, 2002) were purchased from Cell Signaling (Danvers, MA). Mouse monoclonal anti-phospho tyrosine (anti-PY) antibody (clone 4G10) (Navarrete et al., 2015) was purchased from EMD Millipore (Billerica, MA). Peroxidase/conjugated anti-mouse IgG were obtained from Jackson Immunoresearch (West Grove, PA) and peroxidase conjugated anti-rabbit IgG from GE Healthcare (Pittsburg, PA). AlexaFluor647-conjugated anti-mouse secondary antibody was purchased from Invitrogen (ThermoFisher Scientific) (Grand Island, NY). His-Tag recombinant PYK2, FAK and FER were purchased from Invitrogen (Grand Island, NY).

6.2 Methods

6.2.1 Mouse Sample Preparation

Animals were culled following the guidelines of the Animal Care and Use Committee (IACUC) at University of Massachusetts, Amherst. Caudal epididymal murine spermatozoa were collected from CD1 retired male breeders (Charles River Laboratories, Wilmington, MA), from young adult C57 (7–8 week-old mice), *Pyk2*$^{-/-}$ (Shen et al., 2011), *Fer*$^{DR/DR}$ (Craig et al., 2001) mice and their respective wild type littermates. After collection, each cauda epididymis was placed in 500 μl of a modified Krebs-Ringer medium (TYH's HEPES-buffered medium) (Kito and Ohta, 2008): NaCl (100 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), Glucose (5.5 mM), Pyruvic acid (0.8 mM), $CaCl_2$ (1.7mM), HEPES (20 mM (concentrations given in

parenthesis). Collection of sperm in this medium does not promote the onset of capacitation. Following 10 min incubation at 37°C (swim-out), tissue debris from epididymis were removed, and cells suspension were adjusted to a final concentration of $1–2\times10^7$ cells/ml in non-capacitating medium before dilution of four times in the corresponding medium. Sperm were then incubated at 37 °C for the length of times indicated in each r experiment, either in the absence (NON) or in the presence of 15 mM $NaHCO_3$ and 5 mg/ml BSA (CAP). The effect of various inhibitors was tested on sperm incubated under the capacitating conditions described above together with increasing concentrations of the various inhibitors used, as indicated for each experiment. For time dependent analysis of phosphorylation events associated with capacitation (pPKAs and PY), sperm were incubated in capacitating condition in presence or absence of various inhibitor for different amount of time as described for each experiment in the results. For *in vitro* fertilization (IVF) assays, sperm were first incubated in modified TYH medium (without HEPES) containing 25 mM $NaHCO_3$ and 4 mg/ml BSA. The medium was previously equilibrated in a humidified atmosphere of 5% CO2 at 37°C (Wertheimer et al., 2008).

6.2.2 Human Samples Preparation

15 healthy donors (20–35 yearls old) with no fertility issues were included in the study. Ejaculated semen was collected by masturbation and all samples were treated in accordance to World Health Organization reccommendations (Organization, 2010). After liquefaction motile spermatozoa were collected by

standard swim-up technique in absence of HCO_3^- and diluted to a concentration of 1×10^7cells/ml. Sperm were then incubated in modified Biggers, Whitten and Whittingham medium (BWW) in absence (NON) or presence (CAP) of bicarbonate at 37°C, 5% CO_2. BWW use in the study include: $NaHCO_3$ (25mM), NaCl (94.5mM), KCl (4.8mM), $CaCl_2$ (1.7mM), KH_2PO_4 (1.17mM), $MgCl_2$ (1.22mM), Na-Pyruvate (0.3mM), Na-lactate (25.7mM), glucose (5.5mM) and HEPES (10mM) supplemented with BSA (2.6% p/v). Time of incubation vary between experiments, as described in the figure legend.

6.2.3 SDS-PAGE and Immunoblotting

Human and murine sperm were harvested by centrifugation, washed in 1 ml of phosphate buffer solution (PBS), re-suspended in Laemmli sample buffer (Laemmli, 1970), boiled for 4 min and centrifuged one more time. Supernatants were then supplemented with 5% β-mercaptoethanol and boiled again for 3 min. Protein extracts equivalent to 1–2 10^6 sperm were loaded in each lane, subjected to SDS–PAGE and electro-transferred to PVDF membranes (Bio-Rad, Waltham, MA) at 250 mA for 90 min on ice. Immunoblotting with either anti-pPKAS antibodies (clone 100G7E) or with anti-PY (clone 4G10) was carried out as previously described (Krapf et al., 2010). For immunodetection of murine PYK2, PYK2-PY^{402}, FAK and FER, PVDF membranes were blocked with 5% fat-free milk in TBS containing 0.1% Tween 20 (T-TBS) and the respective antibodies used at 1:1,000 final concentration. Secondary antibodies were diluted in T-TBS (1:10,000) and an

enhanced chemioluminescence ECL plus kit (GE Healthcare) was used for signal detection. Hexokinase, a protein known being constitutively phosphorylated on tyrosine residues in mouse sperm, was used as a loading control (Porambo et al., 2012; Visconti et al., 1995a). When necessary, PVDF membranes were stripped at 65 °C for 20 min in 2% SDS, 0.74% β-mercaptoethanol, 62.5 mM Tris, pH 6.5, and then washed six times for 5 min each in T-TBS prior to re-probing with a different antibody. For the analysis of western blot results, molecular masses are expressed in kDa. Image analysis was conducted using ImageJ (http://imagej.nih.gov/ij). The vertical bar on the left side of the each blot represents the regions of interest (ROI) used for quantification. In all cases, results were normalized arbitrarily considering the CAP lane as the unit value. Images shown are representative of experiments repeated three times (n=3) using different animals.

6.2.4 Analysis of Kinases Solubility

The solubility of the various tyrosine kinases (PY, PYK2, FAK and FER) under investigation `was assessed using the non-ionic detergent Triton X-100. After 10 minutes swim-out in TYH-HEPES media, sperm samples were pelleted by centrifugation at 1,500 RPM for 5 minutes and re-suspended in 1% Triton X-100/PBS buffer containing protease and phosphatase inhibitors for 30 min on ice. Both supernatant (Triton X-100-soluble fraction) and the remaining pellets (Triton X-100-insoluble fraction) were produced by centrifugation at 14,000 RPM at 4°C for 2 min and then examined by western blot. Figures represents experiments repeated at least three times (n=3) using different animals.

6.2.5 Recombinant Kinase Assay

The activity of GST-tagged recombinant kinases PYK2, FAK and FER (60ng/reaction) were assayed in a buffer containing: 25 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μM aprotinin, 10 μM leupeptin, 100 μM $NaVO_4$, 5 mM Nitro Phenyl Phosphate (NPP), and 40 mM β-Glycero phosphate (βGP), with 40 μM ATP and 100 μM Poly(Glu:Tyr)(1:4) used as substrate for 20 minutes in presence of 1 μCi of γ-^{32}P-ATP. Reactions were stopped adding 60% trichloric acid (final concentration 30%), the samples were then chilled on ice for 20 minutes and centrifuged at 10,000 X g for 3 minutes. Thirty microliters (30 μL) of from each reaction tube were transferred to phosphocellulose paper (Whatman P81, Millipore, Bedford, MA) (2 cm x 2 cm). Papers were then immediately incubated with 5 mM phosphoric acid and washed seven times using the same solution. After washes, papers were rinsed for 5 min with 100% ethanol and air-dried (15/20 min). Incorporated counts (cpm) were evaluated using a Beckman counter LS6500 (Beckman, Brea, CA) in vials containing the spotted papers together with 2.5 mL of scintillation fluid. Values represent average of three independent experimental replicates (n=3).

6.2.6 Analysis of in vitro activity of PKA

The activity of PKA was measure using previously described methodology (Hao et al., 2004; Visconti et al., 1997), After PKA extraction from mouse

spermatozoa (CD1, CαII-WT and CαII-M120A), its activity was measured quantifying the amount of ^{32}P transferred from γ-^{32}P-ATPs to the peptide substrate of PKA *kemptide* (Leu-Arg-Arg-Ala-Ser-Leu-Gly). The activity of PKA was then assayed in absence and in presence of different concentration of various inhibitors, as described in the results section.

6.2.7 Human Sperm Motility Assay

Capacitated spermatozoa were loaded on pre-warmed 20μm slides (Leja Slide, Spectrum Technologies, Healdsburg, CA, USA), placed on pre-warmed microscope stage and examined using CEROS computer-assisted semen analysis (CASA) (Hamilton Thorne Research, Beverly, MA) (Mortimer et al., 1998). A minimum of 20 field and 200 cells for each experimental group were analyzed. Parameters taken into account include the following: average path velocity (VAP, mm/s), curvilinear velocity (VCL, mm/s), straight linear velocity (VSL, mm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, mm) and straightness (STR, %) as previously described (Battistone et al., 2013). Analysis of progressive and hyperactive motility was based on already established criteria (Mortimer and Mortimer, 1990). Four independent experiments per treatment were performed (n=4).

6.2.8 Murine Sperm Motility Assay

Sperm suspensions (30 μl) were loaded into pre-warmed chamber 100 μm

slides (Leja slides, Spectrum Technologies, Healdsburg, CA) and placed on a pre-warmed (37°C) microscope stage. Sperm movements were examined using the CEROS computer-assisted semen analysis (CASA) system (Hamilton Thorne Research, Beverly, MA). Default settings used for the analysis of murine sperm included the following: frames acquired: 90; frame rate: 60 Hz; minimum cell size: 4 pixels; static head size: 0.13–2.43; static head intensity: 0.10–1.52; and static head elongation: 5–100. Sperm with hyperactive motility, defined as motility with high amplitude thrashing patterns and short distance of travel, were sorted using the CASAnova software with already established criteria (Goodson et al., 2011). At least 20 microscopy fields corresponding to a minimum of 200 sperm were analyzed in each experiment.

6.2.9 Mouse Eggs Collection and IVF Assays

6–8-week old CD1 and C57BL/6 female mice (Charles River Laboratories, Wilmington, MA) were super ovulated using PMSG and hCG. Metaphase II-arrested eggs were then collected 12 Hours after hGC intra-peritoneal injections as previously described (Wertheimer et al., 2008). Cumulus-oocyte complexes (COCs) were transferred to a dish with 500 μl of TYH media (without HEPES) previously equilibrated in an incubator with 5% CO_2 at 37°C. Murine sperm (2.5×10^5 cells for each experiment) were incubated in capacitating condition for 1h and 20 minutes and then co-incubated with metaphase II arrested oocytes (25–40 eggs per experiment) for 4 h. The eggs were washed to remove sperm in excess and moved to fresh TYH media,

fertilization rate was then evaluated 24 h post-insemination. Eggs were considered fertilized, when cells were able to reach the two-cell stage of development. Values represent average of three independent experimental replicates (n=3), using different animals

6.2.10 Genotype of Pyk2$^{-/-}$ and Fer$^{DR/DR}$

Genotype of *Pyk2*$^{-/-}$ was performed based on Shen *et al* (Shen et al., 2011) using the following primers *Pyk2* wild type forward, 5'-GGAGGTCTATGAAGGTGTCTACACGAAC-3'; *Pyk2* mutant forward, 5'-GCCAGCTCATTCCTCCCACTCAT-3'; *Pyk2* reverse, 5'-CCTGCTGGCAGCCTAACCACAT-3'. Genotype of Fer$^{DR/DR}$ was evaluated as described by Craig et al (Craig et al., 2001).

6.2.11 Genotype of CαII animals

The following primers were used for the genotype analysis of CαII animals: lox1 Forward: 5'-TCTTTGCTCAGGGCGGACTG-3'; CaREC10-2 Reverse: 5'-ACCAGGAGGATTGTGAGCCTAAGAC-3'. The analysis of the genotype for each animal was performed as previously described (Morgan et al., 2008).

6.2.12 Indirect Immunofluorescence

Sperm were collected by swim-up in TYH-HEPES medium, were washed once and re-suspended in PBS (1–2 10^6 sperm/ml), and seeded on coverslips. Sperm was air-died and fixed with 4% PFA freshly made in PBS for 10 min at room temperature, washed with PBS, and permeabilized for 5 min with 0.5% Triton X-100. After permeabilization, sperm were blocked using 10% BSA/T-PBS for 1 h at room temperature and then incubated with the anti-PY antibody (1:1000) diluted in 1% BSA/T-PBS overnight at 4 °C. After primary incubation, sperm were washed thoroughly with T-PBS to eliminate antibody in excess and incubated with the corresponding Alexa 555-conjugated secondary antibody (1:200) diluted in T-PBS containing 1% BSA for 1 h at room temperature. Incubation with the secondary antibody was followed by four washes in T-PBS. Slides were then mounted using Vectashield H1000 (Molecular Probes, Eugene, OR). Epifluorescence microscopy was performed using a TE300 Eclipse microscope (× 60) (Nikon). Differential Interference Contrast (DIC) images were taken in parallel and served as control for sperm morphology. Negative controls using either normal serum or secondary antibody alone were used to check for antibody specificity.

6.2.13 Sample Preparation and Immunostaining for Super-resolution Microscopy

After capacitation, sperm were centrifuged at 800 X g for 5 min., the pellet was fixed in 4% paraformaldehyde for 10 min at room temperature and then centrifuged at 800*xg* for 5 min. The cell pellet was resuspended in PBS and 50 µl of this suspension were placed onto polylysinated coverslips and allowed to set for 10 min. Non-bound cells were removed by washing with PBS. Sperm cells were then permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 3% BSA in PBS for 1 h at room temperature. Primary antibodies were diluted in 1% BSA, and incubated with cells overnight at 4°C in a humidified chamber. Anti-PY antibody (clone 4G10; final concentration 1:1000), and anti-FER (clone 5D2) at a dilution 1:50 were used. After primary antibody incubation, cells were washed with T-PBS (0.5% Tween-20 in PBS) three times for 5 min each, and then stained with Alexa Fluor 647-conjugated anti-mouse secondary antibody diluted in PBS containing 1% BSA (1:1000 for pY and 1:500 for FER) at room temperature for 1 h. Cells were then washed with T-PBS three times for 5 min each and incubated with 50 nm gold nanoparticles (Nanopartz, Loveland CO) that were used as fiducial markers for drift correction. After washing, cells were immediately mounted. The STORM imaging buffer was 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10% glucose, 0.56 mg/ml glucose oxidase, 34 □g/ml catalase, 10% glucose, and 1% β-mercaptoethanol.

6.2.14 STORM Imaging

Image stacks were acquired using Andor IQ 2.3 software in a custom-built microscope equipped with an Olympus PlanApo 100×/1.45 objective (Weigel et al., 2011). Alexa Fluor 647 was excited with a 638 nm laser (DL638-050, CrystaLaser, Reno, NV) under continuous illumination. Initially the photo-switching rate was sufficient to provide a substantial fluorophore density. However, as fluorophores photo-bleached, a 405 nm laser was introduced to accelerate the photo-switching rate. The intensity of the 405 nm laser was adjusted to control the density of active fluorophores. A cylindrical lens with a focal lens of 1 m was placed in the detection path in order to achieve 3D resolution as previously described (Huang et al., 2008). The images were acquired in a back-illuminated electron-multiplied charge coupled device (EMCCD) camera (Andor iXon DU-888) operated at −85°C at a rate of 23 frames/s. Fifty thousand (50,000) frames were collected to generate a super-resolution image.

6.2.15 Super-resolution Image reconstruction and analysis

Single-molecule localization, drift correction using gold fiducial markers and reconstruction were performed with ThunderSTORM, an ImageJ plugin (Ovesny et al., 2014). In order to find the molecular radial distributions, we selected regions of interest of the flagellum that were found to lie in a straight line, and the center of the flagellar cross section was calculated in MATLAB with a custom written algorithm. The coordinates of the localized molecules

were then transformed into cylindrical coordinates (Chung et al., 2014), from which the distribution of radial localization was computed.

6.2.16 Statistics

Statistical analyses were performed using the software Infostat 2011 (Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., TabladaM., Robledo C.W. InfoStat version 2011. Grupo InfoStat, FCA, Universidad Nacional de Cordoba, Argentina). All data were verified to accomplish the parametric assumptions: homogeneity of variances and normality. For Western blotting experiments, experiments were repeated at least 3 times and comparison between groups was performed by analysis of variance (ANOVA) in blocks. Data from each Western analysis was considered as a block and all treatments were applied to it. When the ANOVA tests were significantly different between groups ($p<0.05$), multiple comparisons were performed by the Tukey's test. For the IVF experiments, data was analyzed by the Chi-square test. P values ($p< 0.001$, $p<0.01$ or $p<0.05$) were considered to be significant as indicated in the Figure legends.

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