PLASMA MEMBRANE Ca^{2+} ATPase1 (PMCA1) AND INTERACTING PARTNERS: PRESENCE IN CARGOS OF EXOSOMES FROM FEMALE FLUIDS AND ITS UPTAKE BY MURINE SPERM

by

Pradeepthi Bathala

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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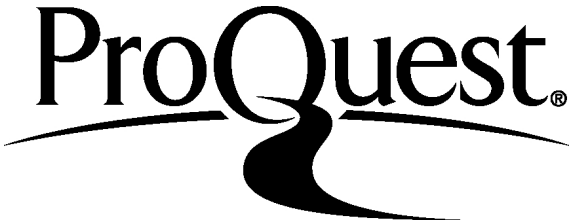

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ABSTRACT

Plasma membrane Ca^{2+} ATPase4 (PMCA4) is the major Ca^{2+} efflux pump in murine sperm. Its deletion leads to infertility in male mice, due to flagellar abnormalities and motility defects. PMCA4 is not only crucial in males but is expressed in females where oviductal sperm, prior to fertilization, acquire additional amounts to efflux increased Ca^{2+} resulting from hyperactivation and the acrosome reaction. However, deletion of *Pmca4* in female mice, has no effect on female fertility, despite Ca^{2+} efflux requirement which also occurs for oviductal ciliary action that is necessary for oocyte transport. As PMCA1 is the other ubiquitous PMCA isoform, it was predicted that it might act as a compensatory mechanism for PMCA4 loss in the female reproductive tract. Here I show that PMCA1 is expressed in tissues, luminal fluids and extracellular vesicles (EV) of the female tract, with increased levels seen in the oviduct during proestrus/estrus when mating occurs. Western analysis showed that PMCA1 is significantly upregulated in *Pmca4* KO oviductal EVs (oviductosomes, OVS) compensating for *Pmca4*'s absence.

Transmission Electron Microscopy (TEM) of pellets obtained from ultracentrifugation of vaginal luminal fluid revealed the presence of EV, dubbed "Vaginosomes". These were confirmed as exosomes/microvesicles based on size and biomarkers such as CD9 and HSC70, and were also visualized in situ using TEM of

tissue sections. Vaginosomes were shown to contain PMCA1 via Western analysis. Western also showed the presence of nNOS (neuronal Nitric Oxide Synthase) and CASK (Ca^{2+}/CaM-dependent Serine Kinase) in OVS, interacting partners of PMCA4 in sperm. Co-immunoprecipitation studies showed that these partners also interact with PMCA1 in oviductosomes.

For the first time, PMCA1 was localized on sperm and shown to co- localize with PMCA4 on the head, midpiece and proximal principal piece. Further, PMCA1 in OVS was shown to be delivered to the sperm surface *in vitro* following co-incubation of sperm in OLF. Further, the level of acquisition of PMCA1 by sperm from OLF was dependent on capacitation states, and is consistent with the presence of PMCA1 on the inner acrosomal membrane, where PMCA4 is also located. Our findings demonstrate that PMCA1 is the compensatory mechanism that accounts for the fertility of *Pmca4* KO females and reveals for the first time the existence of EV in the vaginal luminal fluid.

Chapter 1

INTRODUCTION

1.1 Journey of Sperm during Maturation

Mammalian sperm, produced in the testis, perform the task of fertilizing oocytes. To attain fertilizing ability, sperm undergo three different stages of maturation: 1) Spermatogenesis 2) Epididymal maturation and 3) Capacitation in female reproductive tract (**Fig. 1**) (Reid et al., 2011)

1.1.1 Spermatogenesis

Spermatogenesis is a complex process of producing large numbers of spermatozoa in the testis (Hess and de Franca, 2008). During this process, spherical shaped spermatids are morphologically transformed into elongated spermatozoa consisting of three regions: 1) **Head** comprising of an acrosome with a matrix of enzymes, a nucleus with condensed chromatin in which histones are displaced by protamines for DNA compaction, and cytoplasm with cytoskeletal structures; 2) **Midpiece** with mitochondria, and 3) **Principal piece** with dense cytoskeleton which is used for locomotion (Gadella and Luna, 2014). Due to the presence of very little

cytoplasm (Jones, 1989; Cooper, 1995; Jones, 1998) and compacted nucleus in the head, sperm are transcriptionally inactive (Reid et al., 2011).

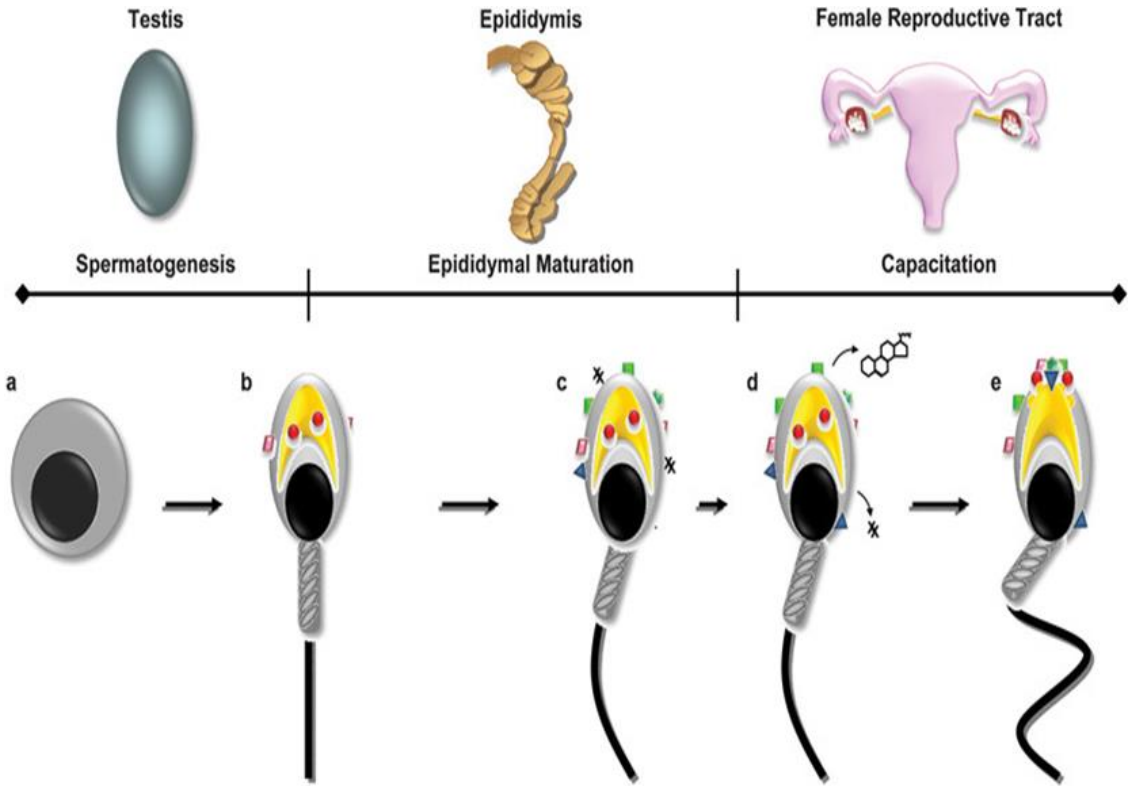


Figure 1.1 Diagrammatic representation of three stages of sperm maturation. Sperm undergoes three stages of maturation: spermatogenesis occurs in testis where sequence of cell divisions and morphological alterations convert **(a)** round spermatids into **(b)** morphological mature sperm, this includes DNA condensation, mitochondria at midpiece and formation of flagella. Following spermatogenesis, spermatozoa undergo epididymal maturation in which sperm enter into epididymis and **(c)** obtain proteins from luminal fluids that are necessary for fertilization. Also, they acquire decapacitated factors to prevent the sperm undergoing premature capacitation. After this, sperm enter into the female tract, where they begin the final stage of maturation called capacitation. During capacitation, sperm undergo **(d)** membrane surface modifications such as efflux of cholesterol and loss of decapacitation factors. Following this, **(e)** sperm interact with the luminal fluids and obtain necessary proteins to become competent for fertilization. (Adapted from Reid et al., 2011)

1.1.2 Epididymal Maturation

When sperm leave the testis, they are morphologically mature but functionally incompetent due to the lack of ability for both forward progressive motility to reach the oocyte and for binding to its zona pellucida during fertilization (Jones, 1989; Cooper, 1995; Jones, 1998; Reid et al., 2011). Sperm obtain functional maturity when

they enter into the long convoluted tubule called the epididymis which consists of three regions: the caput (head), corpus (body) and cauda (tail) (Caballero et al., 2010). In the epididymis, sperm are exposed to large number of essential proteins that are present in the luminal fluid (Cooper, 1998; Jones, 1998). This fluid, the epididymal luminal fluid (ELF), is secreted by epididymal epithelium. During epididymal transit, sperm progress from the caput to cauda and undergo a series of events such as biochemical alterations (Reid et al., 2011) and acquisition of molecular components from ELF (Jones, 1998; Cooper 1998). These components are critical for sperm maturation and for the acquisition of motility and fertilizing ability.

1.1.3 Capacitation in the Female Reproductive Tract

Sperm enter the female reproductive tract and after travelling through the uterus, they reach the oviduct. In the distal segment of oviduct, sperm are stored in the reservoir where lectins present on the sperm membrane bind to fucose residues of the oviductal epithelial membrane. This interaction allows the sperm to become attached to the oviduct (Coy et al., 2012). During ovulation, bound sperm are released from reservoir due to excessive amounts of oviductal secretion, and hyperactivated motility which is characterized by high amplitude asymmterical swimming (DeMott and Suarez, 1992). When hyperactivated sperm are released from the oviductal epithelium, they travel to the fertilization site, the ampullary isthmic junction (AIJ) **(Fig. 2)** (Rodriguez-Martinez, 2007) where they meet oocytes which are transported by

oviductal cilia from the ampulla. During this event, there is an increase in intracellular Ca^{2+} levels in sperm and this is pivotal for hyperactivated motility, the acrosome reaction, and for their ability to penetrate the zona pellucida of oocytes.

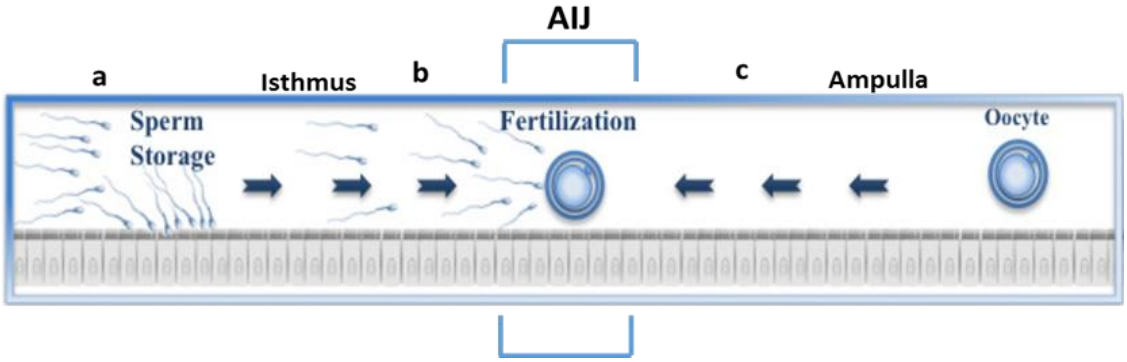


Figure 1.2 Diagram showing the three regions of oviduct: **a)** Sperm stored in reservoir interacting with oviductal epithelium **b)** Sperm released from reservoir moves through Isthmus to reach AIJ while **c)** oocytes from ovary are carried to AIJ through ampulla by ciliary action. (Image adapted from Almiñana, 2015)

Following capacitation, hyperactivation, and acrosome reaction, the increased cytosolic Ca^{2+} must be returned to basal levels to prevent sperm toxicity. In order to regulate the intracellular Ca^{2+} concentration, sperm possess three efflux mechanisms: sodium – calcium exchanger (NCX), mitochondrial uniporter and plasma membrane calcium ATPase (PMCA4) pump. Of these three Ca^{2+} efflux mechanisms, PMCA4 is

the most effective mechanism in sperm, with its high affinity for extrusion of Ca^{2+} from the cytosol (Strehler et al., 2007).

1.2 Plasma Membrane Ca^{2+} ATPase Pumps and their Significance

PMCAs are membrane bound P - type ion transporting ATPases (Pedersen et al., 1987) with molecular weight ranging from 125 to 140 kDa (Strehler et al., 2007). In mammals, there are four isoforms of PMCA (PMCA 1, 2, 3, 4) encoded by four separate genes. Of these isoforms, PMCA1 and PMCA4 are ubiquitously expressed. PMCA 1 and 4 share a similar structure of 10 transmembrane domains, intracellular C and N terminal tails and two cytosolic loops **(Fig. 3)** (Strehler et al., 2007). There are two important functional sites in PMCAs: the catalytic site and the PDZ-binding ligand. PMCA4 plays a vital role not only in calcium clearance but also in signal transduction by interacting with other proteins via their PDZ domains (Strehler et al., 2007).

In sperm, greater than 90% of PMCA expression is due to PMCA4, with the rest attributed to PMCA1 (Okunade et al., 2004). PMCA4 has two splice variants: PMCA4a and PMCA4b (Caride et al., 2007). PMCA4a has a higher basal activity and is more efficient than PMCA4b in bringing the cytosolic calcium to resting levels (Caride et al., 2007). However, PMCA4b has the PDZ-binding ligand on the C-

terminal tail where other proteins interact with the pump and thus it is important in signaling (see Patel et al., 2013).

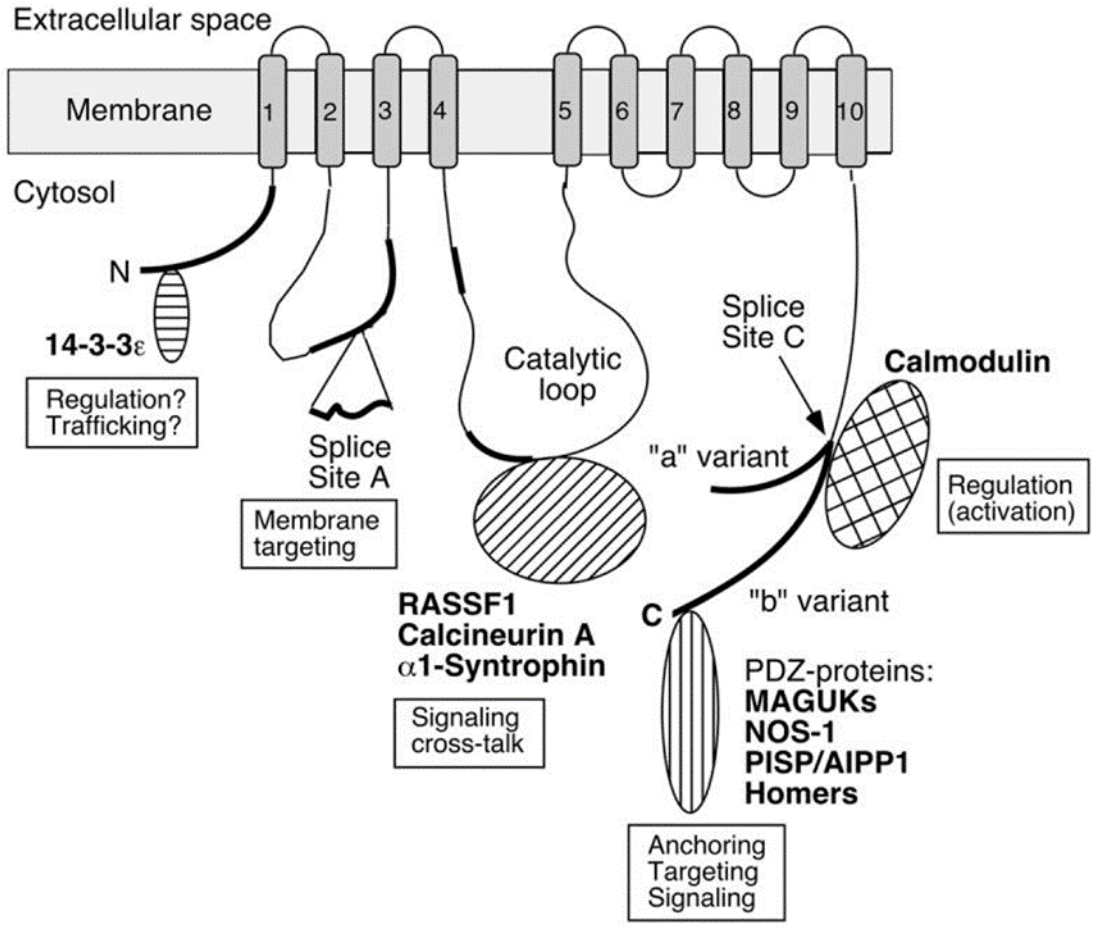


Figure 1.3 Schematic representation of the structure of Plasma Membrane Calcium ATPase4. Important domains are shown, including the catalytic domain, present between membrane passes 4 and 5, responsible for generating ATP; calmodulin binding domain at membrane pass 10 involved in triggering and shutting off the pump and PDZ ligand which is important in signaling events. (Image adapted from Strehler, 2013)

To understand the function of PMCA1 and PMCA4, studies were performed to investigate the phenotypes of mice in which *Pmca1* and *Pmca4* had been deleted. Interestingly, gene deletion of *Pmca1* caused embryolethality suggesting that it is a crucial housekeeping isoform (Okunade et al., 2004), whereas absence of *Pmca4* did not show any evidence of growth retardation, developmental defects or reduced viability. However, it was shown that *Pmca4* knockout male mice are infertile due to the loss of hyperactivated motility and disruption of Ca^{2+} homeostasis in sperm (Okunade et al., 2004; Schuh et al., 2004).

Importantly, for every Ca^{2+} ion ejected from the cell by PMCA4, one H^{+} is taken in, thus PMCA4 regulates intracellular pH (DiLeva et al., 2008). It was reported that with the deletion of *Pmca4*, there is an accumulation of cytosolic Ca^{2+}, leading to Ca^{2+} overload in mitochondria (Okunade et al., 2004). In addition, to this there should be a rise in intracellular pH. Thus in PMCA4's absence there is increased

mitochondrial Ca^{2+} which could reduce the mitochondrial membrane potential and result in decrease of mitochondrial ATP production. A reduction of pH dependent dynein ATPase activity would also be expected. Since this activity is required for triggering the sliding motion between microtubular cytoskeleton which generates flagellar beating (Christen et al., 1983; Giroux-Widemann et al., 1991), the absence of PMCA4 would be expected to result in low flagellar beating and motility defects.

In addition to the above potential mechanism for loss of motility in PMCA4's absence, the DeLeon lab has proposed a mechanism involving the interacting partners of PMCA4, specifically endothelial NOS (eNOS), neuronal NOS (nNOS), and Calcium/calmodulin-dependent Serine Kinase (CASK) which are present in sperm **(Fig. 4)**. nNOS which has a PDZ domain binds to PMCA4 via its PDZ ligand (Kone et al., 2003), while eNOS binds to the catalytic site (Holton et al., 2010). During capacitation and acrosome reaction, when there are elevated calcium levels, eNOS and nNOS which are rapidly activated by Ca^{2+} (Knowles and Moncada 1994) produce elevated levels of nitric oxide (NO). NO is a highly diffusible free radical acting as an essential messenger in both the male (Adams et al., 1992) and female reproductive tracts (Yallampalli et al., 1993; Rosselli et al., 1994), by contributing to the enhancement of sperm motility, induction of acrosome reaction, and capacitation.

During the above processes when Ca^{2+} is globally high, tethering the NOSs to PMCA4 that is actively ejecting Ca^{2+} holds them in a microenvironment of locally low

Ca^{2+}. Thus PMCA4 negatively regulates the NOSs (Holton et al., 2010; Andrews et al., 2015). During low cytosolic calcium in the sperm, CASK (calcium/calmodulin-dependent serine kinase) interacts with PMCA4b at its PDZ binding ligand (replacing nNOS) and facilitates inactivation of the pump (Aravindan et al., 2012) which is autoinhibited (Di Leva et al., 2008). In *Pmca4* nulls when PMCA4 is absent, Ca^{2+} is globally elevated and NO levels are increased to pathological levels. As a result high levels of peroxynitrite are formed, resulting in oxidative stress (Olli, Martin-DeLeon et al., in revision).

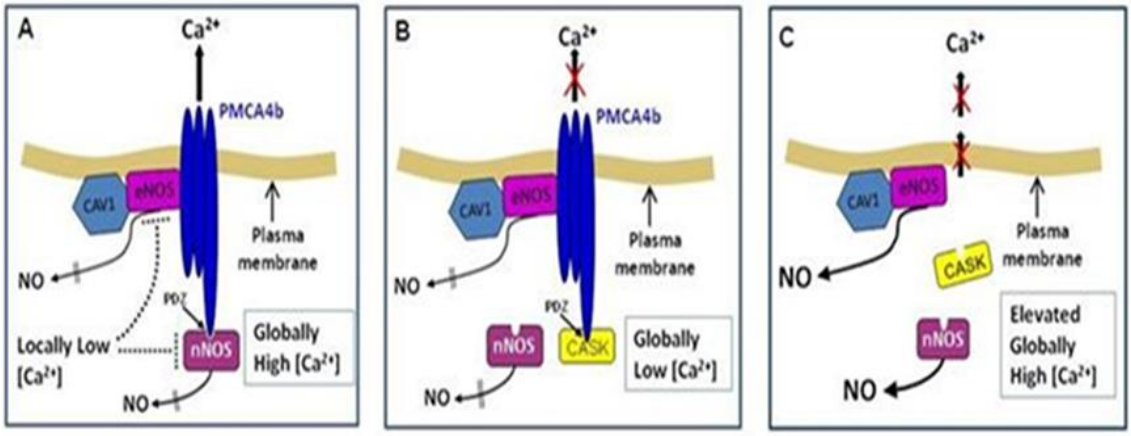


Figure 1.4 Graphical representation of the interactions of PMCA4b with other proteins in sperm. A) During globally high calcium, PMCA4 interacts with the NOSs and they are negatively regulated **B)** During globally low calcium, CASK interacts with PMCA4 and sustains PMCA4 inactivation by displacing nNOS **C)** In the absence of the pump, NOSs are unregulated.

1.3 Role of PMCA4 in the Male and Female Reproductive Tracts

As PMCA4 is the most efficient calcium efflux pump in the sperm and is vital for sperm motility (Okunade et al., Schuh et al., 2004), it has been the focus of our lab for some time. Earlier, it was shown that both PMCA4a and PMCA4b are expressed in the epididymis and are secreted into the epididymal luminal fluid where they are associated together since they could be co-imunoprecipitated (Patel et al., 2013). In the epididymal luminal fluid, PMCA4a was found to be present in extracellular membrane vesicles called epididymosomes and is transferred to sperm during epididymal maturation (Patel et al., 2013). Also, it was reported that there was a 5-fold increase of PMCA4a in caudal sperm compared to caput sperm (Patel et al., 2013) which lack progressive motility due to the presence of high intracellular calcium concentration (Yeung, 2002). This suggests that the uptake of PMCA4a during epididymal transit has an essential role in the acquisition of sperm motility.

Recently, DeLeon and her coworkers validated that PMCA4a is not only acquired by sperm during epididymal transit but also during its transit in the female tract (Al-Dossary et al., 2013). PMCA4a was demonstrated to be present in all three regions of female tract (oviduct, uterus and vagina) and was shown to be secreted in the luminal fluids where it is significantly more abundant in the oviductal luminal fluid (OLF), compared to the uterine luminal fluid (ULF) and vaginal luminal fluid (VLF) (Al-Dossary et al., 2013). Importantly, during the estrus cycle PMCA4 was

shown to be present in elevated levels during proestrus/estrus when mating occurs whereas it was present in only marginal levels during metestrus/diestrus when females do not accept males (Al-Dossary et al., 2013). In addition, PMCA4a was exclusively found in extracellular membrane vesicles of the OLF and the ULF (and absent from the supernatant) were referred to as Oviductosomes (Al-Dossary et al., 2013) and Uterosomes (Griffiths et al., 2008), respectively.

1.4 Oviductal Luminal Fluids – Oviductosomes

Almost all biological fluids consist of membrane extracellular vesicles (EVs) which are of varying sizes. EVs with size of <100 nm in diameter are called exosomes and the ones with 100-1000 nm in diameter size are known as microvesicles (Lakkaraju and Rodriguez-Boulan, 2008; Schorey and Bhatnagar, 2008; Simpson et al., 2008; Simons and Raposo, 2009). They are spherical in structure with an outer lipid bilayer (Tan et al., 2013) and carry components such as nucleic acids, proteins and lipids. EVs deliver these molecular contents to target cells and act as a mode of communication between them (Yamashita et al., 2013). Even though these vesicles have the same components as that of the cell from which they are secreted (Raimondo et al., 2011), their content composition changes after their release from the cell (Yoon et al., 2014). Although there are different subtypes of EVs, they all play a key role in the signaling by cell-cell communication.

1.5 Biogenesis of EVs

EVs are secreted through two different pathways. **A) Apocrine pathway:** In this pathway, blebs are dislodged from the apical membrane of the epithelium and release their components into the lumen **(Fig. 5A)** (Hermo and Jacks, 2002; Caballero et al., 2010). **B) Multivesicular Bodies:** In this process, the outer membrane of multivesicular bodies fuse with the apical membrane and release its interior content into the lumen **(Fig. 5B)** (Thery et al., 2002; Thery et al., 2009; Turturici et al., 2014).

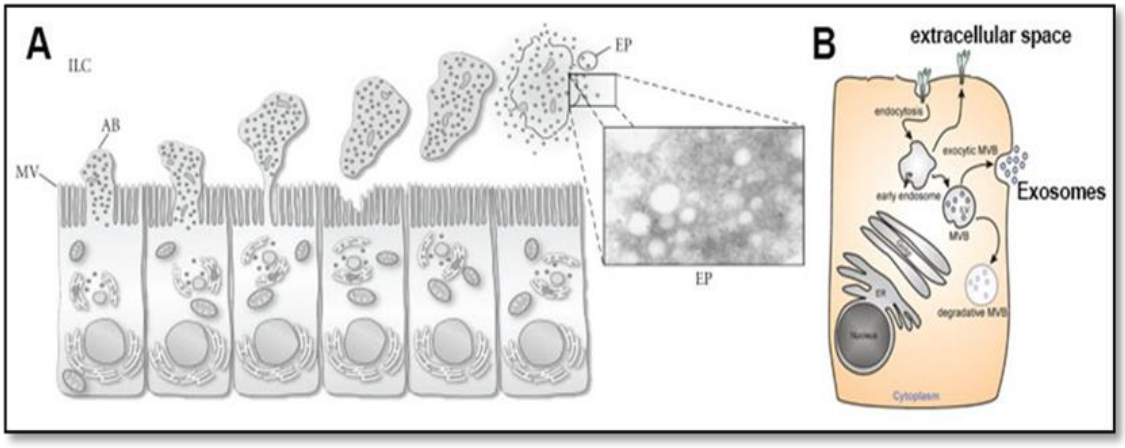


Figure 1.5 Diagrams showing two pathways for extracellular vesicles formation: **A)** Apocrine secretion. The image above represents epididymosomes which are one of the best examples of apocrine secretion. MV, microvilli; ILC, intraluminal compartment; AB, apical bleb; EP, epididymosomes. (Image adapted from Caballero et al, 2010). **B)** Multivesicular bodies (MVBs) (Image adapted from Meckes and Raub-Taub, 2011).

As most of the proteins present in EVs were identified to be membrane transporters, tetraspanins, heat shock proteins and fusion proteins (Lin et al., 2014), EVs can be characterized by the presence of tetraspanins such as CD9, CD63, CD81 and heat shock proteins, HSC 70 on their surface (Lin et al., 2014). However, to date, there is no evidence of the presence of specific markers to distinguish exosomes and microvesicles (Tannetta et al., 2014). Thus exosomes and microvesicles that are secreted into the oviductal luminal fluids (Al-Dossary et al., 2013) and shown to be essential in communicating with sperm (Al-Dossary et al., 2015) were dubbed oviductosomes (Al-Dossary et al., 2013). In unpublished data, our lab has shown that oviductosomes arise by apocrine pathway using tissue section following perfusion of females and analysis by TEM (**Fig. 6**).

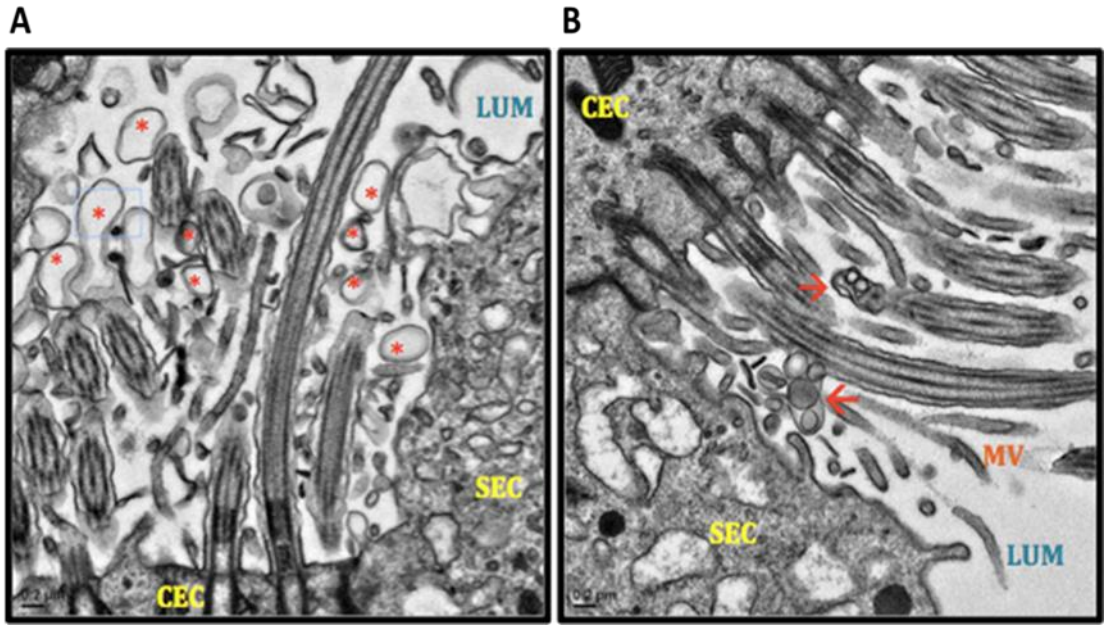


Figure 1.6 TEM image showing the section of perfused oviduct: A) Extracellular vesicles (*) are released from secretory epithelial cells (SEC) into the lumen (LUM). **B)** Red arrow indicating blebs, with exosomes and microvesicles, in the lumen (LUM). These are released by the secretory epithelial cells (SEC). CEC – Ciliated Epithelial Cells and MV – Microvilli. Bar = 0.2 µm

1.6 The Mechanism of Cargo Delivery of Exosomes

The mechanism of delivery of PMCA4 in the oviductosomal cargo has recently been elucidated and shown to involve membrane fusion (Aldossary et al., 2015). In this mechanism, ligands form a connecting bridge between the membranes of the oviductosomes and sperm by binding to CD9 and αV integrin molecules that are

present on those membranes. This formation of connecting bridge brings the membranes close to each other and leads to the formation of a stalk like structure which ultimately results in complete fusion of the oviductosomal membrane with that of the sperm (Al-Dossary et al., 2015).

1.7 Fertility in *Pmca4* null Females:

Despite the abundant expression of PMCA4 in all three regions on the female tract and its presence in the luminal fluids via extracellular vesicles which are able to deliver it to sperm, it is surprising that *Pmca4* KO females, unlike males, are fertile. As mentioned above, the loss of hyperactivated motility and ultimately infertility in *Pmca4* KO sperm is likely due to Ca^{2+} toxicity. High levels of Ca^{2+} were seen to be sequestered in the mitochondria in the midpiece of the sperm flagellum (Okunade et al., 2004) with potential damage to flagellar beat. Thus in the absence of sperm acquisition of PMCA4 from both uterosomes and oviductosomes in *Pmca4* KO females, capacitating sperm would be deprived of their most effective Ca^{2+} efflux mechanism.

More importantly, this efflux mechanism would be expected to maintain normal ciliary function of the oviductal epithelium, since there is a parallelism between the structure and function of cilia and flagella. As oviductal ciliary function is required for transport of oocytes to the fertilization site in the AIJ (Ghersevich et al.,

2015) the inability of the oviductal epithelial cells to extrude Ca^{2+} in *Pmca4* KO females could greatly compromise the ability of the oocyte to be fertilized.

Thus the fertility of *Pmca4* KO female mice is surprising and raises the following questions:

1) In the absence of PMCA4 in the fluids of *Pmca4* KO female, how do sperm that enter into female tract handle the excess of intracellular calcium required for capacitation, hyperactivation, and acrosome reaction?
2) In *Pmca4* KO female mice, how is Ca^{2+} toxicity avoided and ciliary function maintained to effect oocyte transport and normal fertility?
3) Earlier studies have shown that PMCA1 does not compensate for PMCA4 deficiency in testis (Okunade et al., 2004). Thus, due to the lack of a compensatory mechanism to maintain Ca^{2+} homeostasis, *Pmca4* KO males exhibit loss of hyperactivated motility and resulting infertility. Does the fertility of *Pmca4* KO females result from a compensatory expression of PMCA4 in the oviduct?

1.8 Hypothesis and Aims

Based on the above background, the following hypothesis was advanced: PMCA1 acts as a true surrogate for PMCA4 in *Pmca4* KO female mice, explaining their fertility. The following three Aims will test the hypothesis.

Aim 1: Investigate the expression levels of PMCA1 in EV and biofluids secreted by the female reproductive tract at different stages of estrus in WT and *Pmca4* KO female mice. To accomplish this hypothesis, I will use indirect immunofluorescence and Western blot analysis.

Aim 2: Determine the presence of PMCA4 interacting partners in oviductosomes isolated from oviductal luminal fluid and if these proposed partners interact with PMCA1. To investigate this hypothesis, I will perform Western blot and co-immunoprecipitation assays to confirm the presence and interaction of known partners of PMCA4 with PMCA1 in oviductosomes.

Aim 3: Investigate the localization of PMCA1 on sperm, in relation to that of PMCA4, and determine if it can be transferred to sperm from oviductosomes during different capacitating conditions. To examine this hypothesis, I will perform immunofluorescence to attempt to co-localize PMCA1 and PMCA4 on sperm, and flow cytometric analysis after the co-incubation of sperm with oviductal luminal fluid or oviductosomes.

Chapter 2

MATERIALS AND METHODS

2.1 Mice and Reagents

Sexually mature 10-12 week old male and 4-12 week old female mice (FVB/N strain; Harlan, Indianapolis, IN) were used for this study. In addition to these wild type (WT) mice, *Pmca4* null male and female mice were used to obtain testis and oviductal luminal fluid/oviductosomes, respectively for Western blotting analysis. These mutant mice, generated in the laboratory of Dr. Gary Shull (University of Cincinnati), were a generous gift. These mice were bred and genotyped as described previously (Okunade et al., 2004). The studies were approved by the Institutional Animal Care and Use Committee at the University of Delaware and were in agreement with the Guide for the Care and Use of Laboratory Animals published by the National Research Council of the National Academies, 8th ed., Washington, D.C. (publication 85–23, revised 2011).

2.2 Antibodies

Rabbit monoclonal anti-PMCA1 antibody (ab190355) was purchased from Abcam (Cambridge, MA) and used for immunofluorescence, western blots, and flow

cytometric studies. Mouse monoclonal anti-HSC70 antibody (sc-7298), rat monoclonal anti-CD9 antibody (SC-18869) and goat polyclonal anti-PMCA4 antibody, detecting both 4a and 4b isoforms (SC-22080) were purchased from Santa Cruz Biotechnology, Dallas, TX. Rabbit polyclonal nNOS antibody (NOS1) was obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Mouse monoclonal CASK antibody was obtained (#75-000) from UC Davis/NINDS/NIMH NeuroMab Facility (Davis, CA). Secondary antibodies purchased from Santa Cruz Biotech, Inc., Life Technologies or Molecular Probes Inc. (Eugene, OR) were used in this studies. Fluoro-Gel II with DAPI (17985-50), used for immunofluorescence was obtained from Electron microscope sciences, Hatfield, PA.

2.3 Superovulated Females

To induce estrus, 4 to 6 week old female mice were sequentially administered with pregnant mare serum gonadotropin (7.5 i.u.) and human chorionic gonadotropin (7.5 i.u.), 48 h apart. After 13.5-14h from the last hormonal injection, females were sacrificed and their reproductive tissues (oviduct, uterus and vagina) collected for further analysis.

2.4 Identification of the Stages of the Murine Estrus Cycle

Female virgin mice, 8 to 12 weeks old, were used in this experiment. The stages of the estrus cycle were classified based on the proportion of different types of

cells identified in the vaginal secretion (Byers et al., 2012). Oviductal tissues from females in proestrus, estrus, metestrus and diestrus were collected and then processed for Western analysis, as described below.

2.5 Collection of Female Reproductive Tissues and Luminal Fluids

Immediately after sacrificing female mice, oviducts, uteri and vaginas were removed and frozen in OCT at -80^{o}C for immunofluorescence analysis or they were minced in PBS with protease inhibitors to collect luminal fluids. These luminal fluids were clarified by centrifuging at 3,500 x *g* for 10 min to exclude cells and tissue fragments, and then frozen immediately at -80^{o}C for further processing.

2.6 Protein Extraction from Female Tissues

Tissues were homogenized in 300 µl of 1X RIPA buffer with protease inhibitor (Sigma Aldrich, Cat. No. P2714-1BTL) and left on rotor at 4^{o}C overnight. The suspension was then centrifuged at 14,000 x *g* for 10 min and the protein extract collected. Following this, the protein concentration was determined using Bicinchoninic Acid (BCA) assay kit (Pierce, Cat. No. 23225) and spectrophotometry.

2.7 Exosome Preparation

Clarified luminal fluids of oviduct, uterus and vagina (OLF, ULF and VLF respectively) obtained from superovulated female mice were ultracentrifuged at

120,000 x *g* at 4°C for 2h using Beckman Optima 2-70 k ultracentrifuge and a Ti60 rotor. Resulted pellets (oviductosomes, uterosomes and vaginosomes) containing both microvesicles and exosomes were re-suspended in PBS and protease inhibitor for Western blot analysis and transmission electron microscopy (TEM).

2.8 Preparation of Capacitated Sperm

Murine sperm were collected by mincing caudal epididymides of sexually mature males in Human Tubal Fluid (HTF) (Cat #2002, InVitroCare, Frederick, MD) and was left at 37°C for 10 min for sperm to swim out of the tissue (Chen et al., 2006). After gravity settling of tissues, the supernatant was aliquoted into 1.5 ml tubes. To this suspension, capacitation medium (HTF) was added and the samples incubated at 37°C for 90 min.

2.9 Immunofluorescence Staining

Oviductal, uterine and vaginal tissues collected from superovulated females were embedded immediately in optimum cutting temperature media (OCT) (Tissue Tek, Torrance, California) and were frozen at -80°C. Cryostat sections of 20 μm were generated and stored at -80°C until use. Slides were fixed in pre-chilled 1:1 acetone:methanol at -20°C for 20 min and then were air-dried for 10 min before being placed in blocking solution (1% bovine serum albumin (BSA) in 1X PBS) for 2h at RT. They were then incubated in anti-PMCA1 primary monoclonal antibody or rabbit

IgG (negative controls) at a dilution of 1:50 in 1% BSA blocking solution, overnight in an enclosed humid chamber. Following this, slides were washed with 1X PBS (2x, 20min). Sections were then incubated in Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene Oregon) at a dilution of 1:200 in blocking solution for 45 min at RT in the dark, followed by washing with 1X PBS (2x, 20 min). Finally, sections were mounted with fluoro gel II mounting media with DAPI and were cover-slipped. Then slides were visualized using Zeiss LSM 780 confocal microscope (Carl Zeiss, Inc, Gottingen, Germany) using 20X and 40X objective.

2.10 SDS PAGE and Western blot analysis

Samples for electrophoresis were prepared by diluting protein extracts in 5X Laemelli sample buffer and heating them at 99^{o}C for 5 min. Twenty to 40 µg of proteins from tissues and fluids were loaded on each lane of 10% polyacrylamide gels and transferred onto nitrocellulose membrane (Amersham Biosciences). Then blots were blocked for 1h at RT and incubated in anti-PMCA1 (1:2000) primary antibody for overnight at 4^{o}C. The membrane was washed with TBST (20mM Tris, pH 8.0, containing 150mM NaCl and 0.5% Tween 20) (3x, 10min) to remove the non-specific binding of antibody. After this, membranes were incubated in alkaline phosphatase (AP) – conjugated anti-rabbit IgG (Invitrogen, 1:2000) for 1 h at 4^{o}C and was again washed with TBST (5x, 15 min) before detection of chemiluminescence by using ECL

kit (Bio-Rad, Hercules, CA). The membranes were then re-probed with HSC70 antibody which was used as internal loading control for normalization.

2.11 Perfusion of Vaginal Tissue

Vaginas collected from females were immediately after sacrifice were placed in fixative of 2% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer. After placing it in the fixative, vaginal tissue was cut into small pieces of thickness about 1-2 mm^3 and sent to DBI for sectioning and processing for TEM (Zeiss LIBRA 120) analysis.

2.12 Negative Staining of Vesicles for TEM

Nickel TEM grids (Electron Microscopy Sciences) were floated on a drop of VLF pellet suspension. The grids were then washed with several drops of water and stained with 1% uranyl acetate, a phospholipid stain, before being subjected to microscopic analysis. Membrane vesicles present in VLF were imaged using TEM (Zeiss LIBRA 120).

2.13 Co – Immunoprecipitation of PMCA1 and Interacting Partners in Oviductal Luminal Fluid (OLF)

To perform Co-Immunoprecipitation, fifty microliters of PureProteome Protein G magnetic beads (Millipore Corp, Billerica, MA) were washed three times in 0.1% Tween 20 in PBS and recovered by centrifugation at 500 x *g* for 5 min. The beads were then suspended in 100 µl of 1X PBS with 2 µg of specific antibody (nNOS or CASK) for 2h on rotator at 4^{o}C and the controls were treated with the same concentration of either rabbit IgG or mouse IgG. After incubation, the beads were washed with 1X PBS at 500 x *g* and were re-suspended in 500 µg of oviductal luminal fluid (OLF). The volume of suspension was brought to 500 µl with the addition of IP buffer (25mM Tris, 150 mM NaCl, pH 7.2) with 40 µl of protease inhibitor and were incubated on rotator at 4^{o}C overnight. Following incubation, beads were washed with 1X PBS (3x, 5 min) at 500 x *g*. After the final wash, 20 µl of 1X RIPA and 5 µl of dye were added to the beads which were boiled at 100^{o}C for 5 min. These samples were then used for SDS-PAGE and Western blot analysis.

2.14 Super-resolution Structured Illumination Microscopy (SR-SIM) analysis of Immunofluorescence Staining of Sperm

Immunofluorescence staining was performed by fixing the capacitated sperm in 1.5% paraformaldehyde (Cat. #15710, Electron Microscopy Sciences, Hatfield, PA)

in PBS for 1h. Then they were washed with PBS (2x, 15 min) and were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. Following this, sperm were blocked in 2% BSA for 1h and then incubated in primary antibodies (rabbit anti-PMCA1 or goat anti-PMCA4) at a dilution of 1:50, overnight at 4°C. They were subsequently subjected to the appropriate secondary antibodies (Alexa Fluor 568 anti-rabbit or Alexa Fluor 488 anti-goat respectively, 1:200 dilution, 1h) on ice in the dark and washed (2x, 15 min) with PBS. The resulting suspensions were placed on slides and mounted with fluoro-gel II with DAPI and cells were imaged using SR-SIM (Zeiss Elyra PSI SIM, Carl Zeiss, Inc., Germany). Controls were prepared with no addition of primary antibody.

2.15 *In Vitro* Sperm Uptake of PMCA1 from Oviductal Luminal Fluid (OLF)

Caudal sperm isolated from sexually matured male mice were capacitated (See section 2.8 for procedure) or left uncapacitated. Sperm were then incubated in oviductal luminal fluid (OLF) for 3h at 37°C. Following the incubation, sperm were washed with 1X PBS (3x, 15 min) at 500 x *g* and then they were fixed with 1.5% paraformaldehyde for 1h at RT. After incubation, sperm were washed with 1X PBS (3x, 15 min) and were permeabilized with 0.1% triton X-100 for 10 min at RT. They were then blocked with 2% BSA and treated with PMCA1 primary antibody (1:200, Abcam Inc.) followed by Alexa Fluor 488 goat anti-rabbit secondary antibody treatment at a dilution of 1:200. After this, sperm were subjected to flow cytometric

analysis using a FACSAria™ II (BD Sciences, San Jose, CA), equipped with an argon laser with an excitation at 488 nm. The control sperm were incubated in PBS instead of OLF and were treated identically to that of the test samples.

Chapter 3

RESULTS

3.1 Localization of PMCA1 in Female Reproductive Tract

Indirect Immunofluorescence and confocal microscopy of oviductal, uterine and vaginal tissues of WT superovulated virgins revealed that PMCA1 is expressed in all three regions of female reproductive tract as shown in **Fig 3.1**. The results of **Fig 3.1** show the stronger signal of PMCA1 in the oviduct and the uterus where the staining was detected in the luminal epithelial cells and basement membrane. In the vagina, PMCA1 is seen in the epithelial layers at apical membrane and the signal is weaker than in the other regions of the tract.

Superovulated WT and *Pmca4* KO oviductal tissue sections were analyzed for the presence of PMCA1. **Fig 3.2** shows the presence of PMCA1 in the apical and basement membranes of WT and KO oviducts. Interestingly, the intensity of the staining in the KO oviductal tissue was brighter compared to WT. The occurrence of PMCA1 in apical membrane of oviductal, uterine and vaginal epithelia suggests that PMCA1 is secreted into their respective biofluids and exosomes.

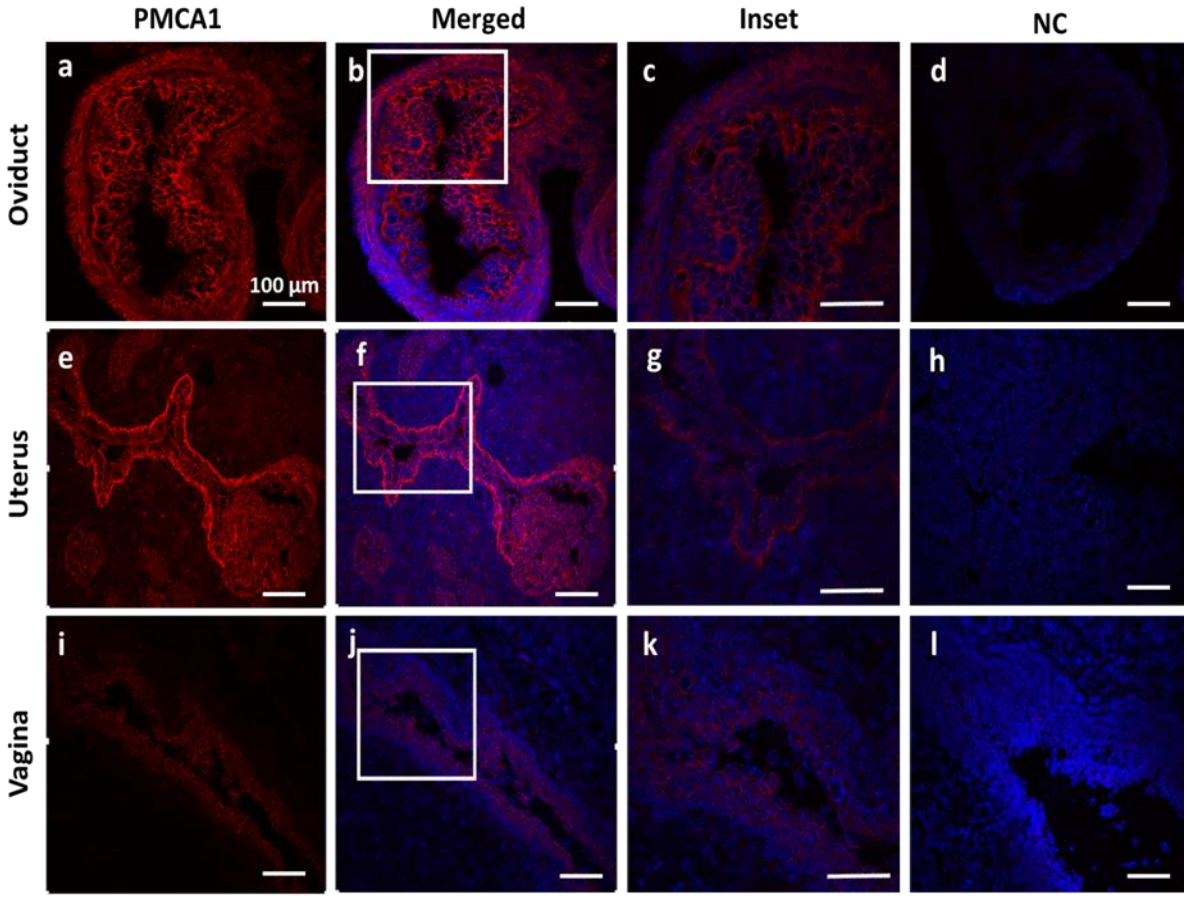

PMCA1
Merged
Inset
NC
Oviduct
Uterus
Vagina
a
b
c
d
e
f
g
h
i
j
k
l
100 µm

Figure 3.1 Detection of PMCA1 in female reproductive tract during estrus. Indirect immunofluorescence shows **(a-c)** a strong staining (red) of PMCA1 in the apical membrane of the oviductal epithelium. **(e-g)** In the uterus, PMCA1 is expressed in the epithelium and on the basement membrane whereas **(i-k)** it is expressed in the apical epithelial membrane in the vagina. Insets of oviduct, uterus and vagina were shown in **c, g and k. (NC; d, h and l)** Negative controls of frozen tissues do not show the staining when they were incubated in primary antibody of rabbit IgG and the corresponding secondary antibody. Bar =100 μm (200 μm for all insets and the same scale for all the micrographs).

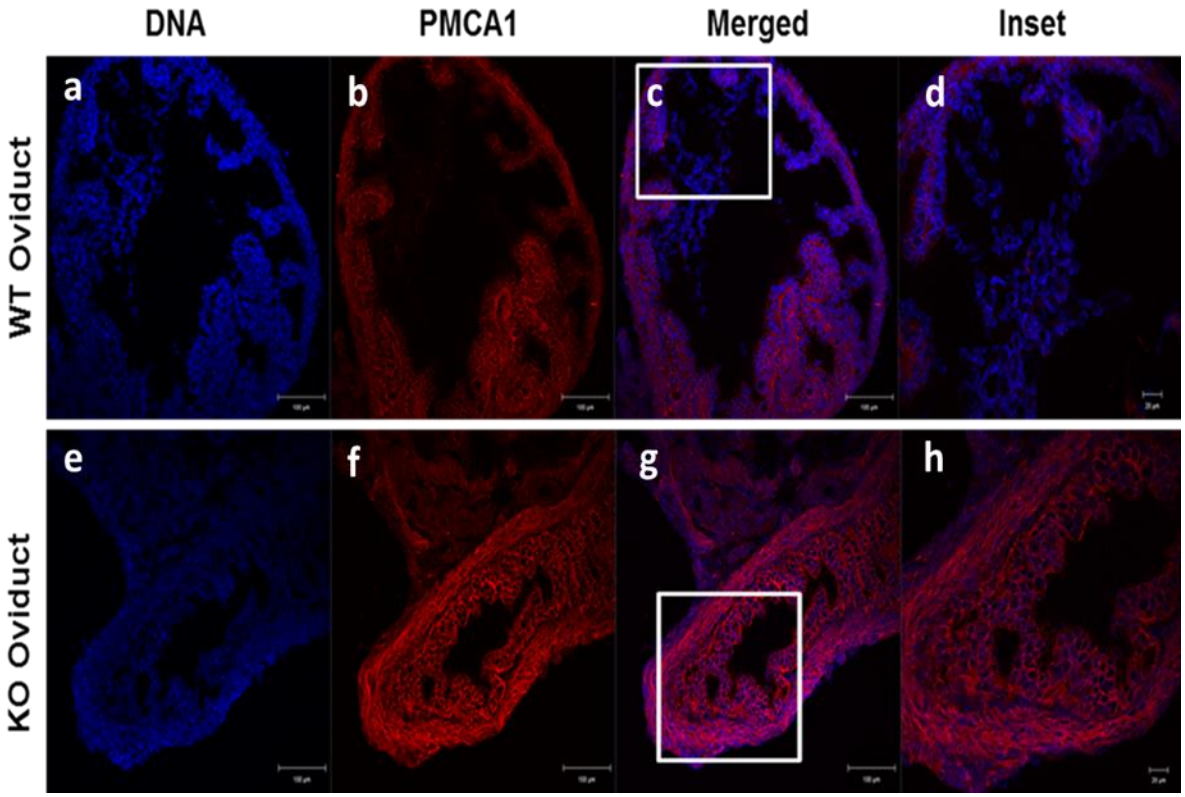
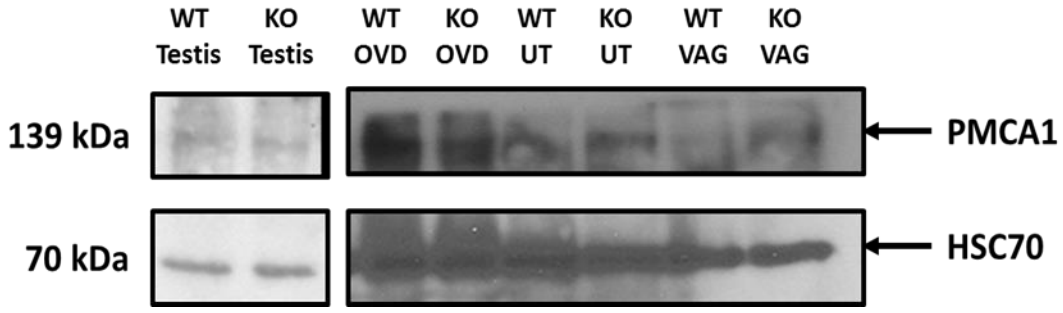


Figure 3.2 Indirect Immunofluorescence of PMCA1 in WT and *Pmca4* KO Oviductal tissues during estrus. The staining of PMCA1 in **(f)** KO oviductal tissue appears to be more intense compared to **(b)** WT. **(a and e)** The nuclei of the oviductal tissues were visualized with DAPI staining (blue). Enlarged images of WT and *Pmca4* KO oviductal are shown of the insets in **d and h**. Bar = 100 µm (200 µm for all insets and the same scale for all the micrographs)

3.2 Analysis of PMCA1 Expression in Female Reproductive Tissues

PMCA1 expression levels in tissues of superovulated females were studied using Western analysis. To determine the level of PMCA1 expression in female tissues, lysates were collected from all three regions of female tract. In the lysates of WT and *Pmca4* KO of oviduct, uterus and vagina, a 139 kDa PMCA1 band was detected **(Fig 3.3A)**, confirming the indirect immunofluorescence findings of PMCA1. Western analysis also revealed that PMCA1 expression levels varied with no significant difference between WT and *Pmca4* KO of both testicular and female tissues (oviduct, uterus and vagina) **(Fig 3.3B)**. Testicular tissue lysates of WT and *Pmca4* KO were used as positive controls.

A)



B)

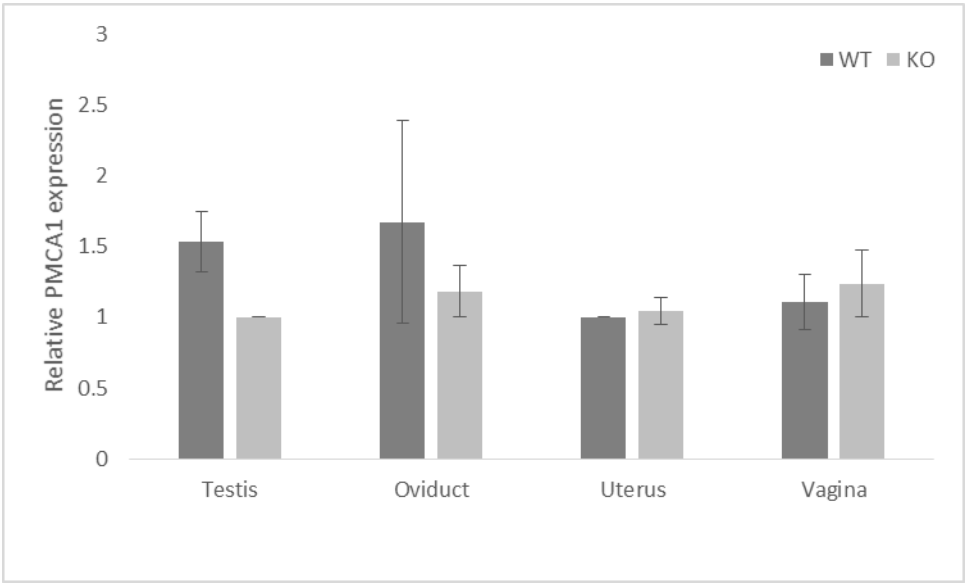


Figure 3.3 Expression of PMCA1 in the female reproductive tissues during estrus. (A) Representative Western blot of female tissue lysate (oviduct, uterus and vagina) of WT and *Pmca4* KO shows PMCA1 bands at 139 kDa in WT and *Pmca4* KO tissues during estrus. WT and *Pmca4* KO testis were used as a positive control and then the membrane was re-probed with HSC70, as a loading control. **(B)** Quantification of Western blot data that are shown in **(A)**. The data represents the mean (±SEM) of three independent experiments and the intensity of the bands in Western blot were quantified, using Image J software and statistical analysis showed no difference of PMCA1 levels between WT and KO tissues.

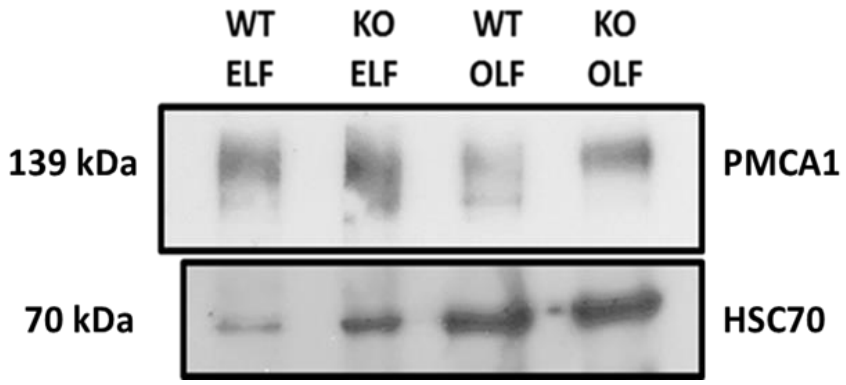
3.3 Detection of PMCA1 in the Secretion of Oviduct

Oviductal luminal fluid (OLF) was collected from oviducts of females that were hormonally induced into estrus. The presence of PMCA1 in WT and *Pmca4* KO OLF was investigated, using Western blot analysis. **Fig 3.4A** shows that PMCA1 is present in WT and *Pmca4* KO OLF as well as epididymal luminal fluid (ELF) which was used as a control. The intensity of the Western bands showed that the amounts of PMCA1 varied with the phenotype. There is an increase of PMCA1 in *Pmca4* KO OLF compared to WT, a trend that is the reverse for WT and KO ELF. Statistical analysis showed that these differences were not significantly different: the P value being 0.07 for OLF and 0.1 for ELF **(Fig. 3.4B)**. However, two-way ANOVA revealed a significant increase of PMCA1 in *Pmca4* KO OLF compared to *Pmca4* KO ELF (P=0.02) **(Fig 3.4B)**.

Luminal oviductal fluids collected during physiological proestrus/estrus demonstrated higher expression levels of PMCA1 in *Pmca4* KO OLF than WT **(Fig 3.5A)**, corroborating the Western findings of PMCA1 in OLF of superovulated female mice where estrus was hormonally-induced. Densitometric analysis showed that PMCA1 levels during proestrus/estrus had a significant increase (P=0.02) in *Pmca4* KO OLF compared to WT, but during metestrus/diestrus there is a significant decrease (P=0.03) **(Fig 3.5B)**. It is important to point out that the increased level of PMCA1 seen in WT OLF at metestrus/diestrus is not significantly different (P= 0.1) from that

collected at proestrus/estrus, as shown by one-way ANOVA. In addition, one-way ANOVA results revealed a significant increase (P=0.01) of PMCA1 levels in *Pmca4* KO OLF at proestrus/estrus compared to at metestrus/diestrus.

A



B)

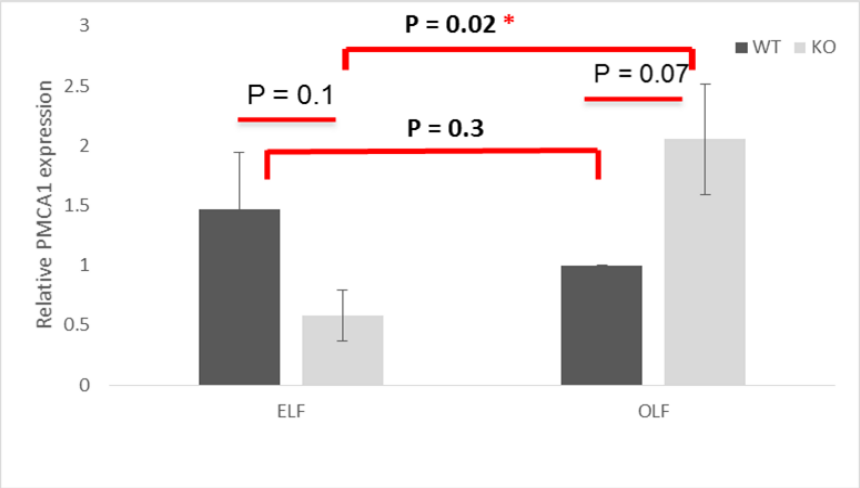
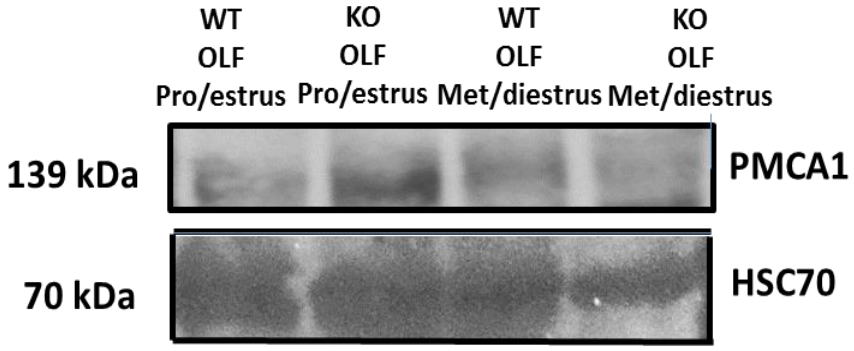


Figure 3.4 Comparison of PMCA1 in the oviductal luminal fluids (OLF) of WT and *Pmca4* KO during induced estrus. A) Western blot of OLF of WT and KO during induced estrus. Increase in PMCA1 was seen in *Pmca4* KO OLF compared to WT and the epididymal luminal fluid (ELF) from *Pmca4* KO and WT were used as controls. The membranes were stripped and re-probed for HSC70 which was used as loading control **B)** Statistical analysis of Western blot data obtained in **(A)** is shown. The data represent the mean (±SEM) of three independent experiments in which the intensities of the bands were quantified, using Image J software. While Student *t*-tests for the mean of WT and KO OLF and for WT and KO ELF did not show a significant difference, ANOVA showed that the increased expression of KO OLF was significantly different from KO ELF (*P=0.02)

A)


WT OLF Pro/estrus
KO OLF Pro/estrus
WT OLF Met/diestrus
KO OLF Met/diestrus
139 kDa
PMCA1
70 kDa
HSC70

B)

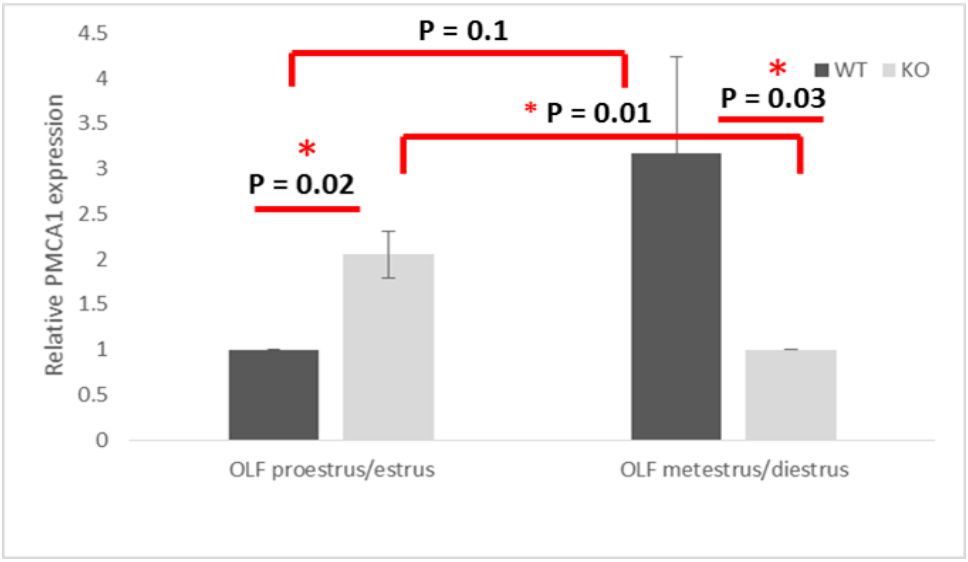

P = 0.1
* P = 0.01
P = 0.03
*
P = 0.02
WT
KO
Relative PMCA1 expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
OLF proestrus/estrus
OLF metestrus/diestrus

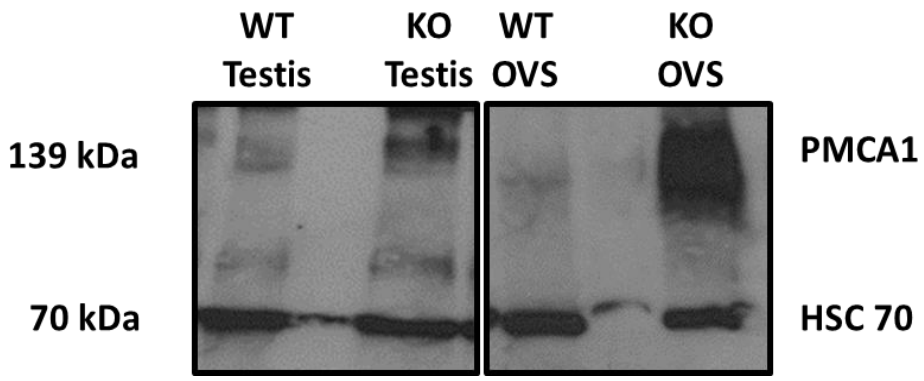
Figure 3.5 Expression of PMCA1 in the oviductal luminal fluids (OLF) of WT and *Pmca4* KO during the estrus cycle. A) Western blots of WT and *Pmca4* KO OLFs collected during proestrus/estrus and metestrus/diestrus show an increase of PMCA1 in *Pmca4* KO OLF during proestrus/estrus while there was a decrease during metestrus/diestrus. The membranes were stripped and re-probed for loading control HSC70. **B)** Densitometric analysis of Western blot data obtained in **(A)**. The data obtained is the average (±SEM) of three independent experiments and the intensity of bands were quantified using Image J software. One-way ANOVA and Student *t*-tests were performed on the mean and the P values were calculated. P values < 0.05 were represented by * and indicate the significance change in expression levels.

3.4 Presence of PMCA1 in Exosomes of Luminal Fluids

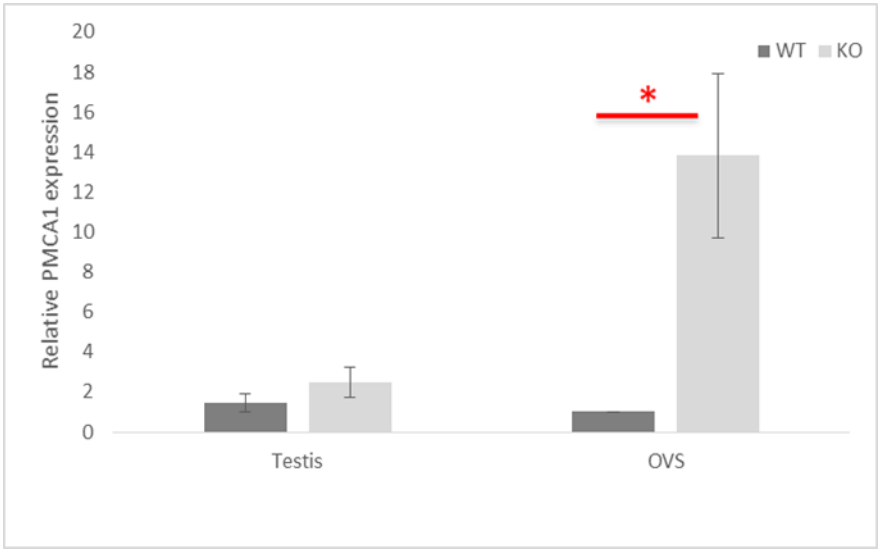
Since membrane proteins in the luminal fluids are carried on exosomes, Western analysis was performed to observe the presence of PMCA1 expression in oviductosomes (OVS) and uterosomes of WT and *Pmca4* KO females. In **Fig 3.6 (A) and (C)**, using WT and KO testis as a positive control, it is shown that WT and *Pmca4* KO oviductosomes and uterosomes carry the 139 kDa PMCA1 with an upregulation of PMCA1 in *Pmca4* KO OVS, compared to WT OVS **[Fig 3.6(A)]**. Western analysis also displayed similar levels of PMCA1 expression in WT and

Pmca4 KO uterosomes **[Fig 3.6(C)]**. Protein quantification studies of OVS revealed a 13-fold significant increase (*P<0.05) of PMCA1 in *Pmca4* KO compared to WT [**Fig 3.6(B)**] while uterosomes exhibit no notable difference in PMCA1 levels between WT and *Pmca4* KO [**Fig 3.6(D)**].

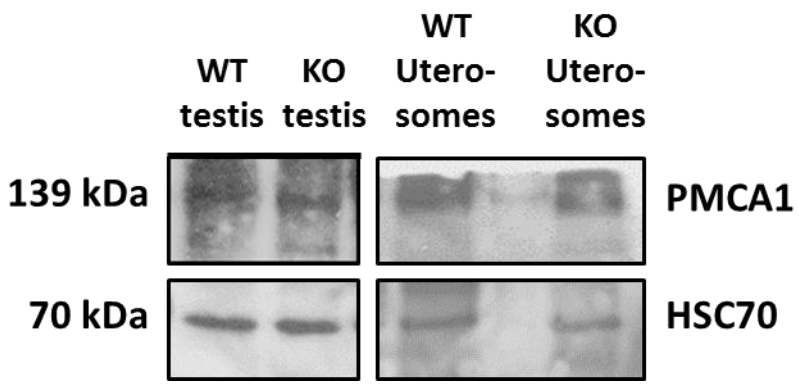
A)



B)



C)


WT testis
KO testis
WT Utero-somes
KO Utero-somes
139 kDa
PMCA1
70 kDa
HSC70

D)

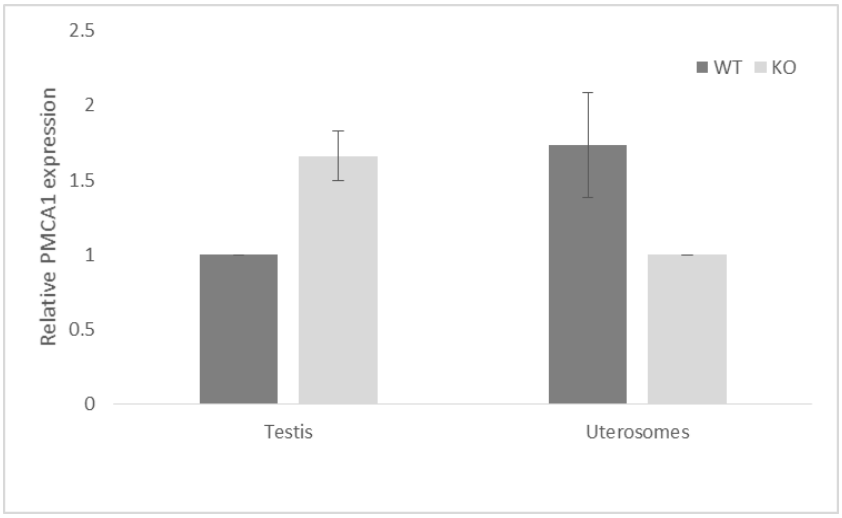

Relative PMCA1 expression
2.5
2
1.5
1
0.5
0
WT
KO
Testis
Uterosomes

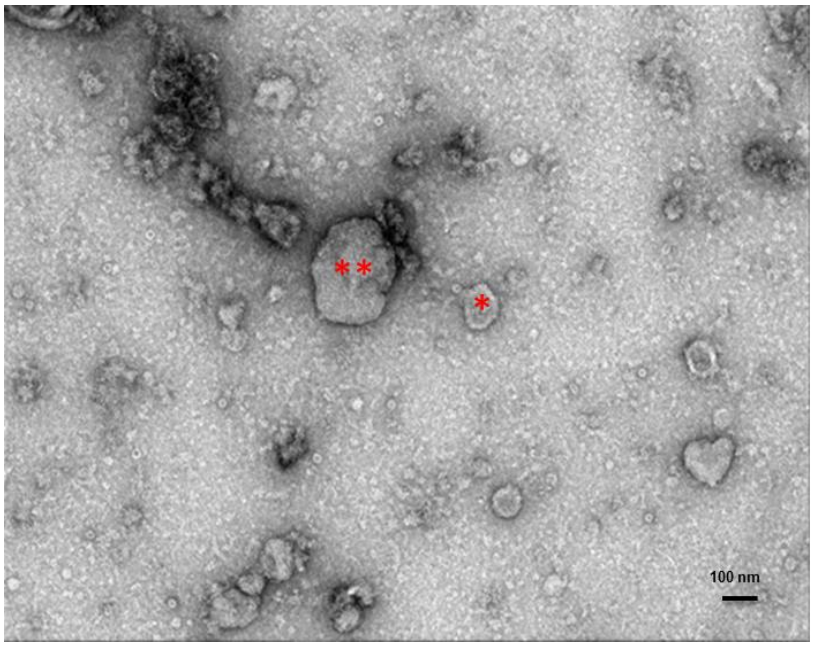
Figure 3.6 Secretion of PMCA1 in oviductosomes (OVS) and uterosomes of WT and *Pmca4* KO during estrus. Representation of Western blot of PMCA1 in oviductosomes **(A)** and uterosomes **(C)** of WT and *Pmca4* KO females. **B)** Quantification of PMCA1 bands of WT and KO OVS (A) was performed using Image J. Student *t*-tests were conducted on the mean of three independent experiments showing a significant increase (*P<0.05) of PMCA1 in *Pmca4* KO OVS. **D)** Statistical analysis was performed on the Western blot data obtained in (C) by using Image J and Student *t*-tests, these showed no difference between the genotypes in the two tissue extracts.

3.5 Characterization of Extracellular Membrane Vesicles in Vaginal Luminal Fluids

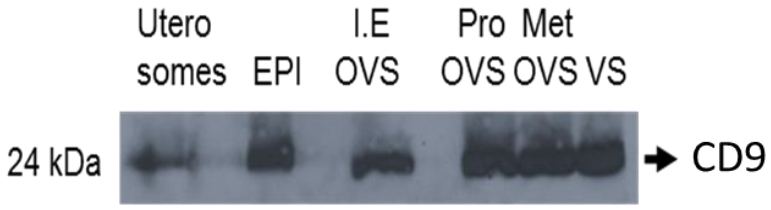
Vaginal luminal fluid was collected from superovulated females and clarified by centrifugation to remove cellular debris. The clarified fluid was then subjected to ultracentrifugation and the pellet was analyzed using TEM. Extracellular vesicles (EVs) consisting of both exosomes and microvesicles were detected in the pellet **[Fig 3.7(A)]**. Since CD9 tetraspanin is the most common biomarker for extracellular vesicles, I characterized the presence of EVs using CD9 in Western analysis. A 24 kDa CD9 band was detected in VLF pellets and also in oviductosomes collected from

different stages of estrus, uterosomes and epididymosomes which were used as positive controls **[Fig 3.7(B)]**. Using the standard size classification for EVs, I show that vesicles in VLF 1) are in the size range of less than 100 nm to 1 µm in diameter, and 2) are enriched with the CD9, biomarker. Therefore, I defined these vesicles present in vaginal luminal fluid as exosomes and microvesicles and named them as "Vaginosomes" (VS). To further confirm the presence of vaginosomes, vaginal tissue was perfused and sections were fixed and prepared for TEM. TEM analysis shows in situ the presence of membrane vesicles of the size of exosomes and microvesicles **[Fig 3.7(C)]**.

A)



B)



C)

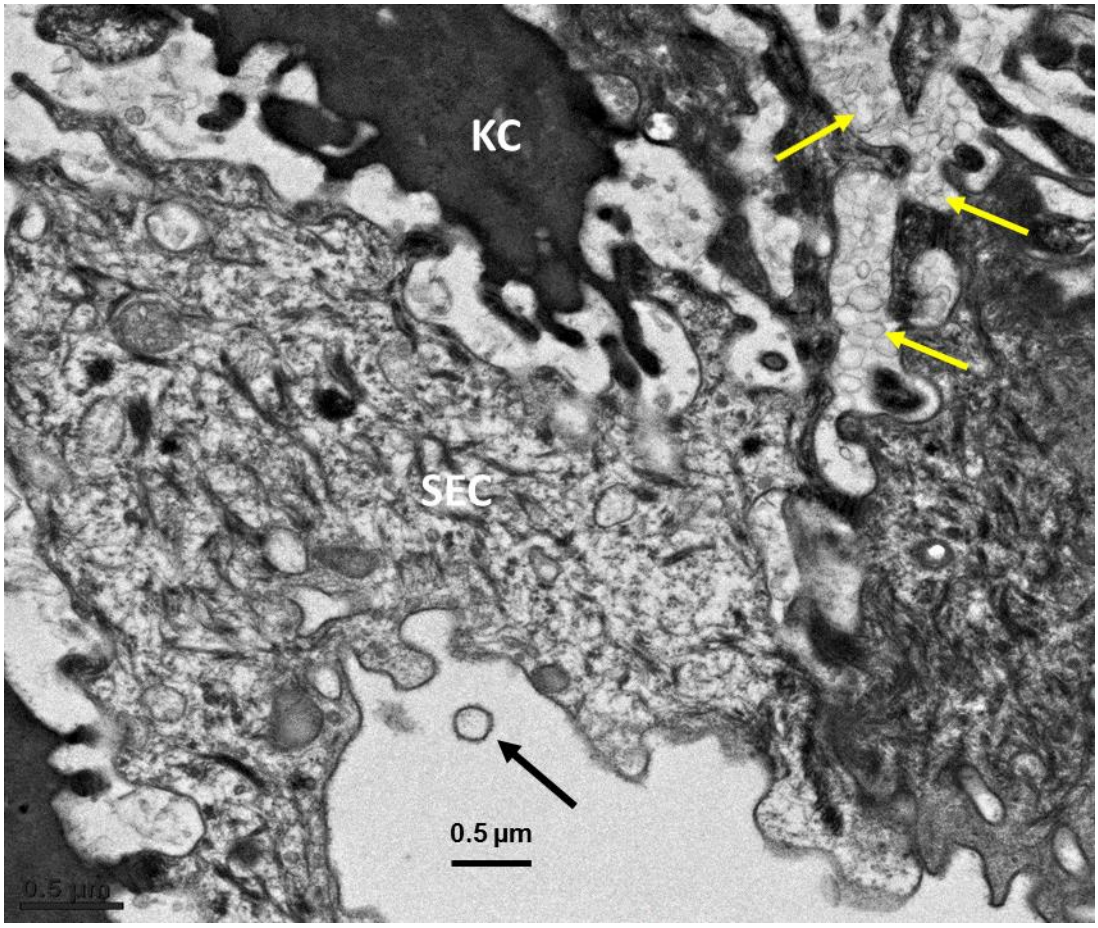
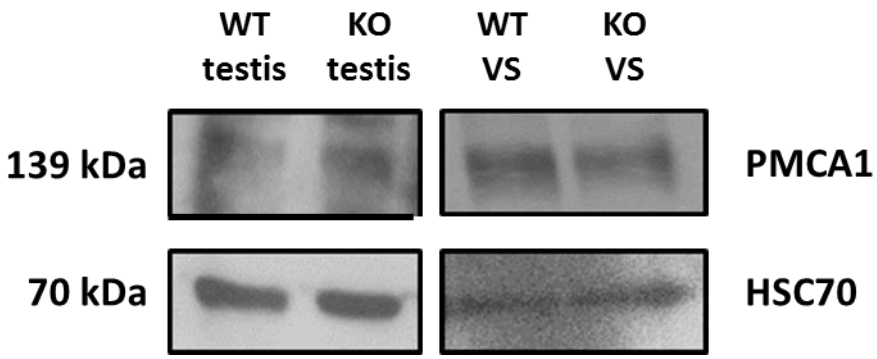


Figure 3.7 Characterization of membrane vesicles in vaginal luminal fluid (VLF) **(A)** Negative staining of TEM for the pellet of VLF reveals the presence of extracellular vesicles of varying sizes *Exosomes of size < 100 nm and **Microvesicles ranging in size from 100 nm to 1µm diameter **(B)** Western blot shows the presence of CD9 on the membranous vesicles of VLF, oviductosomes, uterosomes and epididymosomes. Pro = Proestrus, Met =Metestrus **(C)** TEM image of cross section of the vagina showing the membrane vesicles that are present in between the layers of vaginal tissue, as indicated by yellow arrows. KC = Keratinized cells and SEC = Squamous epithelial cells.

3.6 Determination of the Presence of PMCA1 in Vaginosomes

Samples collected from vaginal luminal fluid revealed the presence of PMCA1 in vaginosomes of WT and *Pmca4* KO superovulated females **[Fig 3.8(A)]**. In **Fig 3.8(A)** WT and KO testis were used as positive controls, while HSC 70 which is one of the biomarkers for EVs was used as a loading control and corroborates the finding that vaginosomes are indeed extracellular vesicles. Analysis (3 replicates) of the expression levels of PMCA1 in WT and *Pmca4* KO vaginosomes reveals no significant difference in PMCA1 levels between WT and *Pmca4* KO vaginosomes **[Fig 3.8(B)]**.

A)


WT testis
KO testis
WT VS
KO VS
139 kDa
PMCA1
70 kDa
HSC70

B)

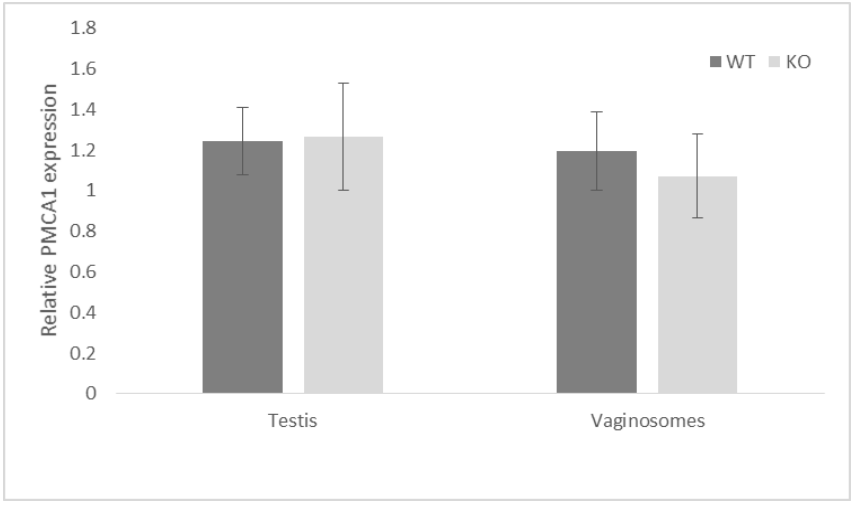

Relative PMCA1 expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
KO
Testis
Vaginosomes

Figure 3.8 Presence of PMCA1 in Vaginosomes. (A) Western blot shows that vaginosomes collected from WT and *Pmca4* KO VLF carry PMCA1. WT and *Pmca4* KO testis protein lysates were used as positive controls, while HSC70 was used as a loading control. (B) Quantification of Western blot data of (A) was performed on three independent samples by using Image J and Student *t*-tests.

3.7 Identification of Interacting Partners of PMCA4 in Oviductosomes

Oviductosomes isolated from oviductal luminal fluids of superovulated females were probed in Western analysis to detect the presence of two known PMCA4-interacting partners. Earlier, it was shown that nNOS and CASK interact with PMCA4b under capacitating and uncapacitating conditions, respectively, of both murine (Olli, Martin-DeLeon. *In Revision*) and human (Andrews et al., 2015) sperm. In **Figure 3.9**, using WT and KO testis as positive control, it is observed that both WT and *Pmca4* KO Oviductosomes carry nNOS (155 kDa) and CASK (100 kDa).

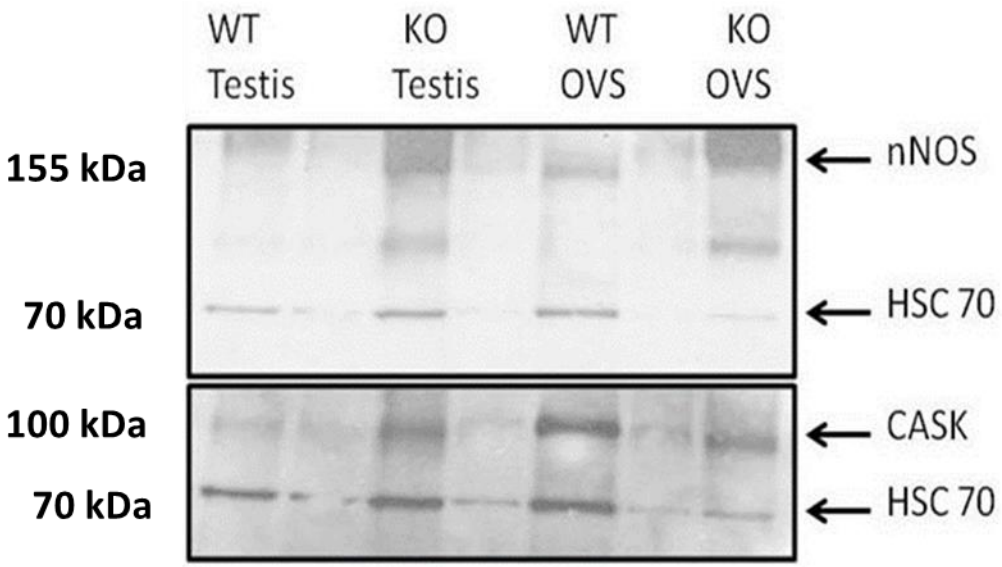


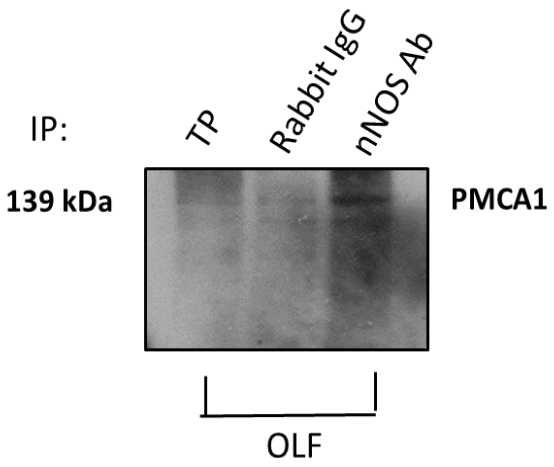
Figure 3.9 Detection of nNOS and CASK in Oviductosomes (OVS). WT and *Pmca4* OVS show a 155 kDa band of nNOS and 100 kDa band of CASK. Protein lysates of WT and *Pmca4* KO testis were used as positive controls.

3.8 Interaction of PMCA1 with nNOS and CASK via Co-Immunoprecipitation analysis

Co-immunoprecipitation was performed to determine if nNOS and CASK are interacting partners of PMCA1 in sperm, as they are for PMCA4. The proteins present in oviductal luminal fluid (OLF) were treated with either nNOS or CASK antibodies. Western of Co-IP **(Fig. 3.10)** for PMCA1 shows that PMCA1 was able to co-immunoprecipitate nNOS **[Fig. 3.10(A)]** and CASK **[Fig 3.10(B)]** while IgG did not

show any bands. This indicates that PMCA1 associates with its interacting partners in oviductal luminal fluid, where oviductosomes are present.

A)



B)

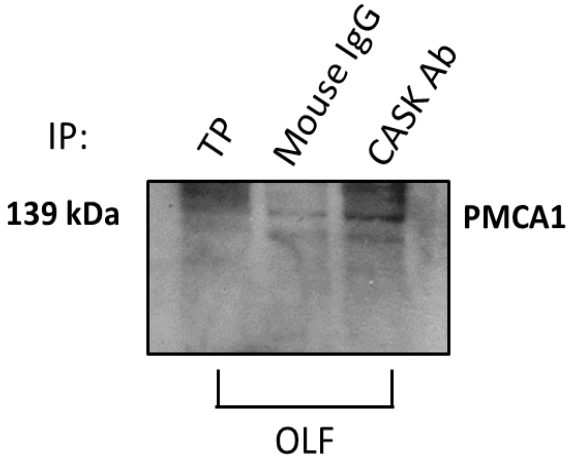
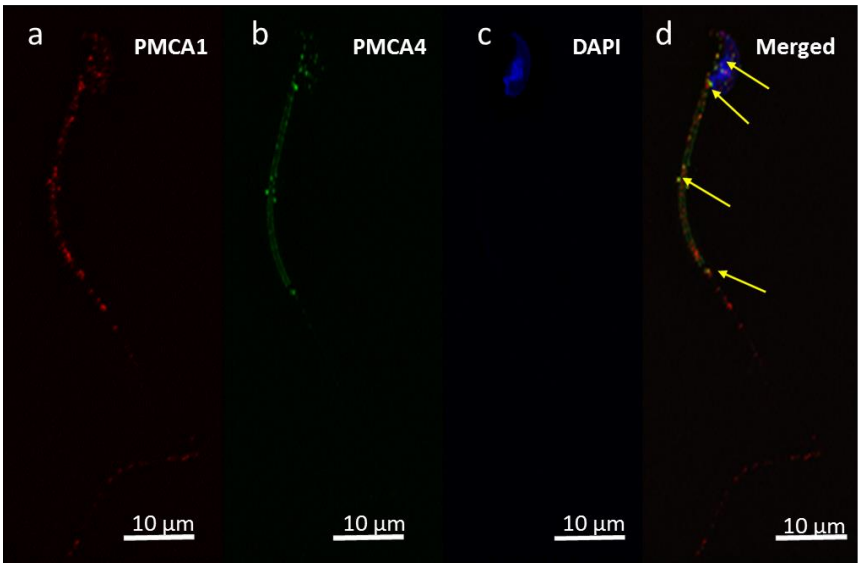


Figure 3.10 Co-Immunoprecipitation of nNOS and CASK with PMCA1 in Oviductal Luminal Fluid (OLF). (A) PMCA1 co-immunoprecipitates with nNOS in OLF showing a 139 kDa band. Total protein (TP) obtained from OLF was used as a positive control whereas rabbit IgG was used as a negative control. **(B)** CASK and PMCA1 co-immunoprecipitate in OLF but not in IgG which was used as a negative control. Total Protein (TP) was used as a positive control.

3.9 Localization and Co-localization of PMCA1 and PMCA4

When capacitated sperm were examined by immunofluorescence and Super-resolution SIM technology, PMCA1 was shown to localize over the head, the midpiece and the proximal principal piece, as shown in **Figure 3.11(A) (Panel a)**. There is more intense staining on the midpiece and weaker staining on the principal piece. Co-localization of PMCA1 and PMCA4 on capacitated sperm revealed that PMCA1 and PMCA4 co-localize and overlap at the head, the midpiece and the proximal principal piece, where the red signal of PMCA1 merges with green of PMCA4 to give a yellow coloration [**Fig 3.11(A) Panel d**]. Thus the distribution of PMCA1 is similar to that of PMCA4. Negative controls are seen in **Fig 3.11(B)**.

A)


a
PMCA1
b
PMCA4
c
DAPI
d
Merged
10 µm
10 µm
10 µm
10 µm

B)

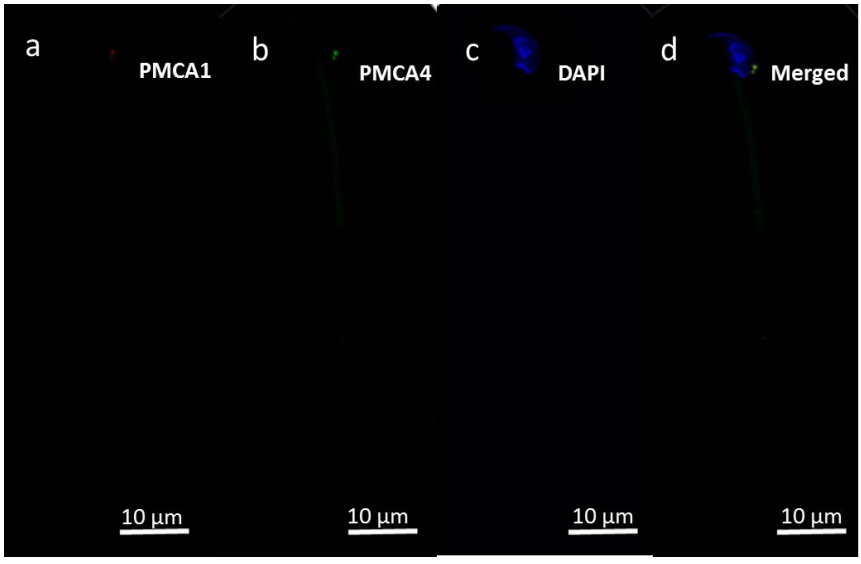

a
PMCA1
b
PMCA4
c
DAPI
d
Merged
10 µm
10 µm
10 µm
10 µm

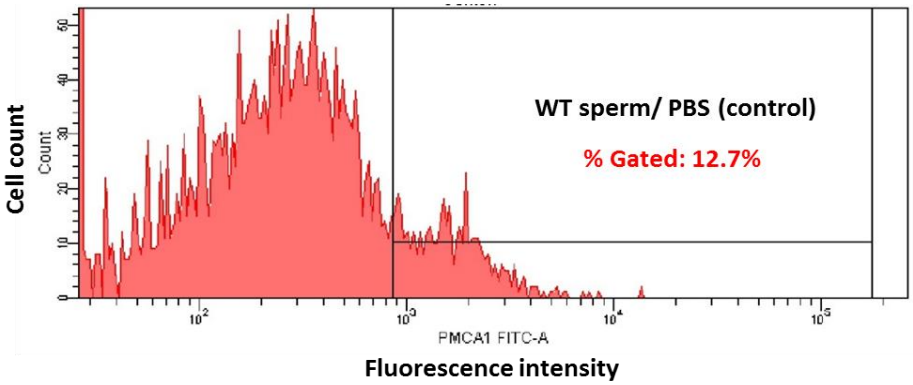
Figure 3.11 Co-localization of PMCA1 and PMCA4 on capacitated sperm. (A) PMCA1 (red) and PMCA4 (green) co-localize on capacitated sperm at the head, midpiece and proximal principal piece (Panel d). Panel (a) shows the red staining of PMCA1 on the head, midpiece and principal piece. Panel (b) shows the green staining of PMCA4 on the head, midpiece and proximal principal piece. Panel (c) is DAPI staining and Panel (d) is merged image. **(B)** Negative control for PMCA1 and PMCA4 where no primary antibody was added. Panel a and b show the absence of a fluorescent signal. Panel c shows DAPI staining and Panel d shows the merged image.

3.10 *In Vitro* Uptake of PMCA1 on Caudal Sperm from OVS via Oviductal Luminal Fluids

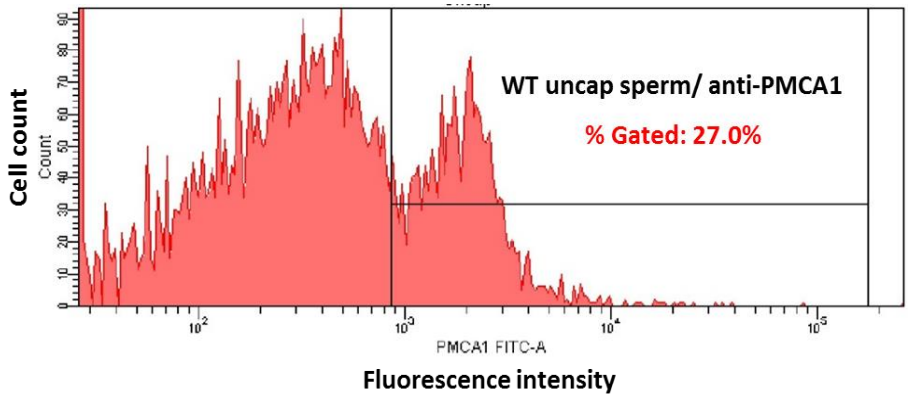
Uptake studies were performed to investigate the potential delivery of PMCA1 from oviductal luminal fluid to sperm, as it was previously shown that sperm acquire PMCA4a from luminal fluids during in vitro incubation (Al-Dossary et al., 2013). PMCA1 present in oviductal luminal fluid was delivered to caudal sperm following the co-incubation of sperm with OLF for 3h **(Fig 3.12)**. Figure 3.12 shows that the amounts of protein in the sperm surface were directly related to the capacitation states of sperm. There is a 2-fold increase in the percentage of uncapacitated cells in the

region marked for the highest fluorescence intensity for PMCA1 following their co-incubation in OLF, compared to those in the PBS control **[Fig 3.12(B)].** On the other hand, there is a 5-fold increase in the percentage of cells in the same fluorescence region when capacitated sperm were co-incubated in OLF **[Fig 3.12(C)]**, as compared to the PBS control **[Fig 3.12(A)]**.

A)



B)



C)

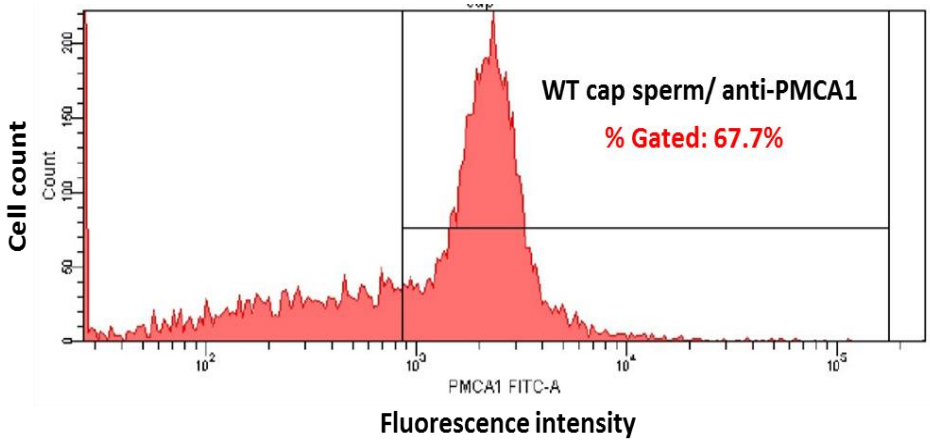


Figure 3.12 Uptake of PMCA1 by caudal sperm following the co-incubation of sperm in OLF. Flow cytometric analysis of ~ 6900 cells treated with PMCA1 primary antibody. **(A)** This graph shows that only 12.7% of the cells showed fluorescence when sperm were co-incubated in PBS. **(B)** When uncapacitated sperm were co-incubated with OLF there was 27% of the cells exhibited fluorescence, reflecting a 2-fold increase, compared to control. **(C)** Co-incubation of capacitated sperm with OLF revealed 67.7% of the cells displayed fluorescence, or 5-fold increase, compared to control.

Chapter 4

DISCUSSION

4.1 Expression and Secretion of PMCA1 in the Murine Female Reproductive Tract

Previous work in our lab showed that PMCA4a is expressed and secreted in the murine female reproductive tract (all three regions) and luminal fluids (Al-Dossary et al., 2013). PMCA4a was expressed in higher levels in the luminal fluids during proestrus/estrus when mating occurs, while only marginal levels were secreted during metestrus/diestrus (Al-Dossary et al., 2013), showing that PMCA4a levels are regulated during the estrus cycle. Further during estrus, of the three luminal fluids, (OLF, ULF and VLF) the highest level of PMCA4a was present in the OLF at the site of fertilization; while the lowest level was seen in the VLF (Al-Dossary et al., 2013). Additionally, PMCA4a was found to be expressed the exclusively in extracellular vesicles of the OLF or oviductosomes (OVS) (Al-Dossary et al., 2013) which deliver it to sperm (Al-Dossary et al., 2013), via a fusion mechanism (Al-Dossary et al., 2015).

Since sperm in the oviduct, particularly at the fertilization site (AIJ), have high levels of cytosolic [Ca^{2+}], PMCA4a that is delivered to them during estrus prevents Ca^{2+} overload and maintains their viability, ultimately resulting in their ability to effect fertilization. However, despite the vital role of PMCA4a in the oviduct, ablation of *Pmca4* in the mouse does not show any effect on female fertility (Okunade et al., 2004). The basis of the normal fertility in females is unknown, but might be due to compensatory mechanisms for PMCA4 in the female tract. In addition to PMCA4, the only other ubiquitously expressed PMCA isoform is PMCA1, and it is likely that it is also expressed in the female tract. To date, there have been no studies investigating the presence, localization and role of PMCA1, in female tract.

In this study, with the use of immunofluorescence (IF), I show that PMCA1 is expressed in all three regions of female tract: oviduct, uterus and vagina **(Fig. 3.1)**. IF also revealed that the oviduct had the most intense staining of PMCA1. When PMCA1 expression was compared in WT and *Pmca4* KO oviduct, the staining appeared to be more intense in the latter **(Fig. 3.2)**. Notably, all the three regions of the female tract exhibited PMCA1 staining on the luminal side of apical membrane of epithelium. This suggests that PMCA1 is secreted into lumen of these tissues, similar to PMCA4a.

Western analysis was used to test the presence of PMCA1 and to quantify the relative distribution. PMCA1 was expressed in similar levels in all female reproductive tissues of WT and *Pmca4* KO, as it is in the testis **(Fig. 3.3)**.

Interestingly, OLF from *Pmca4* KO females had elevated levels of PMCA1 compared to WT; and in KO males, ELF was significantly lower than KO OLF **(Fig. 3.4)**. This indicates that PMCA1 is elevated only in KO OLF and provides strong support for our hypothesis that PMCA1 compensates for PMCA4 loss.

In naturally cycling females, OLF collected during metestrus/diestrus displayed a significant decrease of PMCA1 in *Pmca4* KO OLF compared to WT; but during proestrus/estrus, there was a significant increase **(Fig. 3.5)**. As there was no significant difference in PMCA1 between proestrus/estrus and metestrus/diestrus of WT OLF, it appears that PMCA1 is not regulated during the estrus cycle, unlike PMCA4 (Al Dossary et al. 2013). This suggests that these proteins are not redundant, but have different functional profiles.

The data in Fig. 3.5 indicate that PMCA1 levels vary only among the genotypes of each stage. The increased PMCA1 expression in the OLF of KO females during proestrus/estrus is consistent with the mating behavior of females, if OLF PMCA1 is acquired by sperm. The increased secretion of PMCA1 during estrus in *Pmca4* KO OLF is physiological and may be required to extrude the increased intracellular [Ca^{2+}] encountered by sperm in the female, as a substitute for PMCA4.

In this vain, superovulated females showed a significant upregulation of PMCA1 in *Pmca4* KO OVS, in comparison to WT **[Fig. 3.6(B)]**. This reveals that the

data obtained from *Pmca4* KO OLF during estrus is consistent with that for *Pmca4* KO OVS. Unlike OVS, uterosomes did not show an increase of PMCA1 in *Pmca4* KO females **[Fig. 3.6(D)]**. This indicates that PMCA1 is selectively secreted via OVS from the apical surface of the secretory cells of the oviductal epithelium. This observation is consistent with the notion that OVS are crucial for the final stages of capacitation in the female tract. Thus, the data suggest that PMCA1 is compensating for the loss of PMCA4 in the oviduct, in *Pmca4* KO in order to maintain Ca^{2+} homeostasis in sperm following capacitation, hyperactivation and acrosome reaction and therefore these sperm remain viable and fertile.

It should be noted that PMCA1 is less efficient in its activity and is less stable than PMCA4 (Guerini et al., 2003). Thus the 13-fold increase of PMCA1 in *Pmca4* KO OVS **[Fig. 3.6(B)]** may be necessary to compensate for the more efficient and stable PMCA4 in the oviduct where calcium extrusion is vital for the fertilizing sperm and for ciliary action in the oviductal epithelium. To my knowledge, this is the first report showing a significant upregulation of a PMCA isoform (PMCA1) in the absence of another.

4.2 Isolation, Characterization and Identification of Cargo of Extracellular Vesicles in the Vaginal Luminal Fluid

Extracellular vesicles (exosomes/microvesicles) were identified for the first time in the vaginal fluid and characterized based on TEM negative staining, size and biomarkers such as CD9 and HSC70 **(Fig. 3.7, 3.8)**. They were termed "Vaginosomes". For the first time, I identified that vaginosomes carry PMCA1 **[Fig. 3.8(A)]** and that there is no difference in PMCA1 levels between WT and *Pmca4* KO vaginosomes **[Fig. 3.8(B)]**, as is the case for uterosomes. These vesicles are likely to play a role in communication, such as the vaginal epithelial cells and sperm.

4.3 PMCA4 Interacting Partners are present in OVS and they interact with PMCA1

Recently, we postulated that PMCA4 interacts with eNOS, nNOS, CASK and CAV-1 in both murine (Olli, Martin-DeLeon et al., *in revision*) and human (Andrews et al., 2015) sperm. As these interacting partners are essential to maintain calcium homeostasis in sperm and to prevent oxidative stress, I investigated the presence of these proteins in OVS. Here, I show that nNOS and CASK, interacting partners of PMCA4, are present in both *Pmca4* KO OVS and WT **(Fig. 3.9)**. This suggests that nNOS and CASK would be delivered to sperm via OVS.

With the identification of interacting partners of PMCA4 (nNOS and CASK) in OVS, Co-IP studies were performed to see if PMCA1 interacts with nNOS and CASK in OLF. It was revealed that PMCA1 in OVS interacts with nNOS and CASK **(Fig. 3.10)** similar to that of PMCA4 in the sperm (Andrews et al., 2015). This observation suggests then, when PMCA1 is delivered to sperm, via OVS, this occurs in a complex with NOS or CASK. This efficient method of protein transfer for OVS was recently reported for epididymosomes (Patel et al., 2013, Martin-DeLeon, 2015).

4.4 PMCA1 Co-localizes with PMCA4 on Sperm

From earlier studies it was known that PMCA4 is present on the membrane over the acrosome and proximal principal piece of flagellum (Aravindan et al., 2012, Al-Dossary et al., 2015). In addition to this, with the use of nanoscopy our Lab showed that PMCA4 is also present on the inner acrosomal membrane (IAM), posterior head and the neck of murine sperm (Al-Dossary et al., 2015). With the help of super-resolution SIM technology, for the first time, PMCA1 was localized on murine sperm at the head, midpiece and principal piece **[Fig. 3.11 (A)]**. I also show that on capacitated sperm, PMCA1 co-localizes with PMCA4 on the head, midpiece and proximal principal piece **[Fig. 3.11 (A)]**, suggesting that PMCA1 have a similar function that of PMCA4.

4.5 Transfer of PMCA1 from Oviductal Luminal Fluids to Sperm during different Capacitating states

Since sperm do not undergo transcription and translation, they obtain key molecules from the environment to maintain their viability and functionality. Oviductosomes carrying PMCA4 were shown to have the ability to transfer it to sperm in order to reload them. It is highly likely that other molecules that are crucial for their function are delivered to sperm. Sperm require additional PMCA4 to sustain their motility which is pivotal for fertilizing an oocyte. Likewise, in this study, I detected that PMCA1 is transferred from OVS, via OLF to caudal capacitated and uncapacitated sperm **(Fig. 3.12)**.

Capacitated sperm exhibited a greater than 2-fold increase in the amount of PMCA1 on the sperm surface after they were co-incubated in OLF **[Fig. 3.12(C)]**. The increase in capacitated sperm may have resulted from two factors: 1) Capacitated sperm are likely to have a subpopulation of AR sperm in which the IAM is exposed to the anti-PMCA1 antibody. As mentioned above PMCA4 was shown to reside on the IAM. 2) The IAM membrane acquires PMCA1 from OVS. It is likely that both occur. Hence, these data imply that sperm acquire PMCA1 from OLF as that of PMCA4 and also suggests that PMCA1 is present in inner acrosomal membrane, similar to PMCA4.

Altogether, my study demonstrates that when PMCA4, a key element of Ca^{2+} homeostasis is absent, PMCA1 is significantly upregulated in the female, compensating for *Pmca4*'s loss. Also, PMCA4's interacting partners are present in OVS and interact with PMCA1 which is transferred to sperm via OVS from OLF (similar to PMCA4), demonstrating that PMCA1 is an effective surrogate for PMCA4.

4.6 Conclusions

Based on the results of the current study, the following conclusions can be made:

1) For the first time, the expression of PMCA1 in the female reproductive tissues, luminal fluids and exosomes was identified.
2) During estrus PMCA1 expression levels are increased in *Pmca4* KO oviductal luminal fluid (OLF) and significantly upregulated in *Pmca4* KO oviductosomes (OVS) compared to WT showing that PMCA1 is selectively secreted in OVS.
3) Extracellular membrane vesicles in vaginal luminal fluid were identified and characterized for the first time and dubbed as "Vaginosomes" (VS), and shown to contain PMCA1.
4) Unlike oviductosomes, PMCA1 is not elevated in uterosomes and vaginosomes in *Pmca4* KO females during estrus, suggesting that PMCA1

acts as a compensatory mechanism for PMCA4's loss in the oviduct where sperm undergo increased intracellular [Ca^{2+}] environment and where ciliary action is required for oocyte transport.

5) Interacting partners of PMCA4, such as nNOS and CASK, in sperm are present in OVS and interact with PMCA1, similar to PMCA4 in sperm.
6) Unlike CASK nNOS, which is regulated by PMCAs, is upregulated in *Pmca4* KO OVS. This is parallel to the upregulation of PMCA1 in OVS of the nulls.
7) I localized PMCA1 for the first time on the head, midpiece and principal piece of sperm and showed that it co-localizes with PMCA4 on the head, midpiece and proximal principal piece during capacitation, suggesting a similar function of PMCA4 and PMCA1 in sperm.
8) Similar to PMCA4, PMCA1 is acquired from OLF by sperm following *in vitro* co-incubation. The pattern of uptake indicates that PMCA1, in addition to being on the sperm plasma membrane, resides on inner acrosomal membrane of sperm, similar to PMCA4.
9) Overall the data show that PMCA1 acts as a true surrogate for PMCA4 in *Pmca4* KO females and might be one of the reasons for normal fertility in *Pmca4* KO females.
10) PMCA1 is not regulated during the estrus cycle, unlike PMCA4, indicating that these proteins are not redundant, but may have different functional profiles.

4.7 Future Directions

Several questions emerged from the study and should be considered in the future.

1) To determine if *Pmca1* and *Pmca4* are responsible for normal fertility in female tract by performing double Knockout of *Pmca1*/*Pmca4*.
2) To determine if PMCA1 and its interacting partners are delivered to sperm from OVS as a complex by performing co-localization assays.

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

APPROVAL

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

RECEIVED
OCT 8 2015
IACUC JH

Title of Protocol: Mechanism of Sperm motility in *Pmca4* null mice and he Role of Oviductosomes	
AUP Number: 1181-2015-2	← **(4 digits only)**
Principal Investigator: Patricia A. DeLeon	
Common Name: Mouse **Genus Species:** *Mus Musculus*	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: *(Note change of categories from previous form)*

Category	Description
☐ **B**	Breeding or holding where NO research is conducted
x ☐ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glenn Tallman, DVM

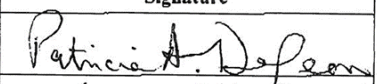
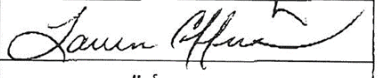
Date of Approval: 12/1/15

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances. Patricia A DeLeo Signature of Principal Investigator 10-08-2015 Date

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Patricia A. DeLeon	
2. Lauren Coffua	
3. Pradeepthi Bathala	
4. Tori Mallardi	
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4. **Project Personnel:** Have there been any personnel changes since the last IACUC approval?

x ☐ Yes ☐ No

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Amal Al Dossary	Click here to enter text.
2. Kathie Wu	Click here to enter text.
3. Rebecca Pollak	Click here to enter text.
4. Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

Preliminary data were used for the submission of an RO1 NIH grant application
We made a major discovery of oviductosomes and a manuscript has been submitted for publication.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

An adverse effect has been seen with the mating of the animals—due to the MRI building construction we have detected a cessation in mating of the animals all summer. Within the last two weeks they have begun to mate again. But we lost precious time and money during the summer.