Mechanisms of oocyte activation in the presence and absence of PLCζ

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A thesis submitted for the degree of Doctor of Philosophy

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Abstract

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Mammalian oocyte activation is thought to be caused by a sperm borne PLC subtype, called phospholipase C zeta1 (PLCζ). PLCζ RNA and protein microinjection and knockout studies in a mouse model have confirmed its primary role in releasing calcium (Ca^{2+}) in the oocyte at fertilisation. Studies revealed that a few delayed Ca^{2+} transients can still occur in the oocyte after fertilisation with PLCζ knockout sperm, resulting in a reduced embryo formation rate and increased polyspermy, but otherwise apparently normal development to term, indicating the presence of a secondary factor(s) which can stimulate Ca^{2+} release in the oocyte. In this study, an attempt was made to identify such a secondary factor(s). *In silico* analysis of transcriptomic data revealed that mature sperm cells carry transcripts of PLCβ, PLCγ, PLCδ, and PLCζ subtypes. In MII oocytes, PLCβ1, PLCβ3, PLCγ1, and PLCγ2 were reported to be expressed. The mRNA and protein expression analysis showed reorganised expressions in the PLCζ knockout sperm cells. This reorganised expression levels of IP_3 generators led to investigate PLC subtypes as potential secondary factors of oocyte activation. Proteomic analysis of PLCζ knockout sperm cells suggested that some proteins showing differential expression that are overexpressed in PLCζ knockout sperm participate in acrosome reaction, fusion and binding of gametes. Pharmacological interventions and Ca^{2+} dynamics investigations suggest that interference with Gαq (Gq) mediated PLCβ activation has a significant impact on Ca^{2+} release as well as fertilisation indicators, for both PLCζ knockout and wild type sperm, in distinct ways. Blocking Gq mediated PLCβ activation also blocks secondary Ca^{2+} transients triggered by PLCζ deficient sperm. Collectively, these data suggest that gametes express a number of PLC isoforms with differential expression in PLCζ knockout sperm, and PLCβ isoforms can act as potential secondary factors during oocyte activation thorough the Gq pathway, in parallel to PLCζ.

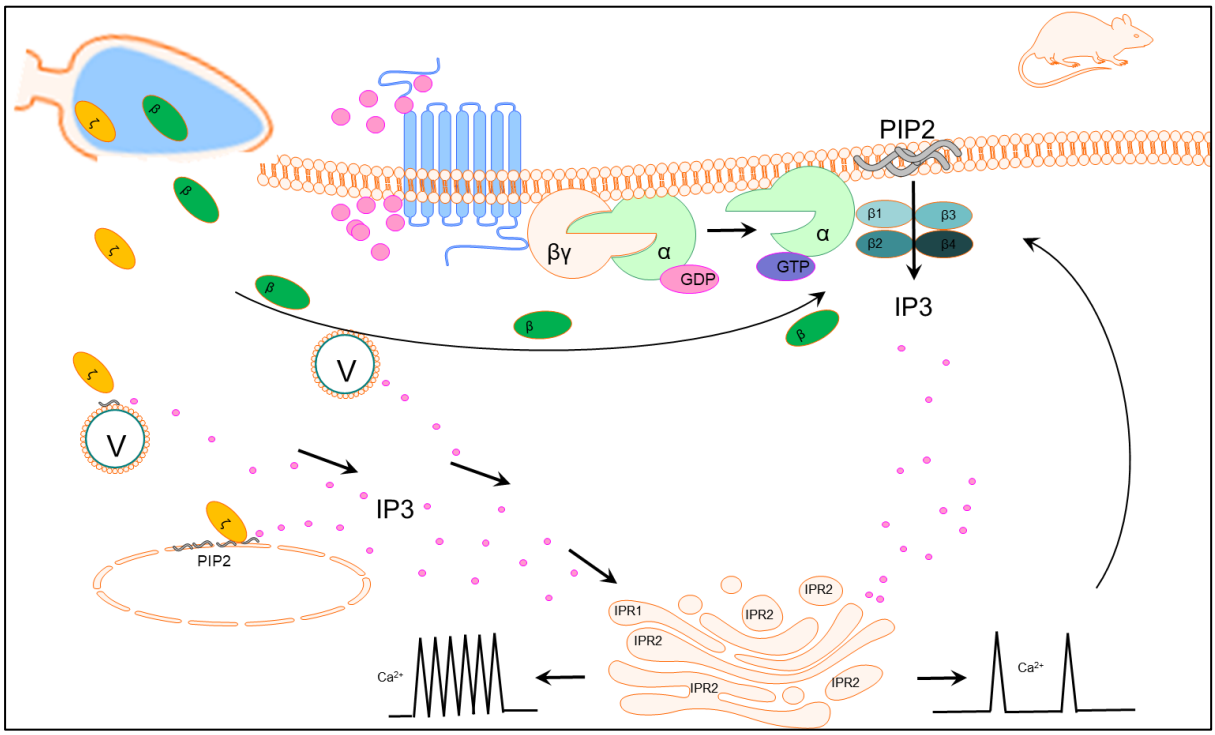


Figure 1 Proposed mechanism of action of secondary factors implicated in oocyte activation.

Fusion of sperm and oocyte membranes possibly activating G-proteins linked to Gq on the oocyte's membrane which partially stimulates PLCβ isoforms. Post-fusion the sperm releases its cytoplasmic PLCζ and other PLC isoforms into the oolemma. PLCζ translocates to vesicular and nuclear membrane where it targets PIP_2 to generate IP_3. The IP_3 generated by PLCζ triggers the typical Ca^{2+} oscillations in MII oocytes and may be supplemented by activation of Gq stimulated oocyte or sperm PLCβ.

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Abbreviations

The following is a list of abbreviations used throughout this thesis. Less frequently occurring abbreviations are described in the text where appropriate

APC	anaphase promoting complex
BLAST	Basic Local Alignment Search Tool
BSA	albumin, bovine serum
cADPR	cyclic ADP-ribose
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
cDNA	complementary DNA
CICR	calcium-induced calcium release
COCs	cumulus-oocyte-cells
DAG	diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole dye
DBTMEE	database of transcriptome in mouse early embryos.
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
FPKM	fragments per kilobase of transcript per million fragments mapped
GNAQ	guanine nucleotide binding protein [G protein]
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidylinositol
Gαq	Gq alpha subunit
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HET	heterozygous
HTF	human tubal fluid medium
IP3	inositol 1,4,5-trisphosphate
IVF	*in vitro* fertilisation
JPEG	joint photographic experts group file format
LFQ	label-free quantification intensity
MAPK	mitogen-activated protein kinase
MII	metaphase II
MPF	maturation promoting factor
NaCl	sodium chloride
ORAI	calcium release-activated calcium channel protein

PAWP	WW domain-binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI(4,5)P2	phosphatidylinositol 4,5-trisphosphate
PIP2	phosphatidylinositol 4,5-trisphosphate
PLCs	phospholipase C isoforms
PLCXD	phosphatidylinositol specific phospholipase C X domain containing isoforms
PLCβ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta isoforms
PLCγ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma isoforms
PLCδ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta isoforms
PLCε	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon isoforms
PLCζ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta
PLCζ KO	phosphodiesterase zeta knockout
RT	reverse transcription
RT-qPCR	quantitative reverse transcription PCR
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
STIM	stromal interaction molecule
WT	wild-type
ZP	zona pellucida

Chapter 1: General Introduction

Fertilisation, a biological process of gamete fusion (*Fertilization | Definition of fertilization in English by Oxford Dictionaries*, 2019), also called syngamy, conception and fecundation, is a natural biological process involving fusion of gametes to achieve the formation of a new organism. In mammals, it occurs internally in the female body but can be achieved externally by artificial methods. Fertilisation is the essential, first phase of the physiological process of embryogenesis which eventually leads to a completely new organism. It differs from species to species in terms of its various stages but the endpoints of each series of processes are the same and can be grouped as five stages, namely gamete recognition, membrane binding, membrane fusion, oocyte activation, and fusion of the male and female pronuclei. Recognition and contact between the gametes ensures that they interact with those of the same species. Once the gametes recognise each other, next follows membrane binding/fusion which results in release of the contents of the sperm into the oocyte. Such a membrane fusion event is followed by oocyte activation which ensures the blockage of further fusion of sperm with the oocyte, and this process also facilitates fusion of the male and female pronuclei and the creation of a zygote (Gilbert, 2000).

1.1 Stages of fertilisation

Maturation of the gametes is an essential prerequisite of fertilisation. An oocyte is mature if it has successfully developed to the metaphase II (MII) stage from a primordial oocyte with a polar body. In contrast a sperm cell is considered mature after it has undergone *capacitation* which is achieved *in vivo* when a sperm cell passes through the female reproductive tract.

When first discovered, sperm cells were believed to be parasitical microbes that survive in the semen. Anton Leeuwenhoek first reported mobility character of sperm cells in 1670s when viewed them under the microscope, hence they were named spermatozoa. As our understanding of the male gamete has evolved, our knowledge of its morphology has been updated in the course of time. Consisting of a haploid nucleus and losing most of its cytoplasm during spermiogenesis, the sperm cell also contains a sac of digestive enzymes called the acrosomal vesicle, and of course a tail, also known as the axoneme, to facilitate its mobility. A developed sperm is thus composed of a head, an acrosomal vesicle on top of this underlain by a packed nucleus; a mid-piece full of mitochondria; and a comparatively very long tail which extends to the very end of the sperm (Gilbert, 2000).

An oocyte is an advanced and complex cell system that provides an environment for formation of the zygote which develops following a successful fertilisation, and subsequently allows further development of the embryo. Morphologically, a mature oocyte is quite like other, somatic cells except for its outer protective envelope (known as the zona pellucida), ploidy and size. Compositionally, an oocyte contains nutritive proteins, a huge quantity of ribosomes and tRNAs, morphogenetic factors, and protective chemicals (Gilbert, 2000).

1.1.1 Recognition and contact of oocyte & sperm

Before membrane fusion, gamete interactions ensure the right selection of cell and species. Gamete interaction comprises of chemoattraction, binding of sperm to the oocyte zona pellucida, exocytosis of acrosomal enzymes and tunnelling of the sperm through the zona pellucida, and finally binding/fusion of the gamete membranes (Gilbert, 2000). Several studies have confirmed that oocytes are able to release chemical signals into the extracellular environment to activate relevant receptors on sperm cells that facilitate gamete interaction (Inamdar et al., 2007; Metz, 1978; Y L Miao et al., 2012; Seifert et al., 2015). In echinoderms, the directional signal is provided by sperm-activating peptides e.g. resact, a potent peptide that leads to the movement of sperm cells towards oocytes (Metz, 1978). *In vitro* injection of resact confirmed its active role in chemoattraction and its specificity towards sperm cells of the same species (Ward et al., 1985). In mammals, capacitation of sperm cells (Cohen-Dayag et al., 1995) and an optimum concentration of chemo-attractants are prerequisites for gamete responsiveness (Strünker et al., 2015). Some of the reported biochemical sperm chemo-attractants comprises beta-defensin 126 (Narciandi et al., 2016), alluring (Burnett et al., 2012), nitric oxide (Miraglia et al., 2007), and progesterone (Lishko et al., 2011; Villanueva-Diaz et al., 1995).

1.1.2 Membrane fusion

Once the sperm interacts with the oocyte's zona pellucida, sperm cells are ready to carry out the acrosome reaction which is a necessary preliminary to association

of the sperm and oocyte membranes. The acrosome reaction is a two-stage reaction that starts with exocytosis of the acrosomal vesicle and is followed by the second stage in which the mixture of acrosomal enzymes are released onto the zona pellucida (Gilbert, 2000). Sperm G protein-coupled receptors (GPCRs) and pertussis toxin-insensitive receptors interact with the zona pellucida proteins (ZP1, ZP2, ZP3) and progesterone. Stimulation of these receptors raises intracellular calcium (Ca^{2+}) levels in sperm cells in a signalling pathway involving phospholipase C beta1 (PLCβ1) and adenylate cyclase which further activates IP_3 receptors on the acrosomal membrane (Simons & Fauci, 2018). Stimulation of the acrosome reaction results in release of enzymes that allow penetration of the zona pellucida by the sperm. Following crossing of the zona layer, the sperm can take part in gamete membrane fusion, which involves proteins on the sperm and oocyte (Schultz & Williams, 2005). The acrosome reaction also triggers binding of sperm protein izumo to the oocyte protein juno to loosen its tight packing. Izumo knockout sperm showed an inability to fuse with the oocyte membrane and were therefore unable to initiate fertilisation but bypassing the fusion step allowed fertilisation suggesting an essential role for the izumo/juno interaction in gamete binding/fusion (Inoue, 2017). Other proteins reported to play important roles in sperm-oocyte binding/fusion are sllp1, integrins, CD46, tetraspanins, CD9, CD81, folate receptor 4, GPI anchored proteins, and sas1b (Klinovska et al., 2014).

1.1.3 Oocyte activation and zygote formation

Following successful gamete membrane fusion, the next step is the release of the sperm contents into the oocyte. But the oocyte also has to prevent multiple sperm

cells from entering it, the so-called block to polyspermy. This barrier system is part of a process that is initiated after gamete fusion which is called oocyte activation. The term oocyte activation can also be understood by counterposing it to the oocyte's metaphase arrest phase when it is inactive. Once the sperm fuses with the oocyte it initiates a series of biochemical reactions that allow release of the oocyte from its arrested phase and its subsequent ability to begin the process of mitotic division and embryo development. Release of the sperm nucleus into the oocyte's cytoplasm initiates the fusion of genetic materials from each gamete. Each gamete contributes a haploid genome to the zygote while the mitochondrial genome is entirely sourced from the oocyte. The sperm centrosome's microtubules carry out the dissolution of the nuclear envelope, allowing the nuclei to combine. The chromatin forms chromosomes that align along the mitotic spindle. The fused state of the genetic material upgrades a fertilised oocyte to the zygote stage in which the pronuclei last for around approximately 12 hours and then the first mitotic cleavage occurs (Gilbert, 2000).

1.2 Electrophysiological behaviour of oocytes at fertilisation

Some studies have investigated the electrophysiology of oocytes during the fertilisation process and how this changes. Findings from such studies suggest that the membrane potential of the oocyte changes as the fusion process proceeds. Pharmacological studies suggest that several voltage-dependent and -independent channels show on-off behaviour during the fertilisation process. Studies on mature metaphase II human oocytes using the voltage-clamp technique indicate that oocytes have a resting potential at 14 ± 10 mV with no current flow at control medium injection. Following injection of sperm extract, oocytes showed a current flow at 400-1000 pA which was similar to that observed in the presence of

Ca^{2+} ionophore, A23187, and IP_3 (Dale et al., 1996). This current is also known as the fertilization current. During the activation process, the oocyte hyperpolarises with a Vd from -20mV to -60mV which takes around an hour, and then returns back to its resting -20mV. Another study showed the fertilization current rise to be up to 300 pA which reached its peak one-hour post insemination. This current was reported to be Ca^{2+}-dependent and showed no significant rise in the presence of EGTA which suggests a relationship between cytosolic free Ca^{2+} and membrane potential which is not common to all mammalian species, since human and hamster oocytes show a Ca^{2+} dependency of the membrane potential whereby mouse oocytes are independent. Gating of Ca^{2+}-activated K^+ channels and nonspecific channels was also observed (Gianaroli et al., 1994). There is a possible involvement of membrane potential in the block to polyspermy that still needs to be explored.

Can membrane voltage change stop the further entry of sperm into the oocyte? Recently, a study investigated the effect on activation current of entry of more than one sperm in sea urchin oocytes. The first Ca^{2+} signal was observed near the site of fusion and then spread throughout the egg. A Ca^{2+} current and accumulation was observed at the site of fusion which represents Ca^{2+} influx through the sperm membrane or sperm cytoplasm. This accumulation was over the egg's Ca^{2+} buffer level. The findings indicated a correlation between fusion site accumulated Ca^{2+} sparks and failure of fusion of the second sperm, this latter process being Ca^{2+}-dependent (Ivonnet et al., 2017) and Na^+ independent (Limatola et al., 2019) which suggests that the electrophysiology of the oocyte membrane changes only after fusion of the oocyte with the first sperm and Ca^{2+} entry. To address whether the

same mechanism of polyspermy block might be operational in mammalian oocytes will require further investigation (Ivonnet et al., 2017).

1.3 Oocyte activation- a multi-task process

Oocyte activation is initiated by changes in intracellular Ca^{2+}in the oocyte cytoplasm that occur following sperm-oocyte binding/fusion. In mammals these changes usually occur in an oscillatory manner, hence they are called Ca^{2+} oscillations. Oocyte activation encompasses resumption of meiosis, cortical granule exocytosis and initiation of maternal mRNA translation (Gilbert, 2000). At the molecular level oocyte activation involves release of Ca^{2+} from intracellular stores, apparently primarily through the IP_3 pathway, suppression of activity of mitogen-activated protein kinase (MAPK) protein, activation of anaphase promoting complex (APC), and inactivation of maturation promoting factor (MPF) which is composed of cdk kinases and cyclin B. Overall alteration of the above-mentioned proteins allows resumption of the meiotic cell cycle and subsequent initiation of the first mitotic cell division. These changes are essential for embryogenesis to begin, and impairment in any step can lead to developmental arrest. Successful fertilisation both degrades some maternal mRNAs and changes the proteome of the zygote with the synthesis of new mRNAs and proteins simultaneously (Krauchunas & Wolfner, 2013).

1.3.1 Calcium (Ca^{2+}) oscillations

Intracellular Ca^{2+} is a universal second messenger responsible for regulating numerous physiological reactions in diverse cell types. Mammalian ocyte activation is also one of those reactions that are Ca^{2+}-regulated and is stimulated after a dramatic rise in cytoplasmic Ca^{2+} concentration. A primary source of this

Ca^{2+} is the oocyte's endoplasmic reticulum as in many somatic cells and requires the IP_3 dependent pathway as a central mechanism in releasing Ca^{2+} from the ER stores. The Ca^{2+} rise mainly starts from the sperm-oocyte fusion site and spreads throughout the oocyte uniformly. This rise is followed by a dramatic fall which restores the Ca^{2+} concentration to its resting state. This rise and fall is subsequently repetitive with a similar amplitude and frequency, collectively called 'Ca^{2+} oscillations'. Studies of oocyte Ca^{2+} oscillations are grouped into two categories comprising first, the upstream source and generation of the Ca^{2+} oscillations and second, the downstream effects of the Ca^{2+} oscillations on the fertilized egg. While the initial site of emergence of the Ca^{2+} rise is the site of fusion, later it propagates from the vegetal hemisphere (Shunichi Miyazaki, 2006; Stricker, 1999). Factors responsible for generating these Ca^{2+} oscillations have been extensively investigated. The essentiality of IP_3 receptors and for the Ca^{2+} oscillations have been confirmed indicating the involvement of phospholipase C (PLC) isoforms and the Ca^{2+}-induced Ca^{2+} release pathway (Furuichi et al., 1994; Shunichi Miyazaki et al., 1993; Z. Xu et al., 1994).

1.3.2 Cortical granule exocytosis

Preventative mechanisms make the fertilisation process successful and the block to polyspermy is one of them. Cortical granules are major players in this mechanism. These granular shaped secretory organelles reside in the cortex of oocytes. Structurally, cortical granules are small packets of conformation changing proteins that are released into the perivitelline space after their exocytosis. Exocytosis of the granules is triggered by the sudden rise in the oocyte's intracellular Ca^{2+} levels. This process of cortical granule exocytosis is called the 'cortical reaction'. Exocytosis of the cortical granules is spontaneous, Ca^{2+}-

dependent, and occurs even before completion of fertilisation. A sudden rise in Ca^{2+} triggers exocytosis of a large number of granules to intensify their effect during the fertilisation process and to block polyspermy (Liu, 2011). The signalling pathways that regulate exocytosis of these granules from the oocyte membrane have been recently investigated. Cortical granule movement is mediated by Rab27 and myosin Va through actin filaments and a Rab11a vesicle associated pathway (Cheeseman et al., 2016). Once cortical granules reach the outer membrane, they release hexosaminidase which eliminates the ZP3 attraction towards sperm receptors and inhibits further sperm binding and fusion (Gong et al., 1995; Miller et al., 1992).

1.3.3 Resumption of oocyte cell cycle

Exocytosis of cortical granules and release of the oocyte from the MII arrested state are simultaneous processes and triggered by Ca^{2+} oscillations. Resumption of meiosis and synthesis of proteins from maternal RNAs to initiate embryo development are direct downstream effects of the Ca^{2+} rise during fertilisation. Resumption of the cell cycle is initiated by the degradation of cyclin B1 which is present in a complexed form with cdk kinase; this combination is also called maturation promoting factor (MPF). The degradation of cyclin B1 is achieved by proteasome-mediated proteolysis. High levels of intracellular Ca^{2+} are enough to activate calmodulin and thereby stimulate calmodulin-dependent kinase II (CaMKII) (Lorca et al., 1993). Interestingly, this stimulation of CaMK II is repetitive and happens every time the Ca^{2+} rises in the fertilised oocyte cytoplasm (Markoulaki et al., 2004). Activation of CaMKII promotes anaphase-promoting complex (APC) which leads to poly-ubiquitinated proteolysis of cyclin B1. Before CaMKII action, APC remains inactive because of the effect of cytostatic factor

(CSF). An insufficient concentration and frequency of Ca^{2+} transients may have adverse effects on zygote formation. A few Ca^{2+} transients may allow the oocyte to exit the MII arrest state but can leave it stuck it this state and also have inhibitory effects on MAPK which is essential for pronucleus formation (Ducibella et al., 2002).

1.3.4 Proteome ups and downs during the oocyte-to embryo transition

Degradation of stored mRNA in oocytes during the fertilisation process is observed in many species including mammals. Once the degradation of stored maternal mRNAs is underway, a wave of newly synthesised transcripts emerges at the late stage of fertilisation which helps re-structuring of the fertilised egg's proteome (Krauchunas & Wolfner, 2013).

The fusion of genetic material and transition to the mitotic cell cycle are the very next steps after fertilisation. At molecular levels, these processes are performed by making the change in the structure and composition of the whole fertilised egg. Proteome studies have shown major changes during the transition from oocyte to embryo (Krauchunas & Wolfner, 2013). Surprisingly, these changes are mostly post-translational and post-transcriptional. Quantitative proteome profiling of zygotes suggests that a zygote has only around 60% of the total proteins in unfertilised oocytes which confirms that there is enormous degradation of proteins during zygote formation (Pfeiffer et al., 2011; Shufang Wang et al., 2010). A potential reason for this significant level of degradation could be the release from APC control during meiotic cell cycle resumption. Functionally, APC is a ligase that targets proteins for degradation by the 26S proteasome. Extensive phosphorylation of proteins is likely to happen during zygote formation. CaMKII

and calcineurin are reported to be the main modulators of these changes. The repetitive nature of the Ca^{2+} oscillations during mammalian fertilisation increases the levels of active CaMK II and calcineurin phosphatase multiple times and maintains their effect for a long time providing ample time to phosphorylate proteins.

On the other side, new proteins are synthesised in the zygote that are not found in the mature oocyte prior to fertilisation. The origin of such proteins is maternal mRNAs that are present in mature oocytes in a repressed state and translated during oocyte activation. Recruited to the polysome, this new group of proteins are highly functional and are involved in cell cycle regulation, metabolism, and transcription. These new proteins are synthesised to prepare the zygote to form an embryo and for regulation of further development (Potireddy et al., 2006).

1.4 Theories of oocyte activation

Factors involved in oocyte activation are considered to be the first stage players in the process of the beginning of life. Several candidates have been identified and their roles have been investigated. It is worth mentioning the theories of oocyte activation before listing the associated factors and extent of involvement. Several hypotheses have been put forward about the source of the Ca^{2+} oscillations and the potential triggers of oocyte activation.

Oocyte receptor binding theory: It is universally accepted that cellular interactions at all levels of the animal kingdom including microorganisms are carried out by cell-cell interactions, mediated by chemical signals, followed by receptor activation leading to protein conformational changes and then activity. It is worth noting that a mouse oocyte starts showing Ca^{2+} oscillations 1-3 minutes

after gamete fusion (Kashir et al., 2014) and these oscillations are sustained for hours after this fusion event. From the perspective of the receptor theory, initiation of Ca^{2+} oscillations triggered by a sperm ligand-oocyte receptor interaction in that short time period seems possible. A heterotrimeric guanine nucleotide-binding regulatory protein (G-protein)-coupled receptor (GPCR) mechanism is one candidate for this hypothesis (Z Macháty et al., 2005; S Miyazaki, 1991; Moore et al., 1993; J Tesarik, 2002; Williams et al., 1992). The essentiality of the IP_3 pathway for oocyte activation suggested that Ca^{2+} oscillations induced at fertilisation could be the consequence of activation of an oocyte version of one of the PLCβ isoforms, which are triggered by GPCRs. An alternative possibility would be that an oocyte version of PLCɣ might be activated by a sperm-oocyte interaction that stimulated a tyrosine kinase signalling pathway, as this pathway activates PLCɣ isoforms.

Soluble sperm factor theory: Several experiments support the hypothesis that oocyte activation is triggered by a soluble sperm factor. Researchers have tested this hypothesis by injecting sperm extract/cytosolic contents from several mammalian species including humans, into oocytes of various mammalian species. Possible signalling pathways have also been studied with relation to the mechanism of action of a soluble sperm factor and this has implicated the IP_3 pathway, which suggests potential involvement of a PLC from sperm that triggers repetitive IP_3 production in the oocyte, resulting in Ca^{2+} oscillations (Ducibella & Fissore, 2008; Lee et al., 2006; K Swann, 1990). Extensive investigations have identified the sperm-specific PLCζ as the dominant candidate for the physiological trigger of oocyte activation (Kashir et al., 2014). PLCζ has received great attention over the last decade and has clinical relevance, since mutations in this protein have been linked to human infertility (Hachem et al., 2017; Kashir et al., 2012,

2018; Nozawa et al., 2018). However, several other factors derived from sperm have been reported to be involved in the oocyte activation process, for instance tr-kit and post-acrosomal sheath in perinuclear theca sharing N- terminal homology to WW domain-binding protein 2 (PAWP) (Aarabi et al., 2014; C Sette et al., 1997; Tavalaee & Nasr-Esfahani, 2016).

Sperm Ca^{2+} content/conduit theory: This hypothesis began with the proposal that sperm release Ca^{2+} into the oocyte during gamete fusion. Later, the hypothesis was modified to the suggestion that the sperm acts as a conduit to funnel extracellular Ca^{2+} into the oocyte cytoplasm. This initial Ca^{2+} stimulus then sets off a regenerative Ca^{2+} rise. The regenerative nature of the Ca^{2+} rise is assumed to be due to IP_3 receptors or ryanodine receptors by the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) (Tosti & Boni, 2004). In mammals, studies on increase of cytoplasmic Ca^{2+} in oocytes failed to regenerate further rise/oscillations (Ozil & Swann, 1995). However, a second wave of Ca^{2+} rise was observed in hamster oocytes (Shun-ichi Miyazaki et al., 1992). Imaging studies have also confirmed that no Ca^{2+} rise was observed at the site of fusion in mouse oocytes (Jones et al., 1998). Collectively, investigations have confirmed that in mammals there is no such Ca^{2+} conduit by sperm in the oocytes and this this mechanism seems unlikely because of a lack of strong evidence. As such, the sperm soluble factor(s) and oocytes-sperm receptor binding theories are most likely to explain how the sperm triggers a Ca^{2+} Ca^{2+} rise at fertilisation

1.5 Calcium recruitment at oocyte activation

In mouse and human oocytes, Ca^{2+} oscillations of a prolonged duration are the first indicator of successful oocyte activation during normal fertilisation. It is known that this free Ca^{2+} is released from endoplasmic reticulum (ER) or sarcoplasmic

reticulum (SR) stores following the action of several stimuli (some known and some controversial) and must be refilled to sustain oscillations for hours. Ca^{2+} reuptake by these stores must involve continuously triggering pathways of such reuptake to ensure a continuous supply. In *Xenopus* oocytes cyclic ADP-ribose (cADPR) has been shown to be involved in Ca^{2+} sequestration by modulation of SERCA pump activity (Yamasaki-Mann et al., 2009); however its role has yet to be properly investigated in mammalian cells. It might be possible that several other Ca^{2+} ATPases are acting on the ER/SR to refill these stores. It is worth noting that, at the cellular level, the Ca^{2+} refilling these stores is also recruited from outside the cell by the machinery of voltage-sensitive and -insensitive channels including TRPM and Ca V3.2 (Bernhardt et al., 2015, 2018). Depletion of the intracellular level of Ca^{2+} is also overcome by an influx of Ca^{2+} from the extracellular medium by the machinery of store-operated Ca^{2+} entry (SOCE). There is a correlation between normal fertilisation and store-operated Ca^{2+} entry which can be bypassed by ICSI (Y L Miao et al., 2012). Stim1, an ER-embedded Ca^{2+} level sensor protein recognises ER Ca^{2+}depletion and triggers refilling of Ca^{2+} in emptied ER stores via an association with plasma membrane-bound orai1 channel protein. This machinery of Ca^{2+}-sensing and refilling is not just utilised at fertilisation but also throughout embryo development (Machaty et al., 2017; C. Wang & Machaty, 2013). The importance of stim1 and orai1 at fertilisation has been established in several recent studies and is regarded as essential for normal fertilisation and development (Bernhardt et al., 2018; Machaty et al., 2017; C. Wang & Machaty, 2013). Contrastingly, stim1/2 single and double conditional knockout (KO) mice and orai1-null female mice have no defects in sperm induced Ca^{2+} oscillations and fertilisation which suggests that oocytes can recruit extracellular Ca^{2+} through trpm

and other membrane channels to achieve Ca^{2+} restoration during fertilisation (Bernhardt et al., 2017), a conclusion which has been supported by similar findings in studies of Drosophila oocyte activation (Hu & Wolfner, 2019).

1.6 Ca2+ releasing pathways implicated in oocyte activation

So far the literature suggests a high level of similarity between somatic and gamete cells in the way that cell signalling regulates intra- and extracellular physiological processes. As a universal signalling molecule, the importance of Ca^{2+} remains the same in gamete cells as well and is an essential ion both for fertilisation and development. However, how fertilisation stimulates Ca^{2+} transients is still a question that is only partially understood (Ciapa & Chiri, 2000). This section covers the basic description of signalling pathways with particular relevance to the findings and conclusions from studies investigating fertilisation to study these pathways' roles in this process.

1.6.1 IP3/DAG Pathway

On stimulation, phospholipase C (PLC) enzymes metabolise phosphatidylinositol 4,5-bisphosphate substrate and generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) in the cytoplasm. IP_3 binds to IP_3 receptors (IP_3Rs) and releases Ca^{2+} from intracellular stores. Pharmacological studies have clearly demonstrated that PLC-mediated IP_3 production is a step during mammalian fertilisation (Stith et al., 1993). A second level confirmation of the involvement of the IP_3 pathway at fertilisation was performed by inhibiting IP_3R1 by several means which successfully blocked fertilisation. A correlation was also observed between the sensitivity of IP_3R1 and the pattern of Ca^{2+} oscillations seen at fertilisation (Mehlmann & Kline, 1994). Recent findings about PLCζ's role during oocyte

activation have also clearly confirmed the centrality of IP_3 production for this process (Hachem et al., 2017; K Swann et al., 2006). Following gamete fusion, the IP_3 levels are maintained above the basal line. This generation of IP_3 in the oocyte assists the release of Ca^{2+} from the stores in a continuous manner. In return, the Ca^{2+} helps to keep the IP_3 levels above the basal level via so-called Ca^{2+}-induced IP_3 production (Matsu-ura et al., 2019).

1.6.2 Cyclic ADP ribose (cADPR) pathway

A number of studies have suggested cADPR involvement in Ca^{2+} release during fertilisation of sea urchin oocytes via an association with ryanodine receptors and linked it to Ca^{2+}-induced Ca^{2+} release (CICR) (Fluck et al., 1999; Leckie et al., 2003; Ramos & Wessel, 2013). But the relevance of this pathway for mammalian fertilisation is something unclear and controversial. Interestingly, microinjection of ryanodine and cyclic ADP ribose in mouse oocytes created favourable conditions for fusion but caused no oocyte activation which weakens their candidacy in the view of fertilisation researchers. Both molecules converted ZP2 to its post-fertilization form ZP2f, possibly by releasing cortical granules, which suggests the release of Ca^{2+} (Ayabe et al., 1995). It is worth noting that in some non-mammals, for instance *Xenopus* oocytes, cADPPR has more involvement in Ca^{2+} ion sequestration by modulating SERCA pump activity rather than RyR receptor stimulation so that it makes more sense to say that cADPR is probably more involved in Ca^{2+} recruitment from ER stores during Ca^{2+} release at oocyte activation (Yamasaki-Mann et al., 2009) and has no impact on IP_3 induced Ca^{2+} release (Kline & Kline, 1994). Collectively, studies showed that ADP ribose pathway may have a role in non-mammalian species but no evidence is available for its role in mammalian oocyte activation.

1.6.3 Ryanodine pathway

In the early 1990s the role of ryanodine receptors was investigated in mammalian and non-mammalian models to understand their role in fertilisation. In the pig, ryanodine-sensitive intracellular Ca^{2+} stores were cited as being essential for oocyte activation as studied pharmacologically by looking at the number of activated oocytes following RyR inhibition (Petr et al., 2002); however the microinjection of ryanodine had no effect on fertilisation in mice (Ayabe et al., 1995). An elevation of the Ca^{2+} response was observed after ryanodine injection in thimerosal pre-incubated pig oocytes suggesting CICR (Zoltán Macháty et al., 1999) and an insufficient rise in Ca^{2+} was observed in rat oocytes after such treatment that led to abortive spontaneous oocyte activation (Premkumar & Chaube, 2014). To fit RyRs into the oocyte activation mechanism, a two-store model was presented that suggested that ryanodine insensitive stores release Ca^{2+} which in turn activates Ca^{2+} release form ryanodine sensitive stores in human oocytes (Jan Tesarik & Sousa, 1996).

1.6.4 Calcium Induced Calcium Release (CICR) Pathways

Early studies of CICR suggested no involvement of the CICR pathway in fertilisation of sea urchin oocytes. Pharmacological studies also suggested that injection of cADPR had no effect in mouse oocytes (Ayabe et al., 1995). In humans, the two store model suggests that CICR mediated Ca^{2+} release from ryanodine insensitive stores can further mediate Ca^{2+} release from sensitive stores (Jan Tesarik & Sousa, 1996). Injection of thimerosal, a sulfhydryl-oxidizing compound, which is known to release Ca^{2+} from intracellular stores causes Ca^{2+}oscillations in hamster oocytes (Karl Swann, 1991), suggesting a potential involvement of CICR,

and another study suggested a similar mechanism in sea urchin oocytes (McDougall et al., 1993). But this hypothesis does not withstand strong scrutiny in the mouse (Kline & Kline, 1994). An IP_3 sensing protein in mouse oocytes has been suggested that remains high in concentration throughout the Ca^{2+} oscillations and the increased level of IP_3 maintained by Ca^{2+} itself could explain why RyRs are not involved in triggering rising Ca^{2+} at fertilisation (Matsu-ura et al., 2019; Sanders et al., 2018).

1.7 Ca^{2+} Oscillations – a prerequisite of mammalian oocyte activation

MII arrested oocytes are rich in cyclin B-cdk complex (MPF) which suppresses the cell cycle and keeps the oocytes in a growth arrested phase. Degradation of this complex is essential for resumption of the cell cycle and subsequent embryo formation and development. The Ca^{2+} increase in oocytes at fertilisation is an important phenomenon which induces several processes that activate the oocyte, including reactivation of meiosis and cell cycle resumption. Sperm fusion leads to release of Ca^{2+} from oocyte stores which is turn activates calmodulin and thereby stimulates calmodulin-dependent kinase II (CaMKII) (Lorca et al., 1993). CaMKII activation degrades cyclin B as well as endogenous meiotic inhibitor 2 (Emi2) levels which releases APC from an inactive state and allows the oocyte to exit from MII phase to form a totipotent embryo (Markoulaki et al., 2004; Medvedev et al., 2014). Ca^{2+} release in oocytes is reparative and lasts for several hours. Every rise in the oocyte's Ca^{2+} levels causes CaMKII activation. This repetitive pattern of Ca^{2+} release and subsequent CaMKII activation depresses the level of cyclin B significantly. Studies of disturbed Ca^{2+} oscillations identified a great reduction in fertilisation success and embryo development. A few Ca^{2+} transients may allow the oocyte to pass the MII arrest but will generally leave it stuck in the MII state, and

also have inhibitory effects on MAPK which is essential for pronucleus formation (Ducibella et al., 2002).

1.8 Sperm derived factors involved in oocyte activation

Having outlined the three main theories of oocyte activation, I will now discuss in more detail the sperm factors that are proposed to mediate the sperm's role in induction of Ca^{2+} oscillations and oocyte activation. This section is a compilation of such sperm derived factors and highlights their possible mechanistic roles in Ca^{2+} oscillations and oocyte activation during mammalian fertilisation.

1.8.1 Oscillin

After the generic proposal of a role for a soluble sperm factor, oscillin, a 33kDa sperm cytosolic protein, was the first candidate to be purified and functionally characterised in 1996. Immunostaining suggests it is localised in the equatorial segment region of the sperm head with less fluorescence in the neck region. Sequencing studies suggested a significant similarity with glucosamine-6-phosphate isomerise (GNPDA) which is primarily involved in metabolic pathways with strong binding to the hexose-phosphate substrate and is a highly conserved molecule (Parrington et al., 1996). Oscillin lost the attention of researchers in the same decade in which it was first discovered due to its inability, in its recombinant form, to cause Ca^{2+} oscillations after injection into the oocyte and no correlation between its known function and oocyte Ca^{2+} oscillations (Parrington et al., 1996; Wolny et al., 1999; Wolosker et al., 1998). However, these findings did not exclude the possibility of post-translational modifications of glucosamine-6-phosphate isomerase protein affecting its activity, and still leave some space for its reinvestigation (J Tesarik, 2002).

1.8.2 tr-kit

A sperm derived truncated and soluble form of the c-kit tyrosine kinase receptor gained some attention when it was shown to apparently activate oocytes parthenogenetically. It has the ability to initiate cortical granule exocytosis, parthenogenetic pronucleus formation and normal zygotic cleavage in its recombinant protein and mRNA forms (C Sette et al., 1997). The missing explanation here was the absence of any obvious relationship with Ca^{2+} oscillation induction in oocytes because no such oscillations were observed/reported in tr-kit studies. A direct mechanism was put forward linking tr-kit with PLCɣ1 phosphorylation and interaction with the Src-like kinase, fyn, which activates PLCɣ1 at its SH3 domain by tyrosine phosphorylation (Paronetto et al., 2003; Claudio Sette et al., 1998, 2002); this could possibly generate monotonic Ca^{2+} rise(s) in the oocyte that would be sufficient to activate the oocyte and allow it to form a pronucleus and achieve cleavage (Ferrer-Buitrago et al., 2018; Y L Miao et al., 2012). Clinical investigations of tr-kit in patients with a history of infertility history presented a direct correlation between infertility, abnormozoospermia, and infertility; however, the lack of a demonstrated direct ability to induce Ca^{2+} oscillations in the oocyte have kept the picture unclear about a role for tr-kit in oocyte activation (Haghighat et al., 2019; Tavalaee & Nasr-Esfahani, 2016).

1.8.3 PAWP/ WBP2NL

A sperm protein called post-acrosomal sheath in perinuclear theca sharing N-terminal homology to WW domain-binding protein 2 (PAWP) is highly and specifically expressed in sperm cells and localised in the head region. PAWP has

been reported to induce pronuclear formation in mammalian oocytes and its inhibition to arrest PN formation and fertilisation (A. T. H. Wu et al., 2007). However, in contrast other studies of PAWP have raised questions about its ability to trigger Ca^{2+} oscillations in the oocyte (Aarabi et al., 2014; Nomikos et al., 2014). Interestingly, expression of PAWP in infertile patients showed it to be low comparatively and this seems to be directly associated with a reduced rate of fertilisation or a possible cause of male infertility. More investigations need to be done to understand the extent of PAWP involvement in fertilisation (Tavalaee & Nasr-Esfahani, 2016).

1.8.4 PLCδ

One of the classes of phospholipase C, PLCδ isoforms are well studied and catalyse the same reaction as other PLCs to generate IP_3 and diacylglycerol (DAG). The presence of a pleckstrin homology (PH) domain in PLCδ suggests its localisation and function at the plasma membrane region where it hydrolyses membrane-bound phosphatidylinositol 4, 5-trisphosphate ()or PIP_2. Functional characterisation of PLCδ isoforms have indicated roles in pathophysiological processes including autophagy, skin homeostasis, and cancer (Mu et al., 2015; Shimozawa et al., 2017; Song et al., 2012; Yudin et al., 2016). PLCδ is less investigated in the context of oocyte activation, fertilisation, and development. Expression studies have confirmed PLCδ expression in both sperm and oocytes in mammals, with the highest expression in testis (Park et al., 2015). A study of the PLCδ4 isoform role at fertilisation highlighted interesting findings using a mouse model. Disruption of PLCδ4 led to 85% reduction in litter size and 65% reduction in pregnancy *in vivo* in homozygous groups whereas a slight reduction of around 15% was observed in heterozygous groups, compared to wild type controls.

Looking into the effect on Ca^{2+} oscillations in the oocyte, PLCδ4 knockout sperm did not induce any Ca^{2+} rise in oocytes leading to a 93% reduction in second polar body formation. However, following ICSI, both PLCδ4 knockout sperm and wild type sperm were shown to be equally effective at inducing Ca^{2+} oscillations and oocyte activation, suggesting that PLCδ4 is important for fertilisation but not for oocyte activation (K Fukami et al., 2001). In fact the study showed that PLCδ4 plays a role in the acrosome reaction, a prerequisite for normal fertilisation.

1.8.5 PLCζ: the primary factor that causes oocyte activation

The discovery of PLCζ was a major breakthrough for those studying the mechanism of mammalian oocyte activation. It satisfied groups who had been hunting for a sperm-derived protein(s) that triggers Ca2+ oscillations in the oocyte (Kashir et al., 2014; Saunders et al., 2002a; K Swann & Lai, 2016; S. Y. Yoon et al., 2008). Functionally, it works quite similarly to other members of the PLC family except for its lack of a PH domain, leading to questions about its intracellular localisation and mode of operation. In contrast to other PLC isoforms, PLCζ appears to act upon a form of PIP2 in vesicular membranes rather than in the plasma membrane, but like other PLC isoforms, PLCζ hydrolyses this to generate IP3 and DAG (Y. Yu et al., 2012). The recombinant form of PLCζ can induce Ca2+ oscillations in human and mouse oocytes (S. Y. Yoon et al., 2012). Extensive studies have been carried out on this protein including investigations of its intracellular localisation and substrate specificity. PLCζ also received significant clinical attention after a correlation was revealed between mutations in the protein and human infertility (Heytens et al., 2009; Escoffier et al., 2016; Kashir et al., 2012; Torra-Massana et al., 2019). But what has been intriguing in recent PLCζ research, is that oocyte activation can still proceed after fertilisation of oocytes with

PLCζ knockout sperm both in vitro and in vivo albeit with reduced efficiency and a delay. Elimination of PLCζ expression has two major effects on oocyte activation, first, a greatly diminished number of Ca2+ transients and second, increased polyspermy in oocytes (18-70%), suggesting insufficient Ca2+ release for cortical granule exocytosis. PLCζ deletion has no impact on the sperm's morphological characters and its ability to fuse with an oocyte, thereby leaving the in vitro fertilisation process unaffected with blastocyst stage development around 70% compared to wild type sperm (Hachem et al., 2017; Nozawa et al., 2018). These findings confirm the importance of PLCζ as a factor responsible for inducing the normal pattern of Ca2+ oscillations but indicate that it is not the sole inducer of Ca2+ oscillations and oocyte activation. An important question now is the identity of the factor(s) that can induce Ca2+ oscillations and oocyte activation in the absence of PLCζ.

1.8.6 miRNAs and endo-siRNAs

Very little is known about non-protein factors involved in oocyte activation, fertilisation and development, and regulatory RNAs are of potential interest in this context. Germline specific conditional knockout of Dicer and Dorsha (classes of ribonucleases) produces significantly less amounts of miRNAs and endo-siRNAs in sperm which in turn resulted in reduced birth rate and litter size. ICSI of siRNA/endo-siRNA knockout sperm into oocytes was associated with reduced formation of two pronuclei and cleavage to two cell stage by 20%, and blastocyst development by 40% (Yuan et al., 2016) whereas miR-449n knockout had a direct impact on cleavage in bovine embryos (M. Wang et al., 2017). The role of regulatory RNAs during oocyte activation is untested but some studies in non-

mammalian models also indicate their potential general roles in fertilisation and development (Han et al., 2009; Pavelec et al., 2009).

1.9 Potential oocyte derived factors involved in its activation

Following the identification of IP_3 as a Ca^{2+} mobilising messenger, those studying fertilisation have sought to explore the potential role of PLC enzymes in oocyte activation, fertilisation and early development. One important question is the expression of different PLC isoforms in the gametes. This is a matter that I will discuss in detail in the following section.

1.9.1 PLCβ

PLCβ (Phospholipase C beta isoforms 1, 2, 3 and 4) is an extensively investigated PLC member among all reported mammalian PLCs. Its role in physiological signalling is universally established and it is known as a prime component of the IP_3 signalling pathway in many somatic cells including neurons. Gq coupled receptors, in response to a stimulus, activate Gq proteins resulting in activation of PLCβ isoforms. These isoforms, once activated, hydrolyse/cleave PIP_2 into IP_3 and DAG. This group of enzymes has left footprints in the literature about oocyte activation. Studies on *Xenopus* and starfish oocytes suggested a role for PLCβ1 in oocyte activation (Shilling et al., 1994; Wilson et al., 1997). These studies support the receptor theory of oocyte activation with PLCβ being activated by receptor stimuli supplied by sperm. In mammals, a 30% suppression of PLCβ1 protein activity in mouse oocytes can affect the peak amplitude of Ca2+ transients by 20% but no major negative effects on successful fertilisation and embryo formation were observed (Igarashi et al., 2007). It is also important to note that injection of G-protein activating analogue, Guanosine triphosphate (GTP) gamma

S was reported to increase Ca^{2+}Ca2+ in the oocytes. GTP is an upstream activating molecule of G-protein that activates PLCβs and this finding suggests a potential involvement of oocyte PLCs (activated through sperm fusion) in triggering Ca^{2+} oscillations at fertilisation (Miyazaki, 1988).

1.9.2 PLCɣ

PLCɣ, which is activated by tyrosine kinase signalling, has been studied in several non-mammalian models of fertilisation, which indicate a potential role in oocyte activation. Its role has been extensively studied in Chaetopterus and starfish oocytes where protein tyrosine kinase-dependent PLCɣ inhibition were shown to arrest both fertilisation and artificial oocyte activation (Carroll et al., 1997; Giusti et al., 2000; Shilling et al., 1994; Yin & Eckberg, 2009). In sea urchin oocytes and starfish oocytes, PLCɣ was shown to be participating in Ca^{2+} release at fertilisation through its SH domains (Giusti et al., 2000; Runft et al., 2004; Tosca et al., 2012; Ueno et al., 2014); however inhibition by tyrosine kinase inhibitors completely failed to affect Ca^{2+} transients and fertilisation in mouse oocytes (G Dupont et al., 1996).

1.10 Receptors and associates at fertilisation

The proposed mechanisms of oocyte activation revolve around Ca^{2+} signalling pathways. Hence, it is worth looking more at the upstream and downstream aspects of these pathways at fertilisation to strengthen our understanding of mammalian oocyte activation. A number of studies have sought to connect oocyte surface receptors to Ca^{2+} release and other oocyte activation events (Z Macháty et al., 2005; S Miyazaki, 1991; Moore et al., 1993; J Tesarik, 2002; Williams et al., 1992). Sperm induced oocyte activation is a naturally occurring event which can

be mimicked by several artificial methods employing physical as well as chemical means. Involvement of guanine nucleotide-binding proteins (G-protein) coupled receptors (GPCRs) was suggested after successful oocyte activation using recombinant muscarinic receptor expression in the oocyte and its activation using acetylcholine. Acetylcholine (Ach), a muscarinic receptor agonist, can activate such an oocyte to form pronuclei, recruit maternal mRNAs, and undergo DNA synthesis and cleavage (Moore et al., 1993). Mechanisms of muscarinic receptor signalling have been established and show that they work through PLCβ activation resulting in release of IP_3 and DAG. Another study supported the role of serotonin receptors in oocyte activation as a synergistic effect was observed using a combination of endogenously expressing 5-hydroxytryptamine receptors (5-HT) and GTP analogue, guanosine-5'-O-(3-thiotriphosphate GTP gamma S, in hamster oocytes (S Miyazaki et al., 1990). These findings were explained by the dissociation and action concept of inactive heterotrimeric G- proteins subunits to their active form i.e. α and βɣ. With α subunits, α_s is known to activate adenylyl cyclase and Ca^{2+} channel activation whereas α_q can directly activate PLCβ (Hepler & Gilman, 1992). Indeed, antibody inhibition of Gq in sperm assisted fertilisation showed partial inhibition of fertilisation and Ca^{2+} oscillations. But the latter study has several loopholes, for instance excessive physical stress on oocytes might have reduced the fertilisation efficiency, and inhibition of Gq using an antibody approach was partial at all stages of the study and inhibiting Gq does not exclude all GPCRs from the picture. A GTP analogue in hamster oocytes was able to activate several GPCRs (S Miyazaki et al., 1990). These findings suggest that GPCRs may be involved in oocyte activation, especially in the process of cortical granule exocytosis. Another interesting finding in sea urchin oocytes was that

surface tyrosine phosphorylation occurred within 20 seconds of sperm interaction which suggests possibilities of several subsequent reactions downstream of fertilisation. Sperm bindin protein potentially initiates this global phosphorylation on the oocyte surface and is a receptor-mediated action. This phosphorylation is essential for zona fusion and might also be directing several downstream functions simultaneously (Abassi & Foltz, 1994; Glabe & Vacquier, 1978). But this finding does not necessarily have any direct relevance for mammalian gamete cell interactions.

β-1,4-galactosyltransferase (GalTase) binds to ZP3 protein O-linked oligosaccharides. This binding facilitates acrosomal release through G protein activation. Aggregation of GalTase increases the intensity of G- protein activation. A relationship of GalTase with $G_{\alpha i}$ was established (Gong et al., 1995) but it may have the ability to induce other G-proteins including the Gαq family which still need to be explored. *In vivo* findings from GalTase null mouse sperm suggest less impact on fertility and greater impact on binding and penetration (Lu & Shur, 1997). The fact that fertilisation is not abolished in GalTase null mice suggests that additional ligands are involved in G-protein activation on the oocyte surface.

Integrins are membrane-bound receptor proteins well known for their roles in cell adhesion, migration and differentiation and they have also been implicated in sperm-oocyte fusion (Hynes, 1992; Sjaastad & Nelson, 1997). As a consequence of fusion, integrins cluster on the oocyte surface to initiate signals which in turn results in recruitment of cytoplasmic tyrosine kinase (Focal adhesion kinase, FAK). FAK proteins cross-phosphorylate each other as well as other proteins and make then sites for Src-family kinases to continue the phosphorylation process (White et al., 2010). This massive phosphorylation could possibly activate several

unknown pathways in an oocyte, an issue that still remains unexplored. However, studies on tyrosine kinase signalling in mouse oocytes suggests their possible participation in the oocyte activation process (Mori et al., 1992).

1.11 Non-protein factors

Looking beyond the potential involvement of fully translated and truncated proteins, mRNA from the sperm cells might be a possible activating factor of the oocyte. Regarded as a 'cryptic activating factor', it is possible that mRNA which remains in sperm and has no translation machinery for it get translated in the sperm, could be translated once delivered to the fertilised oocyte cytoplasm. The translated factor might stimulate IP_3 release (Jones, 2018). This cryptic factor might also activate CICR.

1.12 Secondary factor(s) causing Ca^{2+} release - what is its identity?

PLCζ knockout males are sub-fertile indicating involvement of secondary factors (Hachem et al., 2017; Nozawa et al., 2018). Capacitated fresh PLCζ KO sperm cells can cause a few Ca^{2+} transients following IVF (Nozawa et al., 2018). Interestingly, thawed cryopreserved PLCζ KO sperm is unable to trigger Ca^{2+} transients (both after ICSI and IVF) suggesting that the freezing and/or thawing process has an adverse effect on such secondary factors which are potentially causing Ca^{2+} release in oocytes alongside PLCζ. Such factors need to be investigated in the context of IP_3 and Ca^{2+} release and whether they are delivered as soluble sperm factors or membrane bound ones.

1.13 Conclusions drawn from the literature

Mammalian oocyte activation is a complicated process and the exact mechanism is still unclear. Research has been going on for over a century but it has not been

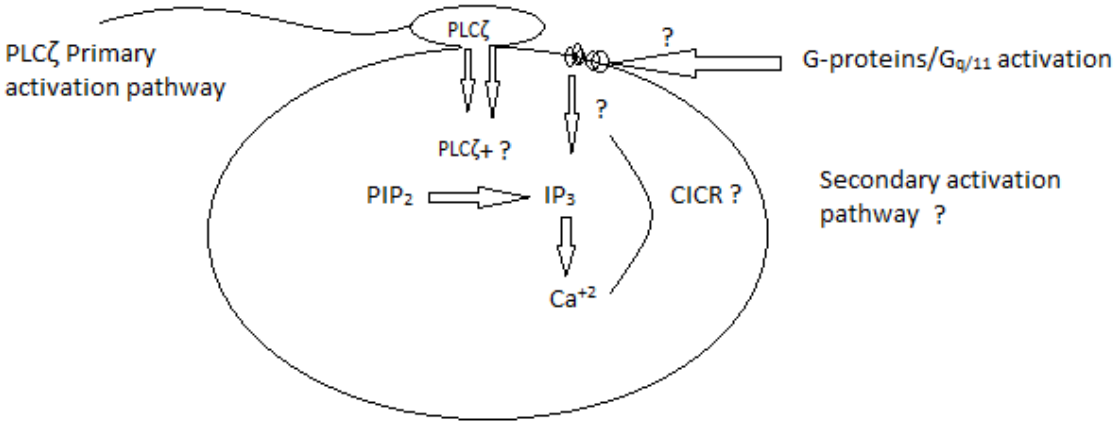
resolved yet. What is the main strategy in oocyte activation research? It is, reporting of the importance of a factor followed by further studies with more robust experimentation to test whether the factor has physiological importance; such studies may be pharmacological or gene knockout studies.

To simplify the story and our understanding of oocyte activation mechanisms, it is important to question what is important and what is not in oocyte activation and then target the main factors to identify them and establish their roles clearly. A high number of studies investigated the pattern of Ca^{2+} signals. It is worth noting that Ca^{2+} oscillations are an indicator of successful oocyte activation (pronuclear formation, cleavage, DNA synthesis, mRNA transcription/protein translation) during normal fertilisation. However, a single rise of intracellular Ca^{2+} in an oocyte can lead to successful fertilisation and normal development and birth of offspring. From the Ca^{2+} oscillation studies it is clear now that PLCζ is the primary sperm derived factor that causes repetitive Ca^{2+} oscillations in the oocyte.

Involvement of the IP_3 pathway in oocyte activation is well documented and evidenced in several animal groups including mammals and is the only pathway that is found to be common in almost all investigated models, ranging from sea urchin to humans (Ducibella & Fissore, 2008; Jones et al., 2000; Parrington et al., 2019). A further confirmation of IP_3 involvement was done by blocking IP_3 receptors or interfering in PLC activity which resulted in no Ca^{2+} transients Ca2+in oocytes (G Dupont et al., 1996). If this signalling pathway is so essential, talking about the involvement of the PLC group of enzymes makes sense. Looking back into the literature, in mammals, several PLCs have been investigated for their roles in different stages such as cell maturation, fertilisation and development (N Avazeri et al., 2000; Genevieve Dupont et al., 1996; Igarashi et al., 2007). In the context of

mammalianoocyte activation, after PLCζ, PLCβ isoforms were commended as potential triggering factors (G Dupont et al., 1996; Igarashi et al., 2007). For remaining PLC subtypes i.e. PLCδ and PLCγ subtypes, there is no direct evidence linked to their involvement in oocyte activation, particularly in mammals. Moreover, a study of PLCγ subtype activation by SH2 domains showed that PLCγ activation is not required to initiate Ca^{2+} transients Ca^{2+}in oocytes (Mehlmann et al., 1998). Blocking PLCs in the oocyte affects theCa^{2+} Ca^{2+} oscillation and fertilisation rates to a great extent in sperm induced fertilisation (H. Wu et al., 2001; S. Y. Yoon et al., 2012). No pronuclei form, no cleavage happens when PLCs of both sperm and oocyte were blocked. This could be because PLCs are not just involved in the rise in Ca^{2+} levels that activates the oocyte but also in embryo development.

On the basis of studies investigating IP_3 and PLC role at fertilisation from the literature, two main conclusions can be drawn. First, PLCζ is involved in activating the oocyte by changing its intracellular Ca^{2+} profile through an IP_3 mediated pathway and second, there are other factors among other PLC subtypes involved in oocyte activation that act parallel to PLCζ. . Essentially, such secondary factors are IP_3 modulators but their identities remain unclear and need to be uncovered. Drawing a conclusion on this basis may not be the right way of explaining the true basis of the underlying mechanisms and keeps the picture unclear. A further intensive validation is needed to understand such secondary factors which are either sperm or oocyte derived working parallel to PLCζ but with a different mechanism of stimulation.



1.14 Need to revisit the theories & factors

PLCζ – The most widely expressed PLC in male sperm cells and testes tissue and gained clinical attention because mutations in PLCζ are highly correlated with infertility or partial fertility (Torra-Massana et al., 2019). The generation of a mouse PLCζ knockout made it possible to test the physiological relevance of PLCζ . Two independent studies showed that loss of PLCζ expression leads to loss of the capacity to induce the normal pattern of Ca^{2+} oscillations and indeed the ability to trigger any Ca^{2+} release following ICSI or IVF with thawed cryopreserved sperm. However, PLCζ knockout sperm can trigger a few, delayed Ca^{2+} transients and oocyte activation following IVF with sperm that have not been cryopreserved. In line with this, PLCζ knockout males are not infertile but only semi-fertile. This raises the question of the identity of the factor that can trigger such Ca^{2+} transients in the absence of PLCζ.

PLCβ1 – Conditional knockdown in oocytes inhibits the amplitude of Ca^{2+} transients at fertilisation and litter size but its precise role is unclear. Anti-Gq studies using antibody inhibition led to the conclusion that oocyte PLCβ1 has no

role but an investigation in the sperm side of things still needs to be done. A more reliable approach, other than via an antibody, for oocyte PLCβ1 inhibition through targeting upstream activators is required to confirm the oocyte's PLCβ1 contribution after its activation. Sperm PLCβ1 is possibly delivered by sperm cells to the oocyte cytoplasm and gets activated in this different environment. Moreover, oocytes have their own machinery to produce Ca^{2+}-induced IP_3 in a systematic and precise manner that releases more Ca^{2+} (a type of CICR) to sustain the transients (Matsu-ura et al., 2019; Sanders et al., 2018).

Collectively, oocytes activation is primarily triggered by PLCζ and its absence can be supplemented by other unclear PLC subtypes. Such secondary factors are either gamete specific or common in both stimulating through a mechanism different from PLCζ. To understand the physiology of oocyte activation it is important to uncover the identities and mechanism of such unclear secondary factors.

1.15 Hypotheses and objectives

In the context of identification of secondary factor(s) and their mechanisms involved in oocyte activation, I have defined objectives to understand oocyte activation factors.

1. *To identify sperm factors causing Ca^{2+} release in the oocyte at fertilisation.*

   It has been revealed that Ca^{2+} oscillations can still happen in an atypical manner in the absence of PLCζ suggesting other sperm or/and oocyte-derived factors are involved. It is important to know the identity of such

sperm derived factors and how they activate oocytes in the absence and/or presence of PLCζ.

2. *Identifying oocyte factors involved in Ca^{2+} release at fertilisation.*

   Studies of oocyte factors have left many unaddressed issues in terms of the role of oocyte derived proteins involved in Ca^{2+} release in the oocyte. Hence it is important to reinvestigate the potential upstream activation of suggested oocyte factors in the literature to study the contribution of the oocyte in its own activation.

3. *Deciphering the mechanisms of actions of identified factors.*

   A better understanding of the mechanisms of Ca^{2+} release during the oocyte activation process is important to solve the underlying basis of this process. Identification of relevant sperm and oocyte factors will help in discovering the mechanisms involved.

Chapter 2: Materials and Methods

2.1 Chemicals

All chemicals used in the studies were supplied by Sigma-Aldrich UK unless otherwise stated. Unless otherwise stated, water used in the preparation of chemicals, reagents solutions, washes, elutions, imaging and general purposes was double-deionised reverse osmosis filtered.

2.2 Physical Conditions

All studies, excluding live cell studies, were conducted at room temperature unless otherwise stated. Live-cell studies were conducted at 37°C in an incubator integrated with a CO_2 supply.

2.3 Animals

2.3.1 Ethics

All experiments were conducted as per the guidelines of the Animals (Scientific Procedures) Act, 1986 implemented in the United Kingdom. Studies and procedures were conducted at the designated study places i.e. the Department of Pharmacology and the Biomedical Services facility of the University of Oxford, Oxford, United Kingdom.

2.3.2 Housing Conditions

Animals were housed in the University of Oxford Biomedical Services animal housing facility where mice are kept in individually ventilated cages (IVG) in a cohort of 1-4 mice per cage. A light cycle was used on a 12-hour light and 12-hour dark cycle at 19-23°C and 45-65% relative humidity. Animals have free access to water and to irradiated standard dry food. All conditions and supplies were arranged and maintained by the Biomedical Services staff.

2.3.3 Strains

The C57BL/6J (Charles River) strain of mice was used to generate *PLCζ*$^{-/-}$ mice which were previously generated by Alaa Hachem in John Parrington's lab using CRISPR/Cas technology. Briefly, the mouse PLCζ sequence (NM_054066.4) was used to identify target sites for single guide RNAs (sgRNAs). Exons 3 and 5 were chosen as targets for functional deletion. Isolated fertilised oocytes were injected with Cas9cRNA/sgRNA to target *PLCζ* exons. Two cell embryos were transplanted to B6CBF1 females (Charles River). F0 mice were further mated with C57BL/6J to begin the process of creating *PLCζ*$^{-/-}$ mice strains. Homozygous male *PLCζ*$^{-/-}$ mice are created by mating male and female heterozygous *PLCζ*$^{+/-}$ mice. The genotype of all mice used in this study was confirmed at two stages of development, once at the age of 3 weeks and then after the isolation of gametes for my studies at the age of 4-9 weeks.

2.4 Genotyping

2.4.1 Ear Clip Digestion

Ear biopsies were used to genotype the animals which were supplied by the housing facility at the age of 2-3 weeks. Genomic DNA was extracted from ear

biopsies by digestion with proteinase K overnight at 55°C in 0.1 ml lysis buffer (100 mM Tris-HCl (pH 8), 5 mM EDTA, 200 mM NaCl, 0.1% SDS, 1% proteinase K). Following flicking of the digested samples, extracts were centrifuged at 1000 rpm for 4 min, and the supernatant was diluted 10× into water prior to inactivation by proteinase K at 95°C for 15 min. After inactivation, a 1 µl sample was used as a template for 10 µl PCR reactions.

2.4.2 PCR Reaction

A PCR mixture was prepared in PCR tubes at a maximum volume of 20 µl using a DreamTaq Green PCR Master Mix (2X) (Thermo Scientific) and water. The primers used to genotype are as follows: F, AGAAAGAAGGGAGGGGAATCTTC; R, TGATGGTATATCTGCATCCTTCTT; wild-type product, 342 bp; mutant product, 325 bp. The templates were amplified over 40 cycles using the programme as shown in Table 1 on an Eppendorf Mastercycler®Gradient PCR machine.

Table 1- Genotyping PCR cycling conditions

95°C	5 min
30x cycles:	
94°C	15s
56°C	30s
68°C	1 min
End cycle	
68°C	5 min
4°C	Infinite store

2.5 Agarose Electrophoresis

Amplified products after the PCR reaction were further analysed using gel electrophoresis. Samples were stored at 4°C until used. Agarose (Sigma A9439) gel was prepared at a strength lying between 1.2-1.5 % in TAE buffer (40mM Tris-acetate, 1mM EDTA; pH 8.0) containing 0.5 µg/ml ethidium bromide (Fisher). Gels

were cast on electrophoresis trays and then immersed in Fischer Brand HU10 electrophoresis tanks containing TAE buffer. PCR amplified samples were loaded as the whole volume in the wells accompanied by a standard 1kb DNA ladder on the first and last wells in any direction. Post-loading, the gel was run at a fixed voltage of 100V throughout the complete resolution. Adding a loading dye for tracing the gel movement was omitted as the DreamTaq Green MasterMix used in the PCR reaction contains a dye to trace movement of samples on the running gel. An illustration of bands of WT, heterozygous and homozygous PCR products is presented below (Figure 2) and was used to identify the genotype in all genotyping experiments throughout the study.

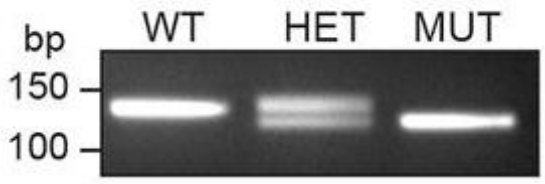


Figure 2 Resolved gel band comparison of wild type (WT), heterozygous (HET) and PLCζ–/– mutant (MUT) (Hachem et al., 2017)

2.6 Isolation of gamete cells

2.6.1 Isolation of sperm cells

Male mice aged 8-9 weeks were used to isolate sperm cells throughout the study. The epididymides were removed and divided into three main regions: caput (regions 1–3); corpus (region 4), and cauda (region 5). Caput and cauda epididymal spermatozoa were collected by puncturing tissues with a needle and allowing the spermatozoa to disperse into phosphate-buffered saline (PBS) (10 mm phosphate, 137 mm NaCl, pH 7.4) containing a protease inhibitor mixture

(PIC) (Mini, EDTA-free Protease Inhibitor Mixture Tablets, cat.no.11836170001, Roche, San Francisco, CA) for 10 min at 37°C. Spermatozoa were purified from contaminating epididymal epithelial cells by filtering through a 10 μm diameter nylon mesh (Medifab washed, cat.no.07-10/2, Sefar Inc, Depew, NY) and the collected spermatozoa were washed two times in PBS/PIC by centrifugation at 500 × g for 5 min at room temperature (RT). The number of isolated spermatozoa was calculated using a haemocytometer.

2.7 Reverse transcription PCR (RT-PCR)

2.7.1 RNA preparation

Total RNA from cells was isolated and purified using a marketed RNA isolation kit (Cat. No. 74104, Qiagen) according to the manufacturer's instructions. Briefly, the cells/tissues were washed 3 times with sterile PBS and collected in 1 ml microcentrifuge tubes. 350 μl cell lysis buffer (buffer RLT), containing 1% β-mercaptoethanol to denature RNase, was added to each tube and transferred to a QIAGEN shredder column. The column was centrifuged (14,500rpm, 2 minutes) to homogenise the cell lysate. An equal volume of 70% ethanol was added to each tube and the contents were then transferred to a QIA mini spin column and centrifuged (10,000rpm, 15 seconds) to bind RNA to the column filter. The bound RNA was washed and treated with DNase I (approximately 30 Kunitz units per column), followed by further washes. The RNA was eluted with RNase-free H_2O (supplied in the kit by QIAGEN) and concentration and integrity were checked.

2.7.2 RNA quantification and integrity verification

The concentration of RNA was checked using a NanoDrop spectrophotometer. The spectrophotometer also provided the ratio of absorbance at 260 nm/280 nm

(A_{260}/A_{280}), giving an indication of the level of protein contamination of the RNA. The A_{260}/A_{280} was found to be between 2.0 and 2.3 for all samples, indicating an acceptably low level of protein in the samples. To check RNA integrity, approximately 0.5 ng of each sample was electrophoresed on a 1.5% agarose gel. The presence of 2 distinct bands corresponding to 28S and 18S ribosomal RNA, in a ratio of approximately 2:1, without smears, indicates good quality RNA. All samples were deemed acceptable according to these criteria.

2.7.3 Reverse Transcription Reaction

cDNA was synthesised from RNA samples using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, a reaction mix was prepared to contain RNA (1.2 µg per sample), deoxyribonucleotides (dNTPs) (4 mM), reverse transcriptase (50 U per sample), 1x random primer mix, and 1x reverse transcription buffer. The reaction mix was incubated at 25°C for 10 min, increasing to 37°C for 120 min, then 85°C for 5 min. The cDNA synthesised was stored at -80°C until analysed. Control reactions were prepared without RNA (to check for RNA contamination from external sources during preparation) and without reverse transcriptase (to check for genomic DNA in the samples affecting qPCR results where intron-spanning primers could not be used). No significant amplification was observed in these control reactions.

2.7.4 PCR reaction and gel electrophoresis

A PCR mixture was prepared in PCR tubes at a maximum volume of 20 µl using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific) and water. All PCR reactions were performed on an Eppendrof Mastercycler®Gradient PCR machine. cDNA primers (Table 2) were selected from the PrimerBank Database (X. Wang et al., 2012), and synthesised and supplied by Thermo Scientific. Primer

sequences were confirmed in the cited literature and tested for binding to the specific cDNA sequence using BLAST+ version 4 databases (dbV4). Amplified products after PCR reaction were further analysed using gel electrophoresis (section 2.5). The resolved gel was illuminated under UV to detect amplified DNA bands.

Table 2- Primers used for mouse PLC isoforms RT-PCR (PrimerBank, update 2012)

PLC Isoform	Forward	Reverse	Amplicon size (bp)
β1	TCGCACAACACCTACCTCAC	TCGTCGTCGTCATCGTCATC	566
β2	TGGATGTCAGATGGTTGCCC	GGTAAGGGCCATGTTGCTCT	502
β3	CAGCCGCATCTACCCTAAGG	CGGATAGCAGAGACAGGCAG	519
β4	GGTGCTACCACGAACATCCA	ATGCGGGTTCGGAACTCTTT	560
γ1	CTGAGGGCAAAAACAACCGG	GTCTCTCATGGTGCTTGGCT	554
γ2	TCTACGGCACCCAGTTTGTC	TCCCGGGAGAACAGATACGT	598
δ1	TATGTGCTGAAACCGGCCTT	AGTTCAGTGGCCTCAAGCTC	571
δ3	CACACGCTCACCTCCAAGAT	GTTGCTGAGTGTTGTGCCTG	550

δ4	ATGGACCTGATGGAGAGCCT	TGAGTGAAGCTGCAGAAGGG	550
ε	CGATTACGAGTCCCTGTCCG	AGTTTCTGAGAACCCCTGCG	552
L1	TCAAGTCAACCTTCCCGAGC	CCAGCATCCTCTTCTGCACA	554
XD1	CAGTCAGAGTTGGTCCCCAC	AAGCCAAGATGACCACCTCG	553
Actin-Control	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC	245
Tubulin- Control	GTCCTGGATCGAATCCGAAAG	CGGAATGCTCTAGGGTGGTG	244

2.8 Real-time quantitative PCR (RT-qPCR)

2.8.1 RNA isolation

RT-qPCR was performed to quantify mRNA expression in gamete cells and reproductive organs. RNA isolation and quantification were carried out as explained in sections 2.7.1 and 2.7.2.

2.8.2 Sample preparation

A PrecisionPLUS OneStep RT-qPCR Master Mix (PrimerDesign) was used in all qPCR experiments as per the manufacturer's instructions for sample preparation and amplification. PrecisionPLUS mastermix contains two enzymes for the RT step and is a mix of M-MLV Reverse Transcriptase (for the RT step) and Taq Polymerase (for amplification) which allows both reactions to be carried out in a single tube. SYBR green was used as a detection dye which comes in the same master mix (PrecisionPLUS OneStep RT-qPCR Master Mix). To avoid protein and RNA instability, all pipetting steps and experimental plate set up was done on ice and centrifugation was done at 4°C. Prolonged incubation with RT was avoided. The following composition was used to prepare samples for qPCR analysis and plated in LightCycler® 480 Multiwell Plates 384 at a maximum volume of 20 µl per well (Table 3).

Table 3- Composition of qPCR reaction mixture

Component	Volume (for 1 reaction)
PrecisionPLUS OneStep RT-qPCR Master Mix*	10 µl
Primers (forward and reverse)	300 nM per 20 µl reaction mixture
Template RNA (25ng)	25ng per 20 µl reaction mixture
RNase/DNase free water	q.s.
Final volume	**20 µl**

* SYBRgreen was used to measure fluorescence, hence, no probe was used

2.8.3 Real Time-qPCR

A Roche LightCycler 480 System was used for the qPCR experiment. Target genes analysed were the same as presented in Table 2. Higher crossing point (Cp) values indicate lower starting cDNA levels, as more amplification cycles are required before this critical point is reached. Cycling conditions are presented in Table 4. Data were gathered using LightCycler 480 SW 1.5 software. Experiments were run in triplicate. The following programme was used to run the amplification for up to 40 cycles:

Table 4- Amplification protocol for SYBR®green detection in the qPCR experiment

Step	Time	Temp
Reverse Transcription	10 min	55°C
Enzyme activation	2 min	95°C
Denaturation	10 sec	95°C
Data Collection	60 sec	60°C
Melt Curve	-	-

2.8.4 Data Analysis

LightCycler 480 SW 1.5 software was used to acquire data and generate graphs measuring the fluorescence of each sample which was further plotted against cycle number, and used to calculate the Cp for each sample. Relative quantification was performed by normalising data of housekeeping genes (calibrator) and fold change was calculated for all the genes of interest (test genes). The ratio was calculated using the standard formula

$$\text{Ratio}_{(test/calibrated)} = 2\text{^}C_{t\,(calibrator)} - C_{t\,(test)}$$

C_t values for the standard curve were plotted for each gene against input concentration on a semi-log scale and used to estimate the relative concentration of cDNA reverse-transcribed from mRNA of each gene of interest in the samples. These figures were adjusted for dilution of the cDNA used for each gene in the PCR reaction. The value for each gene of interest was then normalised to the averaged housekeeping gene value then these normalised values were expressed as values relative to a control.

2.9 Western blotting

2.9.1 Sample preparation

Isolation of sperm cells: 7-9 week old male mice were culled in a CO_2 chamber followed by cervical dislocation for confirmation. Incisions were made to access the cauda epididymis and this was isolated in a pre-heated phosphate buffer saline containing Ca^{2+} and magnesium (PBS, Mg^{2+}, Ca^{2+} Sigma) in a 35 mm dish. Several cuts were made to facilitate free movement of mature and motile sperm into the

media and the sample was incubated at 37°C for 30 min. Post-incubation, cells were separated using a pipette by tilting the dish at 45 degrees to select pure motile sperm cells and then these were transferred to another dish containing pre-heated PBS. Wherever required, dissected tissues were frozen in liquid nitrogen and stored in a -80°C freezer until required for further use.

Isolation of oocytes: 4-5 weeks old female mice were culled in a CO_2 chamber followed by cervical dislocation for confirmation. Incisions were made to access ovaries. Ovaries were isolated in pre-heated phosphate buffer saline containing Ca2+ and magnesium (PBS, Mg^{2+}, Ca^{2+} Sigma) in a 35 mm dish. Wherever required, dissected tissues were frozen in liquid nitrogen and stored in a -80°C freezer until required for further use.

Cells/Tissue homogenisation: Sperm cells and ovaries were homogenised in RIPA buffer using a bead-beating method for three cycles of a high-speed shaker. Homogenised solutions were centrifuged (1500 x 8 min, at 4°C) to separate a supernatant containing soluble proteins.

Protein fractionation: The decanted supernatant was further centrifuged (100,000xg, 1 hour, 4°C) to pellet the membrane protein fraction. The soluble fraction (supernatant) was separated in a microcentrifuge tube. The pellet was re-suspended in hypotonic buffer + 1x PI. Purified fractions were stored in a -80°C freezer until required for further use. The concentrations of fractionated samples were determined by a BCA assay.

2.9.2 Protein Quantification

Bovine serum albumin (BSA) was used to prepare assay standards of 0-2000 µg/ml in NaOH (100mM). Samples were diluted in 100 mM NaOH to test at least

two dilutions per sample (viz. 1:10 and 1:50) in a 96 well plate. Assay reagent was prepared by diluting $CuSO_4$ solution in bicinchoninic acid (BCA) at a ratio of 1:50 $CuSO_4$:BCA. 200 µl of reagent solution was added to 10 µl diluted sample/standard in each sampled well and incubated at 37°C for 30-45 min. Post incubation, absorbance was measured at 595 nm using a Sunrise microplate reader (Tecan). A standard curve was constructed and used to estimate the concentration of samples, considering the dilution factor.

2.9.3 SDS-PAGE

Samples to be analysed for protein resolution by SDS-PAGE were prepared in 2x sample buffer topped with 2-beta-mercaptoethanol (Sigma M3148) (0.3%) and heated at 95°C for 5 min to denature proteins and reduce disulphide bonds. Post-denaturing, samples were brought to room temperature and kept at the same temperature prior to loading. To perform SDS-PAGE, samples were loaded on to 8–16% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad). Sample volume was calculated according to loading conditions with approximately 20 µg protein in each well and the final volume being not over well capacity which is 50 µl. A PageRuler™ Plus Prestained (10-250kDa, from Thermo Scientific) reference protein ladder was also loaded alongside samples on each side of the precast gel plate. To avoid diffusion, the loaded plate was immediately transferred to the gel tank and electrophoresed in running buffer (25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS; pH 8.3) at 100V until complete resolution was achieved (approximately 40-60 min).

2.9.4 Western blot

After successful resolution of protein samples, proteins were transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare) by electroblotting in

ice-cold transfer buffer (25mM Tris-base, 192mM glycine; pH 8.3) at 300 mA for 1 hour. The membrane was blocked overnight at 4°C with PBS/5% (w/v) Marvel milk powder/0.5% (v/v) Tween-20 (for ECL exposure) or PBS/2% (w/v) ECL advance blocking buffer/0.5% (v/v) Tween-20 (for ECL advanced exposure). The blocked membrane was incubated with primary antibody at room temperature for 1 hour, washed five times with PBS/0.5% Tween-20, then incubated with the respective secondary antibody (selected based on species-specific combinations) for 1 hour at room temperature. After secondary antibody treatment, the membrane was further washed five times in PBS/0.5% (v/v) Tween-20. Detection was performed on Li-Cor Odyssey Imaging Systems and the secondary antibodies used are listed in Table 10. The membrane was exposed on a Li-Cor odyssey infrared imaging system for detection of secondary antibody fluorescence. Two wavelengths, that is 700 nm and 800 nm were selected to detect secondary antibodies to cross-check and avoid false-positive signals. Such use of two wavelengths was also employed to detect actin as a loading control in western blot experiments.

2.9.5 Western blot image analysis

Scanned images of the blot were further processed using ImageJ/Fiji software for Windows (Schindelin et al., 2012). Images were initially analysed as 32-bit colour RGB images to enable intensity and density measurements, quantification, and comparative analysis. The processed images were saved as JPEG format files in clear black and white colouring mode. Wherever required, pseudo-colour palettes were used to identify fading bands and stacks overlapping was performed to confirm false-positive bands.

2.10 *In Vitro* fertilisation (IVF)

2.10.1 Isolation of sperm cells

Male mice were culled in a CO_2 chamber followed by cervical dislocation for dead state confirmation. Incisions were made in the lower abdomen to access the cauda epididymis and sperm were isolated in pre-heated EmbryoMax® Human Tubal Fluid (HTF) media supplemented with 5% bovine serum albumin in a 35 mm dish. Several cuts were made to facilitate free movement of mature and motile sperm cells into the media and incubation was at 37°C. Swirling of the plate was performed five times to facilitate cell dispersion into the media. After incubation for 30 min, cells were separated using a pipette by tilting the dish at 45 degrees to select pure motile sperm cells by the swim-up method and these were transferred to another dish containing pre-heated PBS. All sperm samples were isolated and prepared fresh for all the IVF experiments. No freezing of gamete cells was performed and no thawed cryopreserved cells were used in any of the IVF experiments.

2.10.2 Superovulation of oocytes

All procedures were carried out with appropriate approval from the UK Home Office and by a PIL holder. Female mice aged 4 weeks were injected with 0.1 ml containing 5 I.U. of Pregnant Mare Serum Gonadotropin (PMSG) (MSD) around 60 hours before isolation of mature oocytes. Mice were kept in a group of 3 or 4 animals and monitored at intervals for their behaviour. Mice injected with PMSG were further injected with 0.1 ml of human Chorionic Gonadotropin (hCG) (0.1 ml, 5 I.U.). A hCG injection was made 12 hours before isolation of mature oocytes. 12

hours after hCG injections, mice were expected to have superovulated mature oocytes.

2.10.3 Isolation of oocytes

On the day of IVF, mice injected with hormones were culled by the CO_2 method followed by cervical dislocation to confirm their dead state. Incisions were made in the lower abdomen to access ovaries which were cut and transferred to pre-heated EmbryoMax® Human Tubal Fluid (HTF) media supplemented with 5% bovine serum albumin in a 35 mm dish. Ovaries were punctured using a sterilised needle to facilitate oocyte movement out into the media. Oocytes were collected using a pipette and washed in clean media several times, then transferred to 90 µl media covered with mineral oil (Sigma) in a 35 mm petri dish. Isolated oocytes were incubated at 37°C and used within a one-hour incubation. To remove cumulus cells, oocytes were treated with hyaluronidase containing HTF medium (Merck MR 070D) for 10 min. Post-treatment, cells were washed in HTF and placed in fertilisation medium.

2.10.4 Preparation of fertilisation medium (CARD Medium)

A fresh drop of HTF media was supplemented with BSA (10%) and 1 mM glutathione (GSH, from Sigma). Before use, the solution was sterilised using a 0.22 µm syringe end filter. Medium was maintained in an incubator (37ºC, 5% CO_2 in air) for at least 10-20 min before use.

2.10.5 Sperm capacitation

Isolated motile sperm cells were further subjected to capacitation before use in IVF. Sperm cells were incubated in HTF media supplemented with BSA (10%) to

facilitate capacitation in an incubator (37ºC, 5% CO_2 in air) for 45 min to 1 hour prior to IVF.

2.10.6 Insemination and fertilisation

Pre-heated CARD medium was used in all IVF experiments. Isolated oocytes were transferred to CARD medium drops to equilibrate in media conditions for 30-40 min. Zona pellucida was removed, whenever required, before placing oocytes in the fertilisation medium. To prepare denuded oocytes, oocytes were treated with acid Tyrode solution with continuous pipetting for 10-15 seconds and transferred to HTF media to avoid any further cellular digestion. A sperm suspension was added in fertilisation drops at a volume of 5 µl in each of the fertilisation drops and incubated (37ºC, 5% CO_2 in air) for another 3-4 hours.

2.10.7 Post fertilisation incubation

3 hours after fertilisation, oocytes were observed for sperm adherence and cumulus cell detachment under the brightfield microscope. Normal sized oocytes were washed and transferred into pre-heated HTF medium drops in another 35 mm dish and further incubated for 3 hours. 6, 12 and 12 hours after insemination, cells were assessed for fertilisation and development parameters.

2.11 Immunofluorescence

2.11.1 Cells isolation and preparation

Animals were culled by the CO_2 method followed by cervical dislocation. Gametes were isolated as discussed in sections 2.10.1 and 2.10.3. Female mice were superovulated for two days prior to use using hCG and PMSG gonadotropins as discussed in section 2.10.2. Cells were isolated and washed in pre-heated PBS prior to immunofluorescence.

2.11.2 Cell adherence and fixation

Coated coverslips were prepared by incubating coverslips in 24-well plates containing freshly prepared poly-L-lysine (0.1 mg/ml) at room temperature for 1 hour. Coverslips were rinsed in sterile water three times (1 hour each) and allowed to dry. A sperm cell suspension (10^5-10^8 cells/ml) was added to cover coated coverslips in 24-well plates and allowed to dry at room temperature. To prepare oocytes for immunofluorescence imaging, zona pellucida was removed by treatment with acidified Tyrode's solution to facilitate adherence. To avoid any artefacts in imaging no other complex polymer coatings were used.

Adhered cells were fixed using 4% paraformaldehyde in PBS for 10 min at room temperature and washed with ice-cold PBS.

2.11.3 Permeabilisation and blocking

Once fixed, cells were permeabilised and blocked using PBS containing 0.3% Triton X-100 and 10% goat serum (serum selection was performed based on the species used to raise secondary antibodies). Triton X-100 is an extensively used detergent to facilitate antibody permeabilisation whereas goat serum was used to block several non-specific protein sites to avoid non-specific staining/detection. Permeabilisation was carried out for 1 hour at room temperature on a lab platform rocker at a minimal speed followed by washing in PBS.

2.11.4 Immunostaining

Permeabilised cells were further subjected to an overnight treatment of diluted primary antibody solution prepared in PBS containing 1% BSA, at 4°C. The concentration used was chosen by reference to antibody data sheets and optimised wherever required. Following primary antibody treatment, the solution

was decanted and washing performed with PBS followed by goat anti-rabbit Alexa Fluor 546 secondary antibody (Invitrogen) solution (3-4 µg/ml in PBS containing 1% BSA) treatment (dilution prepared in PBS containing 1% BSA) in the dark at room temperature for one hour followed by multiple PBS washings. Stained cells were counter-stained and mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (Thermo Scientific). Mounted slides were stored in the dark overnight at room temperature and then at 4°C.

2.11.5 Imaging and Image Analysis

Stained cells were examined using a Zeiss LSM880 confocal microscope and images were analysed using ImageJ image processing software.

2.12 Ca2+ Imaging

2.12.1 Dye loading into oocytes

Superovulated mouse oocytes were collected as described in section 2.10.2 on the day of imaging. Oocytes were kept and processed in HTF media and a 35 mm dish was used throughout the experiment with the use of a glass bottomed 35mm dish for the imaging step. Collected oocytes were treated with pre-heated HTF medium supplemented with hyaluronidase enzyme for the removal of cumulus cells. The oocytes were kept in the solution for 10 min with 1-2 periods of slight shaking in between to facilitate the detachment of cumulus cell-oocyte complexes (COCs) and to generate oocytes free from these. Sterilised tips were used to handle the oocytes under the microscope and a heating block was used to keep cells outside of the incubator to minimize the impact of temperature change. Following hyaluronidase treatments oocytes were washed and placed in freshly prepared medium supplemented with Fura-PE3 AM (Leak Resistant, Fura 2-AM

from Santa Cruz Biotechnology) at a working concentration of 1 µM in HTF medium and incubated for the next 30 min in the dark at 37°C. Oocytes were then washed in dye-free HTF and kept in an incubator until required for further use. Use of Fura-PE3 allows the dye to remain in the oocytes for a long duration without significant loss of fluorescence.

2.12.2 Imaging dish preparation

To prepare an imaging dish for IVF, oocytes were pre-treated with acidified Tyrode's solution to remove the zona pellucida and then placed aggregately in a HTF-HEPES media (BSA free) drop in mineral oil in the imaging dish. The process of removing the zona layer and placing oocytes in the imaging dish should be completed within one minute to avoid any further damage to the oocytes. The dish was overlayered with mineral oil on a glass coverslip in the imaging dish filled with pre-heated HTF-HEPES media and placed immediately on the heating block until required to be placed on the microscope stage. Once the cells were stuck to the glass surface, HTF was added to reduce any pH change and supplement BSA. The protocol was optimised using standard Ca2+ ionophore (ionomycin, 20 µM) and oocyte activating agents such as Strontium chloride (10-20 mM).

2.12.3 Ca2+ imaging during IVF

The medium used for Ca2+ imaging during IVF was supplemented with glutathione (1 mM). Ca2+ fluorescence was measured at two wavelengths (340 nm and 380 nm) and continued until a stable baseline was observed. A sperm suspension was added (5 µl) to the drop containing attached oocytes to initiate fertilisation and to measure sperm induced Ca^{2+} transients in oocytes. All Ca2+ imaging was done on a heating stage connected to the microscope and controlled manually. A probe was used in the dish to measure real-time temperature throughout the imaging.

Sperm binding was observed visually during the imaging at low bright field light intensity. Images were captured for a duration of 2 hours post insemination. Dark conditions were maintained in all imaging sessions.

2.12.4 Data analysis

Ca2+ was measured in dye loaded cells using a Zeiss Axiovert 200 Epifluoroscence Microscope connected to a CCD Camera (Prinston Instruments). Two lines of images were captured at a magnification of 20x using a 20x lens with UV transmission and NA 0.75 at wavelengths of 340 nm and 380 nm. Real-time fluorescence and ratiometric analysis were performed using MetaFluor v7.0 (MetaImaging Series) and ImageJ. A Multi Measure plugin was used to plot the intensities of regions of interest against time and analysed for two wavelengths ratiometrically. Data were normalised by taking the ratio of each wavelength with a baseline average (F_0) before ratiometric analysis.

2.13 Sperm capacitation and acrosome Reaction

2.13.1 Spermatozoa capacitation

To measure the ability of sperm cells to undergo acrosome reaction in response to various agents, sperm cells were collected as described in section 2.10.1 and washed in HTF medium supplemented with 5% BSA. A sperm count was performed using a haemocytometer and a working suspension containing at least 4 to 8 million sperm/ml was used. To induce capacitation, cells were incubated at 37°C for a period of 30 min in BSA supplemented HTF medium.

2.13.2 Acrosome reaction

The acrosome reaction can be induced artificially by treating sperm cells with Ca2+ releasing agents and easily detected by conjugated peanut agglutinin (PNA). Post

capacitation, sperm suspensions were divided into three groups. The first group was treated with ionomycin (working concentration 10 µM, prepared in DMSO), the second group was treated with progesterone (working concentration 20 µM, prepared in DMSO), and the third group (control) was treated with solvent only. At time points of 0, 30, 60, and 90 min post-treatments, sperm cells were pipetted on to glass slides pre-coated with poly-L-lysine (0.1 mg/ml). Slides were air-dried and cells were fixed using ice-cold methanol (100%) for 10 min. Fixed slides were washed with PBS three times and stained with PNA-Alexa Fluor 594 conjugate (Life Technologies) for 30-40 min. Stained slides were washed with PBS to remove any excess of staining. Stained cells were counter-stained and mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (Thermo Scientific). Mounted slides were stored in the dark overnight at room temperature and then at 4°C. Mounted cells were assessed for acrosomal status (lectin PNA+, unreacted; lectin PNA–, reacted) by imaging using a 40x objective under an Olympus BX40 epifluorescence microscope using the respective channels. Three regions were chosen to assess the reaction and used to calculate the percentage of reacted and unreacted cells. To check the effect of agents on the ability of sperm cells to undergo the acrosome reaction, cells were incubated at different concentrations of pharmacological agents for at least 30 min prior to acrosome induction.

2.14 Phospholipase C protein assay

2.14.1 PLCs

Individual protein assays were performed in the presence and absence of pharmacology agents impacting the fertilisation process. Phospholipase C cleaves membrane lipid PIP2 into the classical second messengers, inositol 1,4,5-

trisphosphate and diacylglycerol. This hydrolysis activity of purified proteins was assessed. The purified proteins (Table 5) were obtained from KXTbio, Inc. and assayed as per the manufacturer's instructions. Proteins were stored in storage buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 5% glycerol and 1 mM DTT) at -80°C.

Table 5- Phospholipase C isoformproperties

Protein	Origin species	Molecular weight (g/mol)	Extension coefficient (m^2/mol)
PLCβ1	*Rattus norvegicus*	139,092	92,600
PLCβ2	*Homo sapiens*	134,427	98,210
PLCβ3	*Homo sapiens*	138,367	80,220
PLCγ1	*Rattus norvegicus*	140,469	179,010
PLCγ2	*Homo sapiens*	139,548	183,010
PLCδ1	*Homo sapiens*	85,806	91,790

2.14.2 Sequence alignment

Protein sequences were aligned to mouse protein sequences using BLAST+ version 4 databases (dbV4) on Protein Blast to check the similarity with mouse sequences. Sequences were aligned as a whole and as catalytic XY domains for all the PLCs.

2.14.3 Functional assay using PLCglow™ and purified PLCs

A PLC assay was performed as described by the supplier. Briefly, the assay buffer was prepared with and without Ca2+ as per the composition presented in Table 6.

Table 6- Assay buffer composition (6x)

Component	**Molecular weight (g/mol)**	**6x concentration (mM)**

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	238.3	300
Potassium Chloride (KCl)	74.55	420
ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	380.35	18
Dithiothreitol (DTT)	154.25	12
Calcium chloride (CaCl2)*	110.98	17.8
Bovine serum album (BSA)	66.5 kDa	300 μg/ml

*not added in Ca2+-free buffer to have inactive PLCs as control

PLCglow, a small molecule biosensor and an analogue of PIP_2 (substrate of phospholipase C), is hydrolysed by phospholipase C enzymes and generates IP_3 and 6-aminoquinoline. 6-aminoquinoline is a highly fluorescent molecule easily detectable at λex/em = 344/530 nm. PLCglow was prepared in 2x assay buffer (+/- free Ca^{2+}). Working protein solutions were prepared at different concentrations (including concentrations suggested by supplier) in 1x assay buffer. Conditions and concentrations were optimised and assays performed in triplicates.

Practically, 37.5 µl of 10 µM PLCglow was mixed with 30 µl of distilled water in a 96 well plate prior to monitoring fluorescence (λex/em =344/535 nm) using Infinite M200 Pro Fluroscence Plate Reader (Teccan) at 25°C to achieve the baseline reads. 7.5 µl of protein solution (of different concentrations) was added to each sample. Working concentrations were: 5 µM PLCglow, 50 mM HEPES (pH 7), 70 mM KCl, 3 mM EGTA, 2 mM DTT,50 µg/ml bovine serum albumin (fatty acid free) and +/- ~10 µM free Ca^{2+}. Protein concentrations were different in individual cases and are presented in Table 7.

Table 7 Optimised dilutions of recombinant PLC isoforms

Protein	Stock concentration (nM)	Working concentrations (nM)
PLCβ1	50, 100	5, 10
PLCβ2	50, 100	5, 10
PLCβ3	50, 100	5, 10
PLCγ1	10, 50	1, 5
PLCγ2	10, 50	1, 5
PLCδ1	10, 50	1, 5

2.14.4 Functional assay using PLCglow^{TM} and sperm extract

Mouse sperm extracts were prepared as homogenates and assays performed to hydrolyse PLCglow to assess the hydrolytic ability of sperm derived PLCs in different conditions. Briefly, sperm cells were washed in Ca2+-free PBS supplemented with protease inhibitors and centrifuged to get sperm pellet. The pellet was then lysed by a freeze-thawing method in liquid nitrogen. Cell lysates were spun down at 100,000 g for 1 hour at 4 °C. The supernatant was concentrated into 120 mM KCl, 20 mM Hepes, pH 7.5 with 200 µM PMSF on an Amicon® Ultra-15 Centrifugal Filter (Amicon Ltd, Gloucestershire, U.K.) at 4°C. The final protein concentration of extracts varied between 10 and 50 mg/ml. Extracts were aliquoted on ice and stored at -80°C. Extracts were used at different concentrations and diluted in ice-cold 2x assay buffer prior to assay experiments to achieve different working concentrations. No sample well contained protein extract at less than 5 mg/ml working concentration.

2.14.5 Fluorescence measurement

For protein assays samples were tested in triplicates in clear 96 well plates (Greiner Bio-One- 655101) and fluorescence was measured using an Infinite M200 Pro (Tecan) at 25°C for 90 min.

2.14.6 Data analysis

Data were analysed and plotted as normalised intensities vs time for one hour in each case. Reads were normalised with the baseline and compared with the test conditions.

2.15 *In vitro* transcription/translation (IVTT) inhibition

Pharmacological inhibition of sperm transcripts was performed to unveil their role in fertilisation. Translation inhibition was performed in both individual gametes and during fertilisation. Different translation inhibitors (homoharringtonine – 0-50 µg/ml; cyclohexamide – 0-50 µg/ml, stocks prepared in DMSO) were used along with the control groups to treat sperm and oocytes before fertilisation. Fertilisation indicators were measured by assessing intracellular Ca^{2+}, meiotic resumption, pronuclear formation, second polar body formation and blastocyst formation.

2.16 Sperm proteomic analysis

2.16.1 Cell lysis and protein sample preparation

Sperm samples to be sent for proteomic profiling were capacitated prior to cell lysis for 1 hour for both wild type and PLCζ knockout sperm. Cells were lysed in NP40 cell lysis buffer as per manufacturer's instructions. Briefly, cell lysis buffer was supplemented with protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at a concentration of 1mM and protease inhibitor cocktail (Sigma, P-2714). Cells were collected as described in chapter 2 and incubated for one hour at 37°C. Post

incubation, cells were washed in PBS twice and centrifuged to collect the pellet. Supplemented NP40 buffer was added on the pellet and incubated for 30 min on ice with vortexing every 10 min. The cell lysate was transferred to microcentrifuge tubes and centrifuged at 4°C at 13,000 rpm for 10 min. Supernatants were separated and samples were sent to the proteomics facility. Multiple freeze/thaws were avoided throughout the sample preparation.

2.16.2 Protein quantification

Bovine serum albumin (BSA) was used to prepare assay standards of 0-2000 µg/ml in NaOH (100 mM). Samples were diluted in 100mM NaOH to test at least two dilutions per sample (viz. 1:10 and 1:50) in a 96 well plate. Assay reagent was prepared by diluting $CuSO_4$ (Sigma C2284) in bicinchoninic acid (BCA) at a ratio of 1:50$CuSO_4$:BCA. 200µl of reagent solution was added to 10µl diluted sample/standard in each sample well and incubated at 37°C for 30-45 min. Post incubation, absorbance was measured at 595 nm using a Sunrise microplate reader (Tecan). A standard curve was constructed and used to estimate the concentration of samples, considering the dilution factor.

2.16.3 LC-MS/MS protein sample preparation and digestion

Sample preparation and digestion protocol was followed as described by Pinto-Fernández et al., (Pinto-Fernández et al., 2019). Brieflly, protein-targeting activity DUB probe samples eluates (immunoprecipitated) were diluted to 175 µl with ultrapure water and reduced with 5 µl of DTT (200 mM in 0.1 M Tris, pH 7.8) at 37°C for 30 min. Alkylation of samples was done with 20 µl of iodoacetamide (100 mM in 0.1 M Tris, pH 7.8) at room temperature for 15 min (samples were prevented from light). Protein precipitation wascarried out using a double methanol/chloroform extraction method. Samples were further treated with 600 µl

of methanol, 150 µl of chloroform, and 450 µl of water, followed by vortexing, and centrifugation at 17,000 g for 3 min. Upper aqueous phase was removed. Proteins were pelleted after the addition of 450 µl of methanol by centrifugation at 17,000 g for 6 min. Extraction was repeated after supernatant removal. After second extraction process, precipitates were re-suspended in 50 µl of 6 M urea and further diluted to <1 M urea and 250 µl of buffer solution (20 mM HEPES, pH 8.0). Protein digestion was performed by adding trypsin from stock of 1 mg/ml in 1 mM HCl to a ratio of 1:100 with rocking at 12 rpm overnight. After digestion, samples were acidified (1% trifluoroacetic acid) and desalted on C18 solid-phase extraction cartridges (SEP-PAK plus, Waters). Further samples were dried and suspended in 2% acetonitrile + 0.1% formic acid for LC-MS/MS analysis.

2.16.4 Protein fractionation

Fractionation was done as described previously by Pinto-Fernández et al., (Pinto-Fernández et al., 2019). Digested samples were fractionated using the loading pump of a Dionex Ultimate 3000 HPLC and a Waters Acquity UPLC Peptide BEH C18 column (300 Å, 1.7 µm, 1 mm × 100 mm, part no. 186005593) over a 65 min gradient using basic pH reverse-phase buffers (A: water, pH 10 with ammonium hydroxide; B: 90% acetonitrile, pH 10 with ammonium hydroxide). The gradient consisted of a 15 min wash with 2% B, then increasing to 35% of B over 30 min, with a further increase to 95% B for 0.1 min, followed by a 9.9 min wash at 95% B and then returning to 2% for 0.1 min, followed by re-equilibration at 2% B for 9.9 min, at a flow rate of 100 µl/min with fractions collected every minute from 0 to 60 min. One hundred µl of the fractions were dried and resuspended in 20 µl of 2% acetonitrile/0.1% formic acid for analysis by LC–MS/MS. Fractions were loaded on the LC–MS/MS following concatenation of 60 fractions into 10, combining fractions

in a 10-fraction interval (F1 + F11 + F21 + F31 + F41 + F51… to F10 + F20 + F30 + F40 + F50 + F60).

2.16.5 Mass spectrometric analysis

LC-MS/MS analysis was carried out using a Dionex Ultimate 3000 nano-ultra high-pressure reverse-phase chromatography coupled to a Q Exactive HF mass spectrometer (Thermo Scientific). In brief, samples were separated using EASY-Spray PepMap RSLC C18 column (500 mm × 75 µm, 2 µm particle size, Thermo Scientific) over an hour gradient of 2–35% acetonitrile in 5% dimethyl sulfoxide (DMSO), 0.1% formic acid at 250 nL/min. MS1 scans were acquired at a resolution of 60,000 at 200 m/z and the top 12 abundant precursor ions were chosen for high collision dissociation (HCD) fragmentation.

2.16.6 Proteomic data analysis and expression validation

DUB proteome in sperm was for the analysis of proteomic data which was assessed by analysing the quantified iBAQ values on Perseus software platform v1.6.0.2. The statistical analysis workflow using Perseus for qualtification is validated by the developers. All raw MS data files obtained after thee HA-IP and high-pH fractionation experiments were analyzed using MaxQuant v1.5.5.1., and searched against the UniProt Mouse database. Intensity values were primarily used to compare against the DUB proteome. Zero values reads were replaced with the value of 1 to allow their scatter plots display. The HA-UbPA probe adduct (112.06 Da) on cysteine residues was used to search PTMs and searched against the UniProt database using customised parameters as followed by Pinto Fernández group (Pinto-Fernández et al., 2019): 10 ppm; fragment mass error tolerance: 0.05 Da; precursor mass search: monoisotopic; enzyme: trypsin; missed cleavages: 2; variable modifications (PEAKS PTM, only the most common listed):

deamidation (NQ), oxidation (M), carbamidomethylation (C), acetylation (K), acetylation (N-term), PA Probe adduct (C), maximal variable PTM per peptide:3.

2.17 Reference with whole transcriptome database (DBTMEE v 1.5.3)

The Database of Transcriptome in Mouse Early Embryos (DBTMEE v 1.5.3)(Park et al., 2015) was referred to in order to analyse the mRNA and protein expression of several proteins including PLC isoforms at pre- and post fertilisation stages. DBTMEE is used to study gene expression and to identify transcription factors associated with genes of interest. Briefly, $1.5x10^5$ MII oocytes were sequenced on a SOLiD system to analyse and understand the changes in gene expression from sperm and oocyte to the zygotic embryo. Total RNA from $\geq 10^4 \times 2$ cells at each stage was isolated and sequenced for quantitative gene expression. Patterns of expression were categorized by clustering. Expression was measured as FPKM (fragments per kilobase of transcript per million fragments mapped) in sperm, oocytes and zygotes. RNA-seq data used to annotate gene expression is available at the DDBJ Sequence read archive (DRA) under the accession number DRA001066. RNA was isolated and prepared for sequencing from non-fertilised oocytes and sperm, parthenogenetically activated oocytes at 4.5, 19, and 43 hours post fertilisation.

2.18 Pharmacological inhibition studies

2.18.1 Gq inhibition

To target Gq, an upstream stimulator of PLCβ isoforms, FR900359 was used which was obtained from Prof Evi Kostenis, University of Bonn. FR900359, a plant-derived depsipeptide, is a selective inhibitor of Gαq/14 and has no effect on other classes of G-proteins (Schrage et al., 2015). A FR900359 stock solution was

prepared in DMSO and stored at -80°C. A stock solution was used to prepare different concentrations, ranging from 1 µM to 10 µM. In live cell experiments, FR900359 was diluted in HTF medium and cells were pre-treated for 30-45 min at 37°C prior to the fertilisation experiment. Post FR900359 treatment cells were washed with HTF unless stated otherwise.

2.18.2 Phospholipase C inhibition

U73122 (Santa Cruz Biotechnology, sc-3574), an aminosteroid phospholipase C inhibitor, was used to carry out PLC inhibition in both sperm and oocytes. To include a negative control group, an inactive form of U73122, called U73343 (Santa Cruz Biotechnology, sc-201422), was used at the same concentrations as U73122 i.e. 5µM to 100µM. U73343 demonstrated no activity towards phospholipase C in several experiments. The main difference between U73122 and U73343 is that the pyrrolidinedione group of U73122 is substituted by a pyrroledione group which makes U73343 inactive.

The use of U73122 is well demonstrated in the literature and has been utilised to understand several biological processes involving phospholipase C enzymes including cancer (Huali Chen et al., 2019; Genevieve Dupont et al., 1996; Neacsu et al., 2019; Stovall et al., 2019; Sun et al., 2019; S. Y. Yoon et al., 2012). In addition to the use of U73122 to understand roles of phospholipase Cs, U73122 specificity experiments were also conducted to determine its ability to inhibit individual PLC enzymes. Functional assays of U73122 mediated phospholipase C inhibition was assessed using the PLCglow^{TM}method as described in section 2.14.3. Studies were also carried out with sperm homogenates to better understand the ability of U73122 to block endogenous phospholipase C activity in sperm cells (as described in section 2.14.4).

2.19 Sperm-oocyte binding patterns

Sperm binding to superovulated mature oocytes was examined as described by Bedford and Kim (Bedford and Kim, 1993). Briefly, oocytes were superovulated and collected as described in section 2.10.2 and 2.10.3. Sperm cells were collected in a similar fashion as described in section 2.10.1.HEPES-buffered human tubal fluid (HTF) was used in the collection and handling of gametes. Oocytes were inseminated with approximately 150,000 spermatozoa in 35 mm dishes containing 0.9 ml of HTF medium. Timely observation of cumulus oophorus was performed in all groups for around 3-4 hours after insemination. PBS washings were added to the fertilised oocytes to remove loosely bound and superficially attached sperm cells. After washing, fertilised oocytes were transferred to glass slides and covered with ProLong Gold Antifade Mountant with DAPI (Thermo Scientific) to stain the nucleus. Cells were preserved in the dark and examined for sperm binding patterns to the oocytes. Patterns were assessed as: no sperm binding; >3 spermatozoa bound per oocyte to the zona surface only; <3 spermatozoa/oocyte at the surface of and/or intruding into the matrix of the zona; and >3 spermatozoa per oocyte bound to and/or intruding into the zona. Bound sperm numbers on oocytes were expressed as group means ± SEM.

2.20 Developmental studies

To understand the effects of several inhibitors, oocytes were examined for several indicators to assess the development from 1-cell zygotic stage to blastocyst stage. Pronuclear and second polar body extrusion were assessed in 1-cell stage fertilised eggs. Cleavage of fertilised oocytes which confirms the successful meiotic division of fused DNA was examined followed by observation of 4-cell stage, 8-cell stage, morula and blastocyst stage. Brightfield microscopic examination was

performed using a heating stage to avoid the effects of temperature change. Cells were separated and stained wherever required.

2.21 Statistical analysis

All data are presented as +/- standard error of the mean (SEM). For most data comparisons, an unpaired 2-tail student's t-test was used (*$p<0.05$; **$p<0.01$; ***$p<0.001$). For multiple comparisons of 3 or more data sets, one-way analysis of variance (ANOVA) was used (*$p<0.01$; ***$p<0.001$). Statistical analyses were conducted using GraphPad InStat 3 software (for ANOVA and student's t-tests).

Chapter 3: Expression of IP_3 producers in gamete cells

3.1 Introduction

During studies of oocyte activation in mammals, the role of the IP_3 pathway and associated proteins in this process has been extensively reported as well as supported in the majority of the literature about mammalian oocyte activation. Inositol 1,4,5-trisphosphate (IP_3) is produced by phospholipase C isoforms (PLCs). Although PLCs have different regulatory mechanisms, they have a similar overall structure and carry out the same function which is the production of IP_3 and diacylglycerol (DAG) (Gilbert, 2000). PLCs currently comprise 13 isoforms which are expressed in a wide variety of mammalian tissues as they are essential to carry out basic physiological functions (Bill & Vines, 2020).

In the context of mammalian oocyte activation, very few PLCs have been investigated which means that their role in oocyte activation is fairly unclear (Igarashi et al., 2007; Krauchunas & Wolfner, 2013). However, the discovery of PLCζ in mammalian sperm cells led to a demonstration of its role in activating the oocyte but its deficiency can be substituted by some unknown factors as shown by studies of PLCζ knockout mice (Hachem et al., 2017; Nozawa et al., 2018).

Since the PLCζ knockout findings a major unresolved issue is the nature of the proteins and other factors that might work either alongside or in the absence of PLCζ to initiate oocyte activation, and partially substitute for its absence. One possible type of oocyte activation factor is one or more of the other PLC isoforms. However, there is a limited amount known about what roles individual PLC isoforms play in sperm and oocytes from their maturation to fertilisation and post fertilisation. Some of the important known functions are highlighted below.

3.1.1 PLC isoforms in oocytes

PLCβ1 – This isoform from the PLCβ group (which comprises β1, β2, β3 and β4 isoforms) is widely expressed in mammalian cells including oocytes and known to have important physiological functions. The role of PLCβ1 in oocytes during the meiotic process is well documented. PLCβ1 is implicated in the control of the G2 to M phase transition and serves as a direct regulator of growth during the oocyte maturation process. Its translocation from the nucleus to the cytoplasm and then the lining of the plasma membrane and its return to the nucleus at GV breakdown, is an important step that occurs at this stage. This translocation has been reported to be crucial for oocyte maturation and any inappropriate localisation may lead to defects in fertilisation (N Avazeri et al., 2000; Nathalie Avazeri et al., 2003; Lefèvre et al., 2007). PLCβ1 has also been suggested to be involved in oocyte activation by playing a role in the regulation of Ca^{2+} oscillations in oocytes (Igarashi et al., 2007). Igarashi et al., attempted to reduce the expression amount of PLCβ1 in mouse oocytes thorough a transgenic RNAi approach and tested their ability to maintain Ca^{2+} oscillations. A decrease in overall PLCβ1 expression resulted in a significant fall in the amplitude of Ca^{2+} transients without affecting their frequency. Moreover, overexpression of the same PLCβ1 isoform through cRNA

microinjection affected Ca^{2+} transients. Specifically, the interval time between two transients was increased by 100% and the frequency was reduced by ~50%. These findings highlights that PLCβ subtypes have some important role in modulating Ca^{2+} oscillations during oocyte activation. Moreover, Ca^{2+} transients are not solely generated by the primary factor PLCζ. This was the first direct evidence reported of a potential role for a PLCβ subtype in regulating Ca^{2+} oscillations at fertilisation.

PLCβ2 and PLCβ3 - Little is known about the role of PLCβ2 in relation to oocytes. There is a lack of evidence for their link to oocyte activation. However, some studies have showed links to embryogenesis, pregnancy, and oocyte competence. A study reported PLCβ2 as a potential biomarker in which the cumulus cells were isolated from oocytes that were successful and unsuccessful at oocyte competence, embryo quality and pregnancy (post-implantation). Moreover, differential expression analysis indicated that PLCβ2 is expressed in oocytes and is involved in Ca^{2+} signalling, and several signal transduction pathways where it interacts with protein kinase (Assidi et al., 2011). In addition, genome-wide association studies of individuals with polycystic ovary syndrome (PCOS) indicated that GNAQ, PLCβ2 and PLCβ3 play a role in cumulus cell-oocyte interactions, oocyte maturation and embryo development (Qiao & Feng, 2011; Shim et al., 2015).

PLCδ4 – Female mice, lacking this gene, are fertile with slightly reduced fertility and litter size (K Fukami et al., 2001; Oliveri et al., 2007). Again, PLCδ4 has not been studied in mammals for its possible role in oocyte activation leaving gaps in our understandings. However, some studies of its role in oocyte maturation, and embryo development have been reported. A novel mutation in PLCδ4 (L696P)

was was reported to be linked to triploidy and has been suggested to be linked to oocyte maturation, activation or second polar body extrusion. This study suggests that the failure of embryonic development due to a mutant form of PLCδ4 mutation in oocytes might be caused by improper oocyte activation/fertilisation (Filges et al., 2014).

3.1.2 PLC isoforms in sperm

PLCβ1 – The acrosome reaction is a crucial step that has to occur to facilitate the interaction of the sperm and the oocyte. Acrosome induction is a Ca^{2+}-dependent reaction and regulated by PLCβ1 through a G alpha q/11 activation and IP_3 release by IP_3 receptors (IP_3R) selectively localised at the acrosome (Walensky & Snyder, 1995).

PLCβ2 - PLCβ2, known as an important member of the bitter taste transduction cascade (including α-gustducin, Ggamma13, PLCβ2, α-gustducin and Trpm5), is also known to be expressed in the testis and present in all phases of spermatogenesis; depletion of this isoform leads to massive spermatid damage. This reveals an important role for PLCβ2 in male germ cell development (F. Li & Zhou, 2012).

PLCδ4- This isoform is highly expressed in testis and known to play important roles in fertilisation and development. PLCδ4$^{-/-}$ sperm cells failed to undergo the acrosome reaction. As the acrosome reaction is a zona pellucida induced reaction, PLCδ4 in sperm cells seems to be regulated by surface recognition and receptor activation to initiate the acrosome reaction. However, PLCδ4 has no ability to induce fertilization-like Ca^{2+} oscillations if transferred into the oocyte by ICSI (K Fukami et al., 2001).

PLCζ – This is selectively expressed in sperm cells and is known as the main factor that triggers Ca^{2+} oscillations in oocytes at fertilisation (Hachem et al., 2017; Kashir et al., 2018; K Swann & Lai, 2016).

Expression of the rest of the PLC isoforms has not been reported at protein levels in sperm cells which leaves a gap in our understanding of their involvement in sperm development and potential role during oocyte activation.

3.1.3 Expression of PLC isoforms in sperm, oocytes and embryo developmental stages

PLCs are known to be expressed in almost all types of mammalian cells including germ cells and have been implicated in carrying out an enormous variety of functions through Ca^{2+} signalling cascades. Their expression in germ cells has been reported in the literature but has not been investigated in a consistent manner. Table 7 depicts a summarised literature review of the expression of phospholipase PLC isoforms in germ cells in the mouse and suggests the gaps that need to be filled in terms of expression analysis before intensive investigation of different PLC isoforms' potential roles in oocyte activation.

3.1.4 Objectives

In sperm and mature oocytes, the expression of PLC isoforms has been under extensively reported, making it harder to form a basis for further studies to understand their potential roles in oocyte activation. To fill this gap, the objective of this chapter is to study the expression of PLC isoforms in sperm and oocytes both at their mRNA and protein levels. mRNA expression dynamics and associated histone modification changes were also investigated to understand the PLC isoforms' expression changes in oocytes from their unfertilised state, through

fertilisation, and subsequently two cell stage embryo development. A second objective of this chapter is to investigate and compare PLC isoforms transcriptional (in sperm cells, ovaries and testes) and protein expression levels (in sperm cells) in wild type and PLCζ knockout mice to identify any up-regulation or down-regulation of these proteins which could throw light on possible overexpression of any PLC subtype, gene compensation or epigenetic changes in the PLCζ knockout animals. This investigation more generally was expected to provide extensive new information about the patterns of expression of PLC isoforms in sperm and oocytes.

3.2 Results

3.2.1 Phospholipase C isoform gene expression

As well-known regulators of Ca^{2+} signalling, at both the mRNA and protein levels, expression of PLC isoforms is widely distributed among major organs and tissues. Publically available data was used to study their expression pattern, as shown in Figure 3. PLCζ is highly expressed in testis and apparently found at trace levels in skin, stomach, kidney and oesophagus. The PLCβ isoforms are expressed in brain, testis, stomach and skin whereas PLCδ isoforms are expressed heavily in the prostate gland, testis, ovary and skin. Looking particularly at the reproductive organs, i.e. testis and ovary tissues, almost all PLC isoforms are expressed in the ovary, except PLCζ. Other PLC isoforms that have never been reported in the context of expression in testis and ovary include XD1, L1 and E1 isoforms.

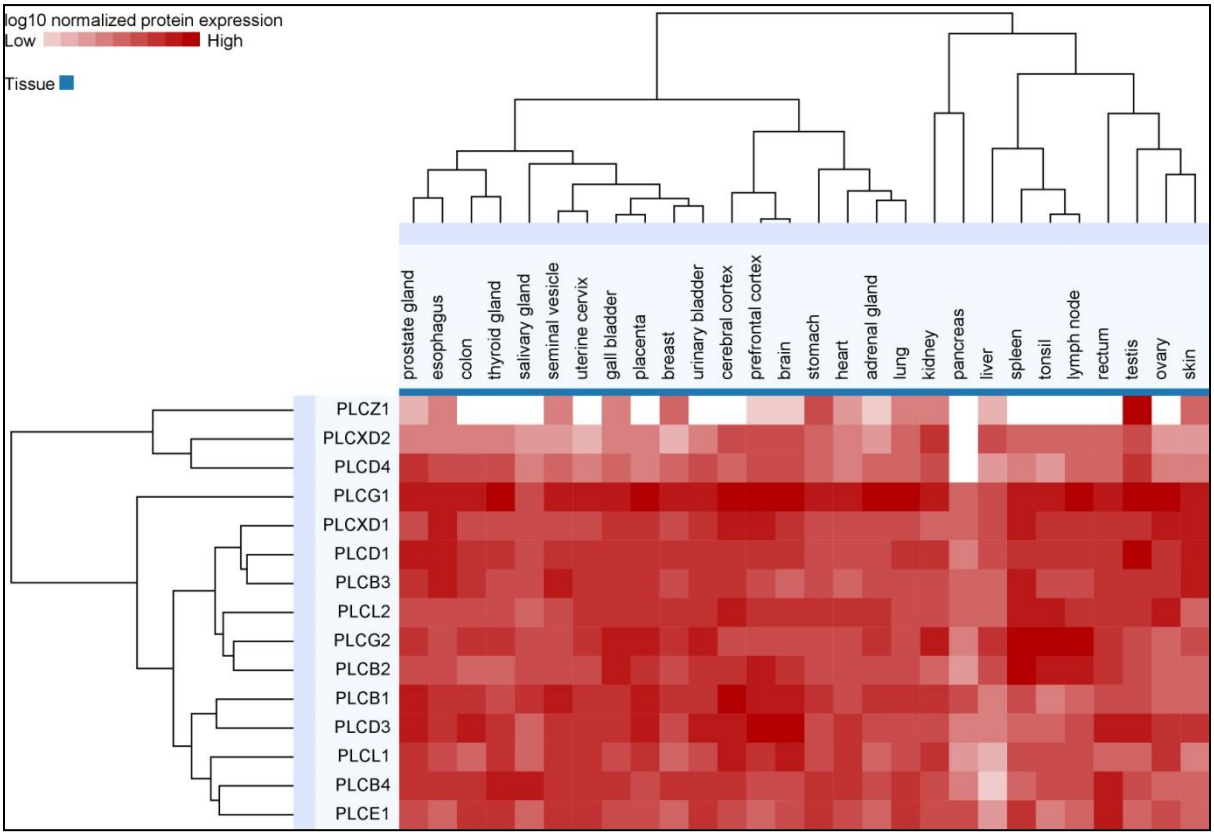


Figure 3 Tissue distribution of major mammalian phospholipase C isoforms.

3.2.2 Gene expression of PLC isoforms in germ cells

An approach of combining several sets of publically available transcriptomic data was adopted to identify the transcription patterns of PLC isoforms and their levels of expression in mature oocytes and sperm cells. The Database of Transcriptome in Mouse Early Embryos (DBTMEE v2.0) was used to extract data and analysed further to identify levels of transcripts of PLC isoforms. In total, 21206 RefSeq genes were mapped in the analysis and compared with gene expression changes during parthenogenetically activated oocyte development. 17486 showed changed levels of expression. It was also possible to trace expression of paternal genes during early embryo development. Data collected from different platforms also

gives an opportunity to compare the expression variation of specific genes of interest.

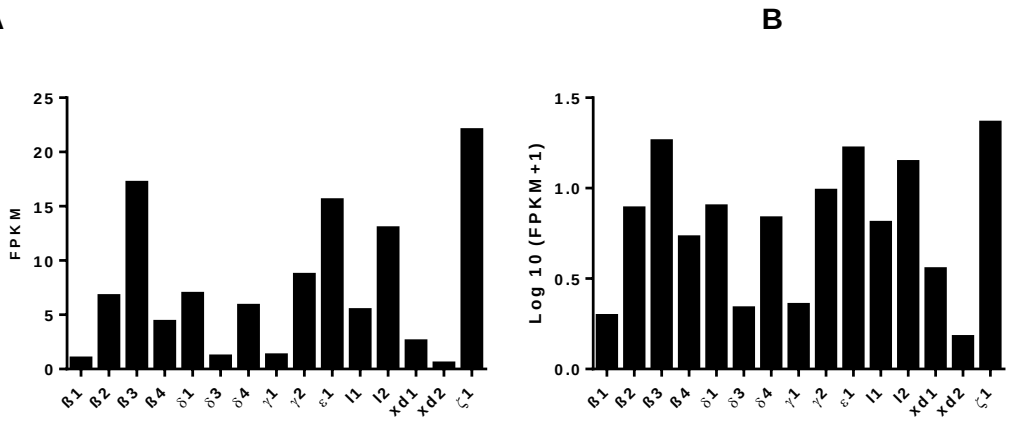


Figure 4 Expression of phospholipase C isoforms in sperm cells through RNA-seq data on SOLiD platform. Data presented as FPKM (fragments per kilobase of exon model per million reads mapped). Isoforms that have not shown any transcript were not presented. A. Data presented as FPKM values; B. Normalised FPKM values. Source: DBTMEE

In sperm cells, as shown in Figure 4, the expression of PLCζ, PLCβ3 and PLCε1 are prominent whereby the expression of PLCβ1, PLCδ3, and PLCɣ1 were found to be comparatively low. A few other isoforms were not detected as transcripts and are not included in the figures. Phospholipase C X Domain Containing 1 and 2 (PLCxd1 and PLCxd2) isoforms share a similar structure with other PLC subtypes, except the Y-domain which is lacking in PLCxd subtypes. Functionally PLCxd1 and PLCxd2, like other PLC subtypes, are PIP_2 hydrolysing enzymes. Overexpression of PLCxd subtypes increases IP3 turnover and endogenous PLC activity in HeLa cell line (Gellatly et al., 2012). Molecular weights of PLCxd subtypes are approximately 36 kDa which is comparatively half of PLCζ proteins. Transcripts of PLCxd1 and PLCxd2 were also detected at small levels in sperm. PLCxd isoforms localise to the cytoplasm and have PIP_2 hydrolysis activity and the lower molecular

weight PLCxd isoform could possibly contribute to IP_3 production once it reaches the oocyte cytoplasm.

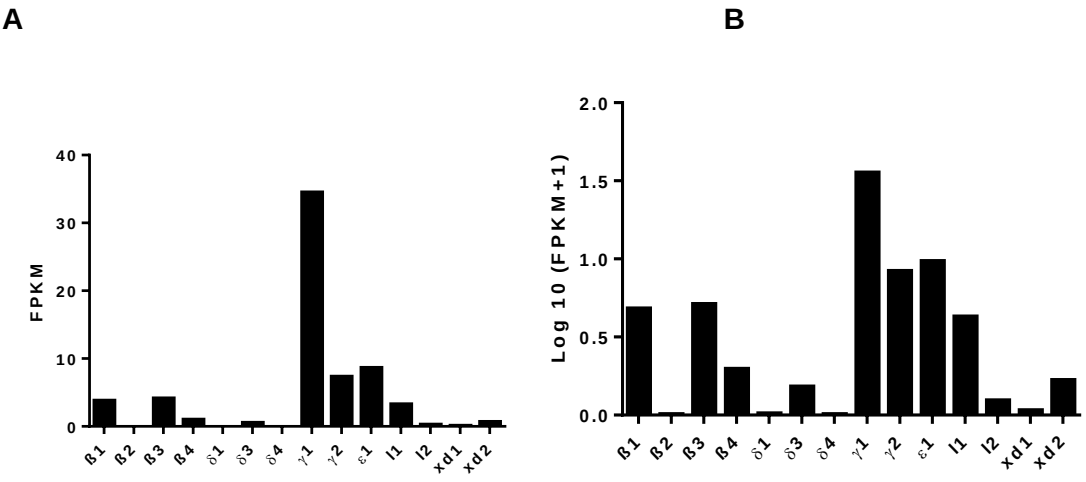


Figure 5 Expression of phospholipase C isoforms in unfertilised oocytes through RNA-seq data on SOLiD platform. Data presented as FPKM (fragments per kilobase of exon model per million reads mapped). Isoforms that have not shown any transcript were not presented. A Data presented as FPKM values; B Normalised FPKM values.

Figure 5 shows the transcription levels of PLC isoforms in mature oocytes which suggests that a number of such isoforms are expressed in oocytes, and that they are expressed at lower levels than sperm PLC isoforms. Interestingly, PLCβ isoforms and PLCδ isoforms were found to be expressed at lower levels in oocytes whereas PLCɣ1 is much more predominantly expressed. PLCɣ1 in oocytes was expressed at even higher levels than in sperm cells. PLCζ was not detected in mature oocytes. Overall, sperm cells showed a higher expression of all major PLC isoforms, with the highest being PLCζ, in comparison to oocyte PLCs expression.

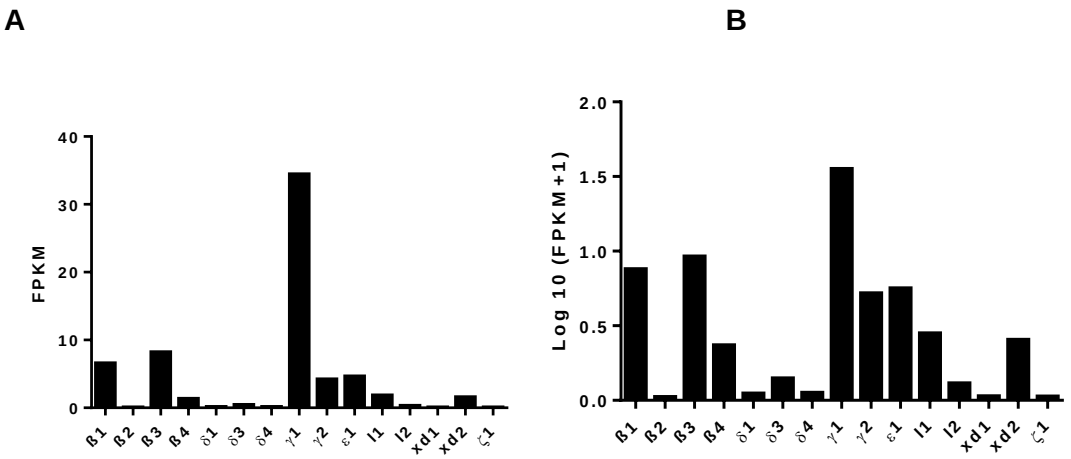


Figure 6 Expression of phospholipase C isoforms in 1 cell fertilised oocyte through RNA-seq data on SOLiD platform. Data presented as FPKM (fragments per kilobase of exon model per million reads mapped). Isoforms that have not shown any transcript were not presented. A. Data presented as FPKM values; B. Normalised FPKM values.

Fertilised oocyte showed a similar pattern of PLCs trancripts at one-cell embryo (Figure 6). Moving to the next stage of embryo development, some PLCs showed a dramatic change in their expression at the two-cell embryo stage. The isoforms that showed a shift in their transcription levels from unfertilised oocyte to two-cell embryo were PLCβ1, PLCβ3, PLCδ3, PLCδ4 and PLCɣ1.

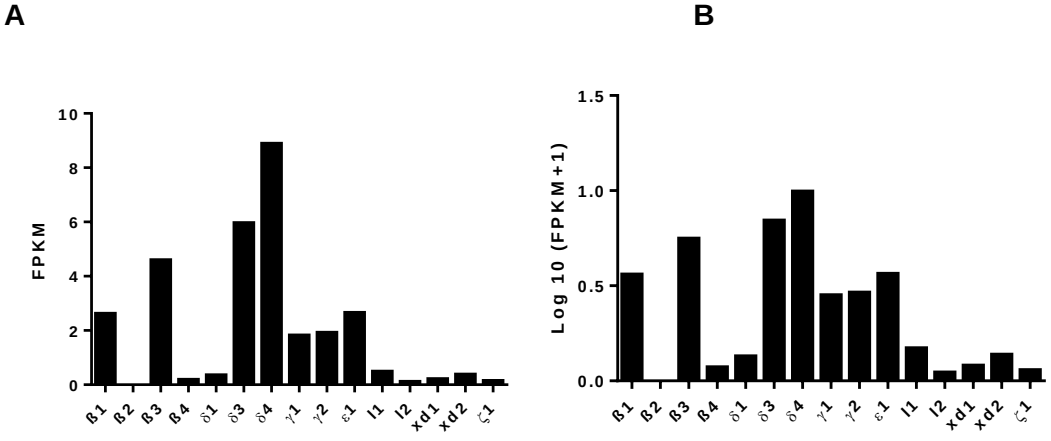


Figure 7 Expression of phospholipase C isoforms in 2 cell stage oocyte through RNA-seq data on SOLiD platform. Data presented as FPKM (fragments per kilobase of exon model per million reads mapped). Isoforms that have not shown any transcript were not included. A. Data presented as FPKM values; B. Normalised FPKM values.

Figure 7 shows that a significant fall in PLCɣ1 expression was detected at the two-cell embryo stage. An overall analysis of the transformation in expression dynamics from the unfertilised oocyte to the two-cell embryo stage would be really useful for understanding the changes in PLC expression. As the activation of the oocyte is highly dependent on IP_3-mediated Ca^{2+} transients, information about changes in the dynamics of PLC isoform expression would potentially shed some light on how IP_3 production changes from the unfertilised oocyte following fertilisation. Figure 8 represents the overall PLC expression dynamics which suggests that changes occur in transcriptional expression of a number of different PLC isoforms in oocytes following fertilisation.

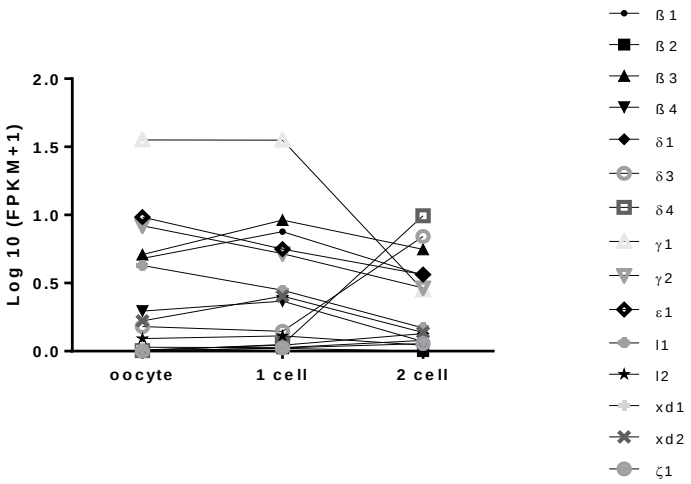


Figure 8 Expression dynamics of PLC isoforms in the early development of the embryo (from unfertilised mature oocyte to 2 -cell stage). Data presented as normalisedFPKM.

Such overall analysis confirmed that at least the mRNA expression of PLC subtypes does not change during the embryogenesis process. Only a few isoforms are seen to change under normal circumstances. Notably, PLCɣ1 which was

expressed at the highest levels in the oocyte among all isoforms decreased dramatically in its level of expression at the two-cell embryo stage. Other isoforms that declined in expression were PLCɣ2, PLCε2, and PLCl1. On the other hand, PLCδ3 and PLCδ4 isoforms showed a rise in expression from the oocyte to the 2-cell embryo. None of the PLCβ isoforms showed any notable change in expression through these stages.

3.2.3 Transcription dynamics and histone modification

Changes in PLC mRNA expression levels from the unfertilised oocyte to the zygote could give an indication of oocyte-derived PLC isoforms that may be involved in oocyte activation and pronuclei formation. To study this issue, the dynamics of gene expression would first need to be explored.

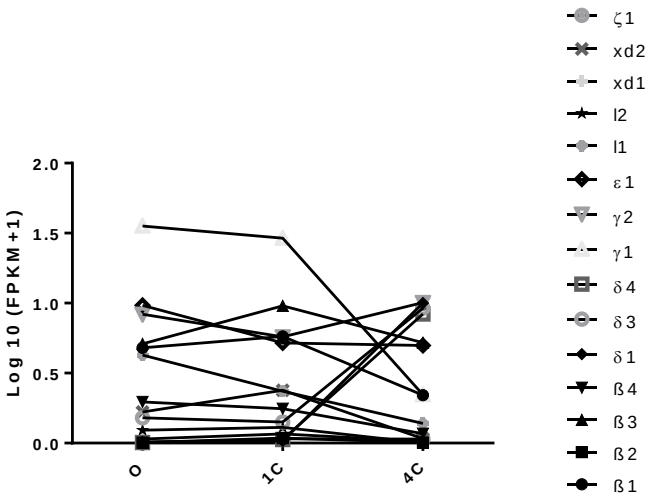
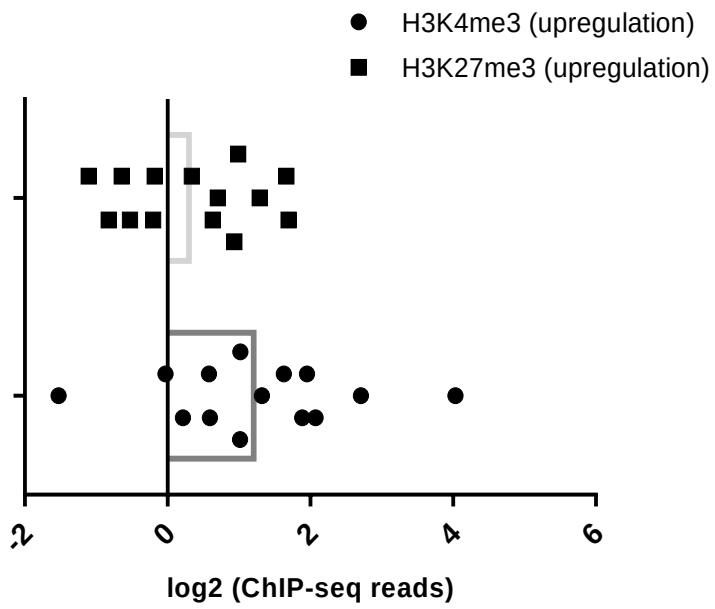


Figure 9 Expression dynamics of PLC isoforms in parthenogenetically activated oocyte up to 4 –cells stage. Data presented as normalised FPKM.

In fact, no significant changes in expression of PLCs were observed in the zygote compared to the unfertilised oocyte. In parthenogenetically activated oocytes a similar trend of PLC isoform expression was observed as after normal sperm induced fertilisation (Figure 9).

A



B

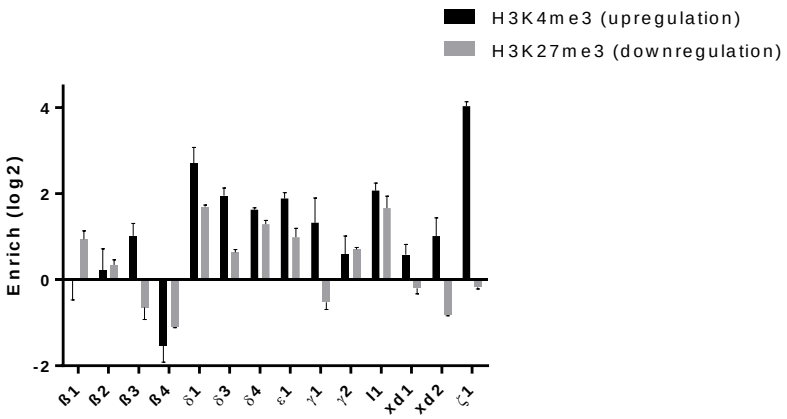


Figure 10 Histone enrichment scores of PLC isoforms in spermatids. A. Group distribution of H3K4me3 & H3K27me3 epigenetic histone modifications to upregulate & downregulate gene expression; B. Individual isoform enrichment score of histone modification at lysine 4 and 27 by 3 methyl groups.

Histone methylation data were extracted from transcriptomic database to understand the transcription regulation of PLCs in sperm cells. Score of trimethylation at 4th lysine (upregulation) and 27th lysine (downregulation) of

histone H3 protein were recorded (Figure 10). Group distribution of 4th lysine suggest that several PLCs are transcribed in mature sperm cells. This data is in line with RNAseq transcription levels of PLCs, as shown in Figure 4.

3.2.4 Protein expression of phospholipase C isoforms in germ cells

Publically available proteomic data for PLC expression is also a potential source of information. Such proteomic analysis of PLC expression suggests that PLCζ is specifically expressed in sperm cells whereas PLCβ, PLCɣ and PLCɣ subclasses are widely expressed in different tissues. Figure 11 depicts a tissue distribution of PLC isoforms.

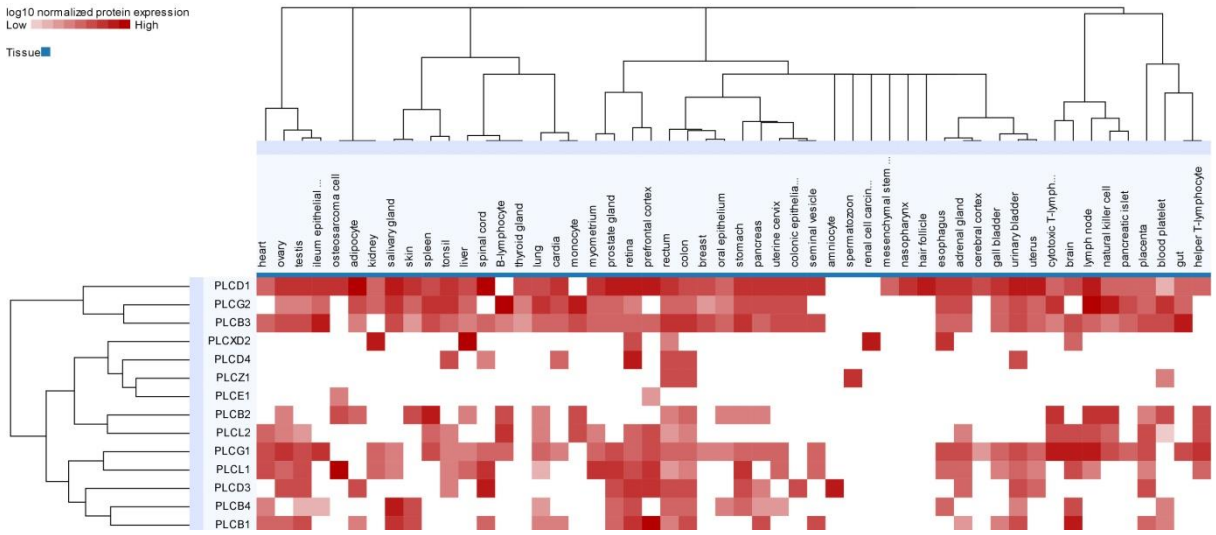


Figure 11 PLC isoform protein expression in different tissues. (Source- Proteomics DB v3.0)

As shown in Table 7 studies of the expression of PLC isoforms in sperm cells remain limited.

Table 8 Expression of phospholipase C isoforms in mouse germ cells

PLC isoform	Sperm	Oocyte	References
β1	?	Yes	(Igarashi et al., 2007)
β2	?	No	
β3	?	Yes	(N Avazeri et al., 2000; G Dupont et al., 1996)
β4	?	Yes	(Yi Liang Miao & Williams, 2012)
δ1	?	Yes	(Igarashi et al., 2007)
δ3	?	No	(Igarashi et al., 2007)
δ4	Yes	Yes	(K Fukami et al., 2001; Kiyoko Fukami et al., 2003; Igarashi et al., 2007; Vicens et al., 2017)
ɣ1	?	Yes	(G Dupont et al., 1996; Mehlmann et al., 1998)
ɣ2	?	Yes	(G Dupont et al., 1996; Mehlmann et al., 1998)
ζ1	Yes	No	(Sanders et al., 2018; Saunders et al., 2002a; K Swann et al., 2006)
L1	?	?	
ε1	Yes	Yes	(Bayram et al., 2016; Igarashi et al., 2007)
ε2	?	?	

Proteomic data from different sources suggests that PLCζ and PLCδ4 are predominantly expressed in sperm cells among all isoforms. A comparison with mRNA expression shows that PLCζ is highly expressed at both mRNA and protein levels whereas PLCδ4 is expressed more at the protein level. PLCε1 was also detected at some levels in mouse sperm (Bayram et al., 2016).

3.2.5 Selection of affinity-purified primary antibodies and secondary antibodies

While publically available data about PLC expression can yield some insights, there is no substitute for carrying out one's own experimental analysis. To this end,

to study the protein expression of different PLC isoforms in sperm, anti-PLC antibodies were selected from the same source to minimise manufacturer variations. A few of them were procured from other sources when antibodies did not work. An optimisation series of experiments were conducted to find out the optimum concentration of primary antibodies. Optimised concentrations, as indicated in Table 9, were used throughout the western blot experiments performed in the project.

Table 9 Primary antibodies information

Protein	Company	Catalog number of antibody	Concentration used (antibody: blocking buffer	Epitope region (Reference sp.)
PLC β1	Santa Cruz Biotechnology, Inc.	sc-136040	1:250	4-159 (Rat)
PLC β1	Santa Cruz Biotechnology, Inc.	sc- 5291	1:250	831-1063 (Rat)
PLC β2	Santa Cruz Biotechnology, Inc.	sc- 515912	1:250	794-818 (Human)
PLC β3	Santa Cruz Biotechnology, Inc.	sc-133231	1:250	1151-1234 (Human)
PLC β4	Santa Cruz Biotechnology, Inc.	sc- 166131	1:250	876-1115 (Human)
PLCδ1	Santa Cruz Biotechnology, Inc.	sc-393464	1:250	71-92 N-terminus (Human)
PLCδ3	Santa Cruz Biotechnology, Inc.	Sc-514912	1:250	517-535 Integral (Human)
PLCδ4	Santa Cruz Biotechnology, Inc.	sc-373875	1:250	1-250 N-terminus (Human)
PLCɣ1	Santa Cruz Biotechnology, Inc.	sc-7290	1:250	1243-1262 C-terminus (Bovine)
PLCɣ2	Santa Cruz Biotechnology, Inc.	sc-5283	1:250	826-985 (Human)
PLC-l	Santa Cruz Biotechnology, Inc.	sc-28328	1:250	121-185 (Human)
PLCxd1	Santa Cruz Biotechnology, Inc.	sc-393244	1:250	42-59 (Human)
p-PLC γ1	Santa Cruz Biotechnology, Inc.	sc-136186	1:250	-Tyr783- (Human)
PLC β1	Proteintech Europe	26551-1-AP	1:200	1162-1216 (Human)
PLCδ4	Proteintech Europe	10589-2-AP	1:250	462-762 (Human)
PLCδ3	Proteintech Europe	16792-1-AP	1:250	441-789 (Human)

Table 10 Secondary antibodies information

Secondary antibody	Source	Working Concentration
IRDye®800CW Goat anti-Mouse IgG	LI-COR, Inc. (926-32210)	1:15,000
IRDye®650 Goat anti-Mouse IgG	LI-COR, Inc. (926-65010)	1:15,000
Goat anti-Mouse IgG (H+L), Alexa Fluor® 488	Thermo Scientific (A-11001)	1 µg/mL

3.2.6 PLC isoforms protein expression analysis in WT and PLCζ knockout mouse model

Western blot studies were performed to identify the expression of PLC isoforms in sperm cells with the aim of establishing the very first systematic study of expression of PLC isoforms in wild type sperm and to identify their pattern of expression in PLCζ knockout sperm. Expressions of several PLC isoforms has been confirmed in brain tissues. Mouse brain tissues lysate was used as control as it was recommended by the primary antibodies manufacturers. Such a comparative study of expression was intended to shed light on any differential expression of these isoforms at the protein level in wild type versus PLCζ knockout sperm, since this might highlight whether another PLC isoform might act as a secondary factor to compensate for the deficiency of PLCζ in the knockout model. Intensity analysis was performed using ImageJ to quantitate analysis and comparison of western blot images. All experiments were performed under the same conditions and detected using the same imaging system.

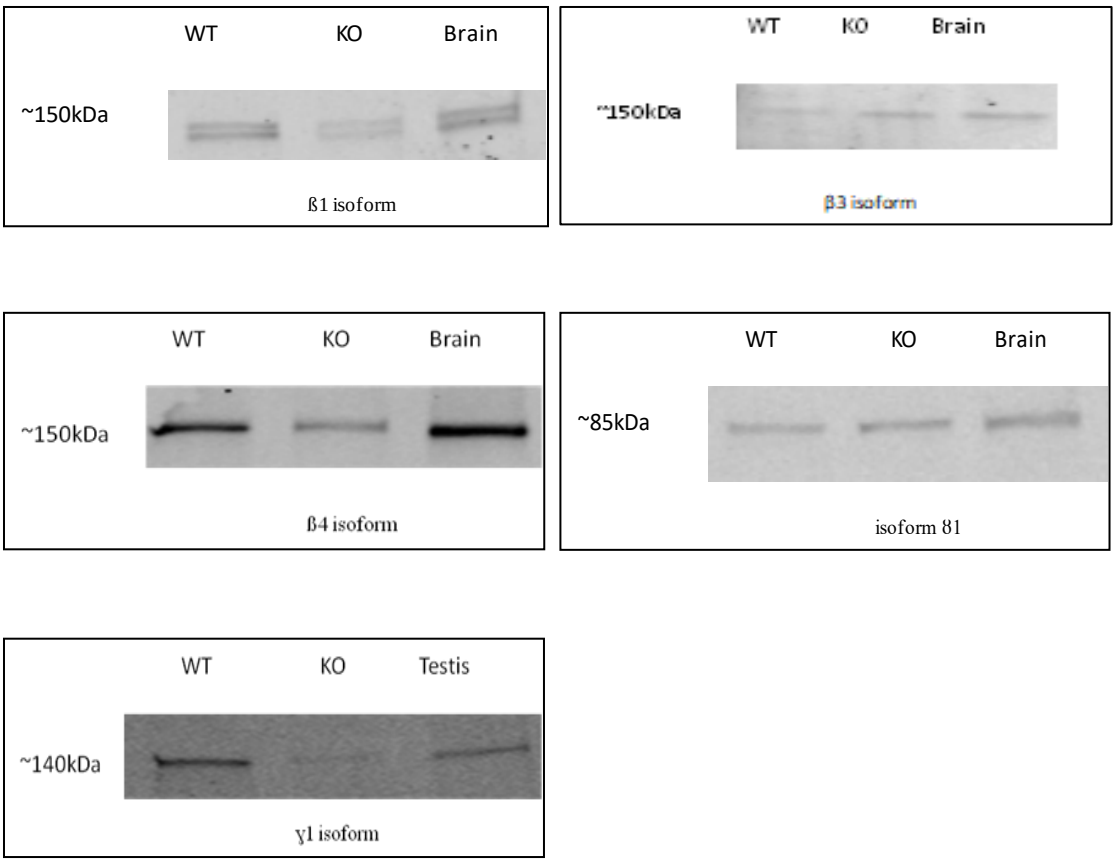


Figure 12 Immunoblot images of PLC isoforms express in mouse sperm. Third band in each image indicates expression in control samples obtained from wild type strain

Expression of five phospholipase C isoforms was confirmed by western blotting and comparative densitometry analysis as indicated in Figure 12. Other isoforms have not been detected with this analysis. Densitometry analysis showed that PLCβ3 and PLCδ1 expressions were increased and PLCβ1 and PLCβ4 and PLCγ1 was decreased, in PLCζ knockout sperm (Figure 13).

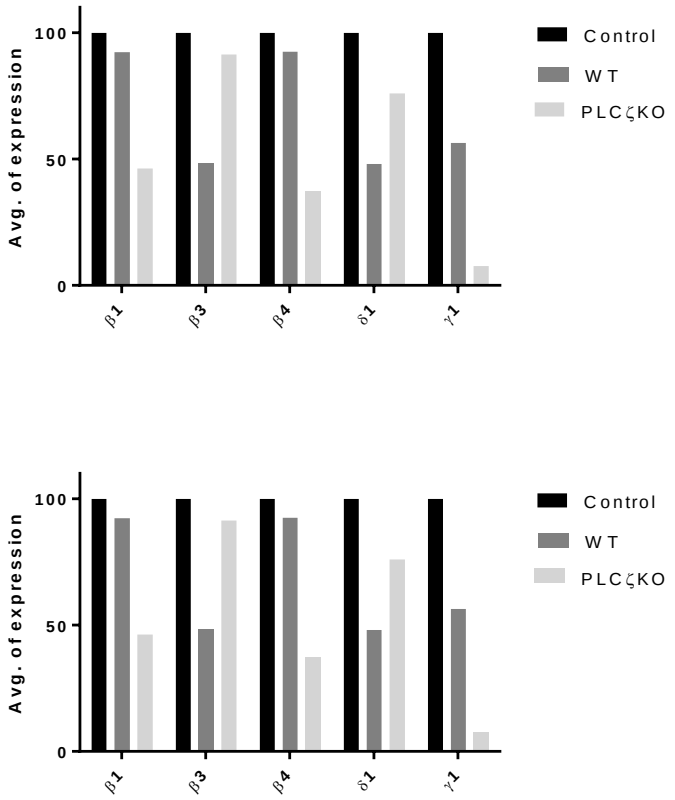


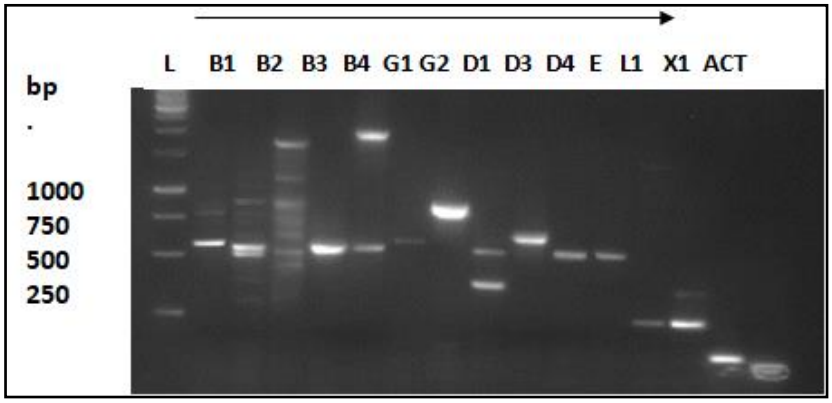
Figure 13 Normalised densitometry intensities of immunoblots of wild type and PLCζ knockout sperm samples

3.2.7 Qualitative gene expression in gametes and reproductive tissues

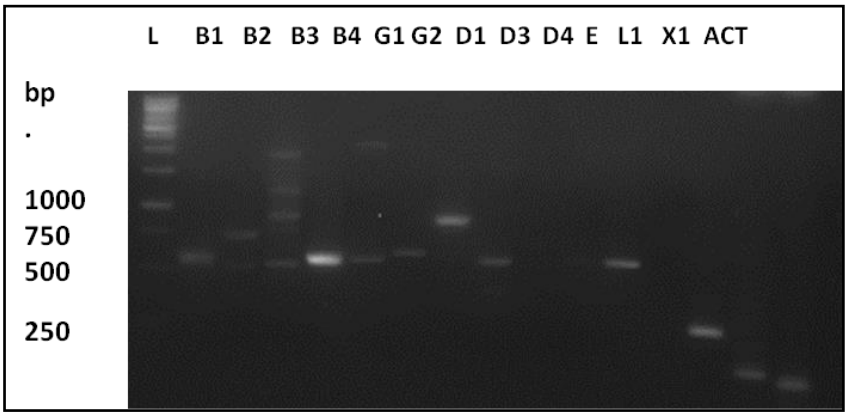
To study the tissue wide expression of reported PLC isoforms in both male and female mice, qualitative mRNA expression was investigated by RT-qPCR. Testis, ovary, and sperm were analysed to compare the expression changes, and to understand whether such changes are gamete specific or not, testes and ovaries were also included Oocytes were not included in this study due to limited animal availability and hormone-induced transcriptional variations. Qualitative mRNA

expression results suggest that testes, ovaries and sperm celss express a all major PLC subtypes (β, δ, γ), as indicated in Figure 14.

A



B



C

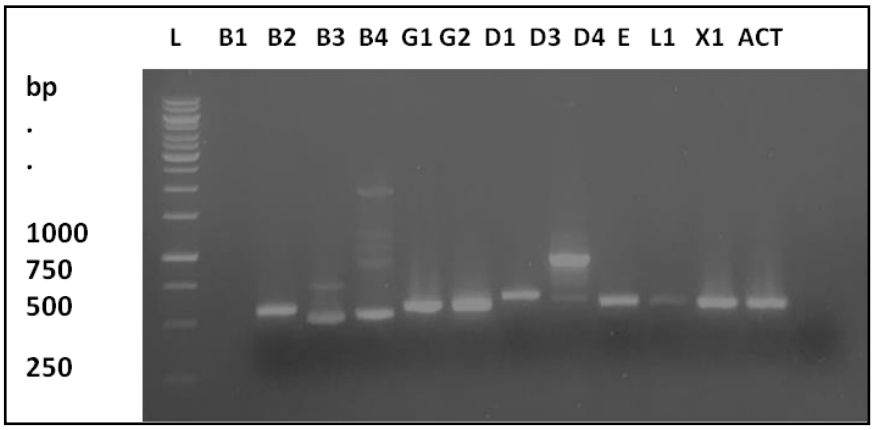


Figure 14 Qualitative mRNA expression of phospholipase C isoforms in mouse A. Testis; B. Sperm; C. Ovary. *L- stands for ladder

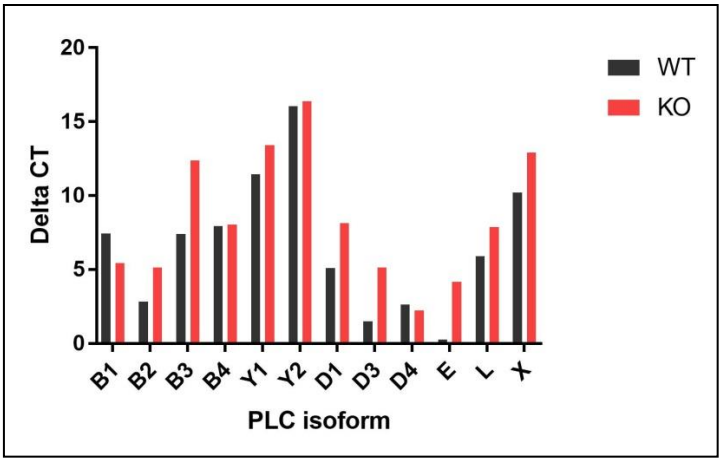
In the pictures above, in testes, lanes B2 and B3 were observed smeared which may be due to excess of primers concentration. Gels for the sperm cells and ovary were ran on different days. The amplification in the sperm samples seems lesser than expected which may be due to less concentration of primers. In sperm and ovary gels, the minor differences in agarose concentration and imaging equipment settings may have caused the variation in resolving time and appearance of the pictures. Nevertheless, the desired bands were observed in all the gels. In testis, PLCxd1 seems not to be expressed. Looking at the sperm profile, a specific trend of PLC isoform expression was observed in sperm cells. All PLCβ isoforms are expressed in sperm but with lower levels of PLCβ2. Both PLCɣ1 and PLCɣ2 are expressed and PLCδ1 and PLCδ4 are present at comparatively lower levels than PLCδ3. PLCxd1 was not detected in sperm. On the other hand, the ovary seems to express all PLC isoforms at a significant level. In comparison to the publically available mouse transcriptomic data, some variation in the expression of PLC isoforms was observed with the current study's own experimental analysis particularly for PLCδ1 and PLCxd1 isoforms.

3.2.8 WT vs PLCζ KO - PLC isoforms gene expression analysis

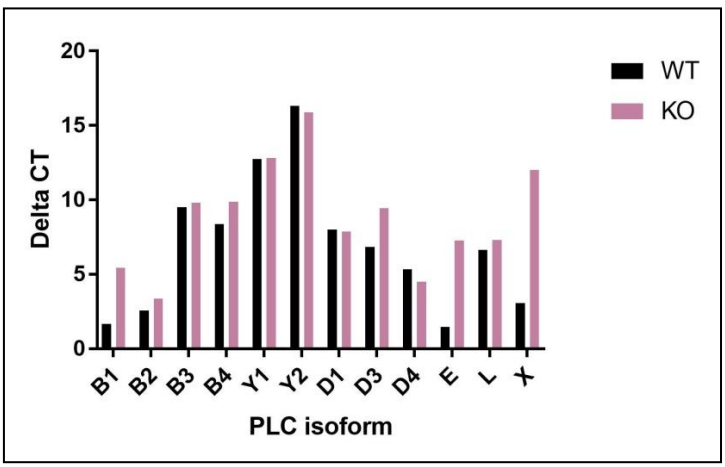
To test the hypothesis that upregulated sperm PLC isoforms potentially contribute to Ca^{2+} signals in the oocyte in the absence of PLCζ expression, a real-time quantitative dynamics of these isoforms' mRNA expression in wild type versus PLCζ knockout sperm was performed. At the same time, mRNA expression of different PLC isoforms in wild type versus PLCζ knockout testis and ovary was also studied. In other words, the tissues selected for this experiment were the same as for the RT-PCR analysis. As in the previous analysis, oocytes were not included in

this study. A real-time quantitative PCR reaction setup was used to identify differentially expressed PLC isoforms in PLCζ knockout mice. Data was analysed at different stages to: 1) determine the fold change of expression of PLC isoforms in PLCζ knockout tissues and 2) expression change (delta CT) in relation of housekeeping gene, actin, within the tissue to correlate with qualitative expression findings (Figure 15).

A



B



C

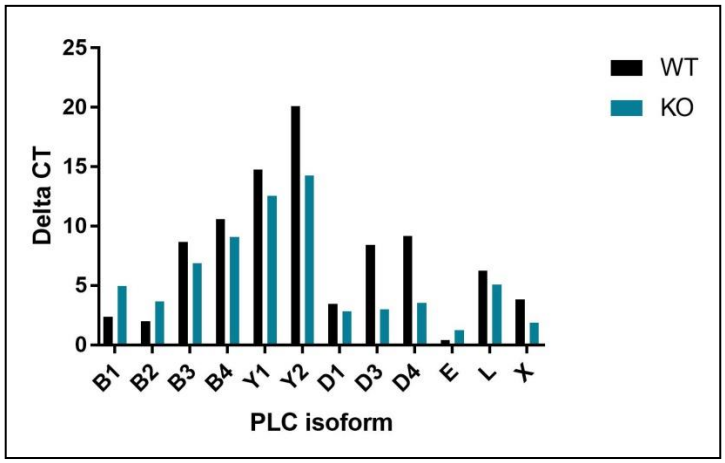
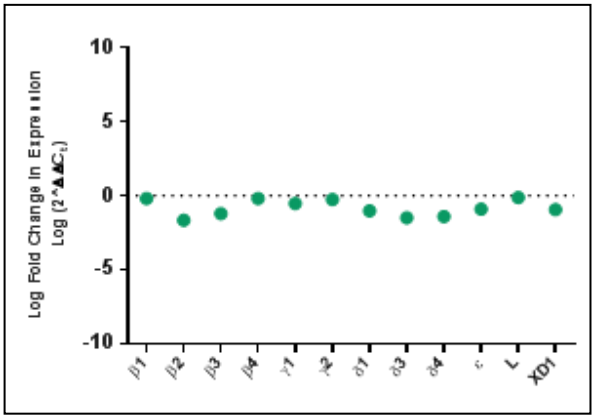
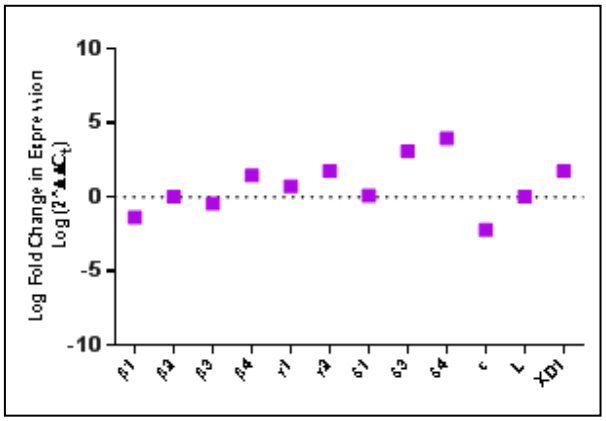


Figure 15 Comparative expression levels of PLC isoforms in relation to actin control (ΔΔCt) in A. Testis, B. Ovary, and C. Sperm

A



B



C

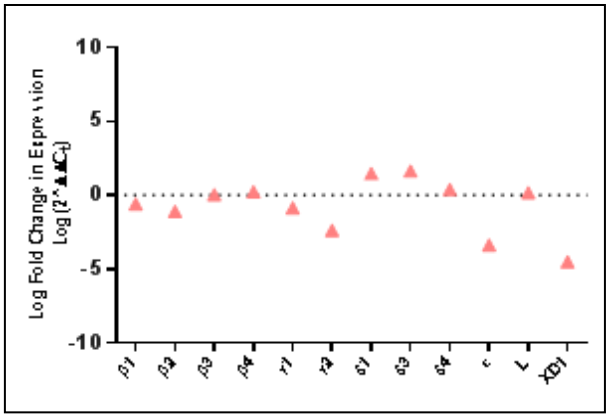
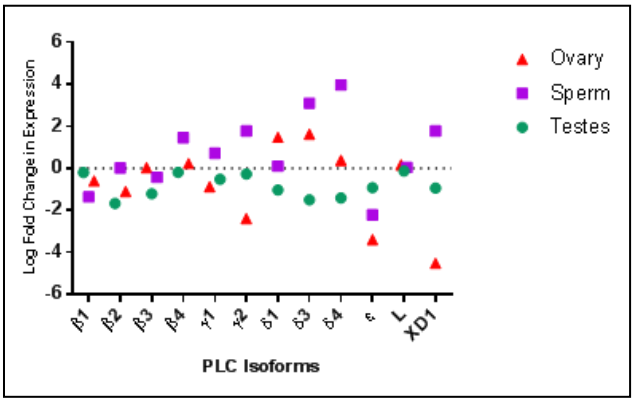


Figure 16 Logarithmic fold change of PLC isoforms in WT vs PLCζ knockout A. Testis, B. Ovary, C. Sperm

The differential fold change, shown in Figure 16 and 17, in expression in the testis showed mostly downregulation of expression of other PLC isoforms in the PLCζ knockout. Thus PLCβ2, PLCδ3 and PLCδ4 were downregulated in PLCζ knockout testis. In sperm cells PLCβ1 and PLCβ2 showed no change in expression in the testis but PLCδ3 and PLCδ4 were upregulated by 2 fold. Other downregulated PLC isoforms in PLCζ knockout sperm are PLCɣ1, PLCɣ2, PLCε1 and PLCxd1.



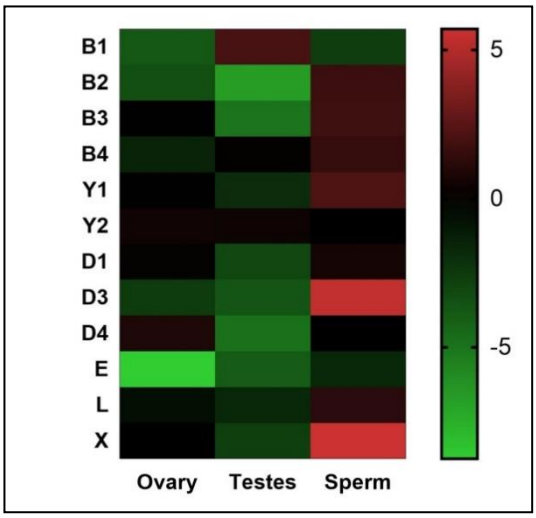


Figure 17 Combined view of the fold change of PLC isoforms in testis, ovary and sperm cells.

In the ovary, most of the isoforms showed upregulation including PLCβ, PLCɣ and PLCxd1 subtypes. PLCβ2 showed some level of down-regulation. PLCδ1 remains unchanged. These results have shown that deleting sperm specific PLCζ brought a range of changes in other member subtypes regulation in non-specific tissues such as ovary. Any deletion or change of a gene may drive genome evolution and leads to secondary mutations which has been proved in yeast model (Teng et al., 2013). This may cause either gain or loss of function. Levels of regulation of affected genes might differ among tissues within the same organism which could be explained by associated regulatory genes in a tissue. However, this theory needs validation for CRISPR/Cas9 generated knockout models.

3.2.9 WT vs PLCζ KO protein expression analysis of PLCβ isoform

To further study the differential expression of PLC isoforms at the protein level, immuno-fluorescence analysis was carried out on selected PLC isoforms to confirm whether there was differential expression in PLCζ KO sperm, and also to see if there were any changes in terms of intracellular localisation. The accuracy and standards of immunofluorescence were not established in this study and the comparative analysis was done based on the intensity measurements of the captured images. Primary antibodies were used as indicated in Table 9. Members of the PLCβ, PLCɣ and PLCδ isoform classes were investigated. PLCβ1, PLCβ2

and PLCβ3 were found to be localised in the sperm head region (Figure 18 and 19).

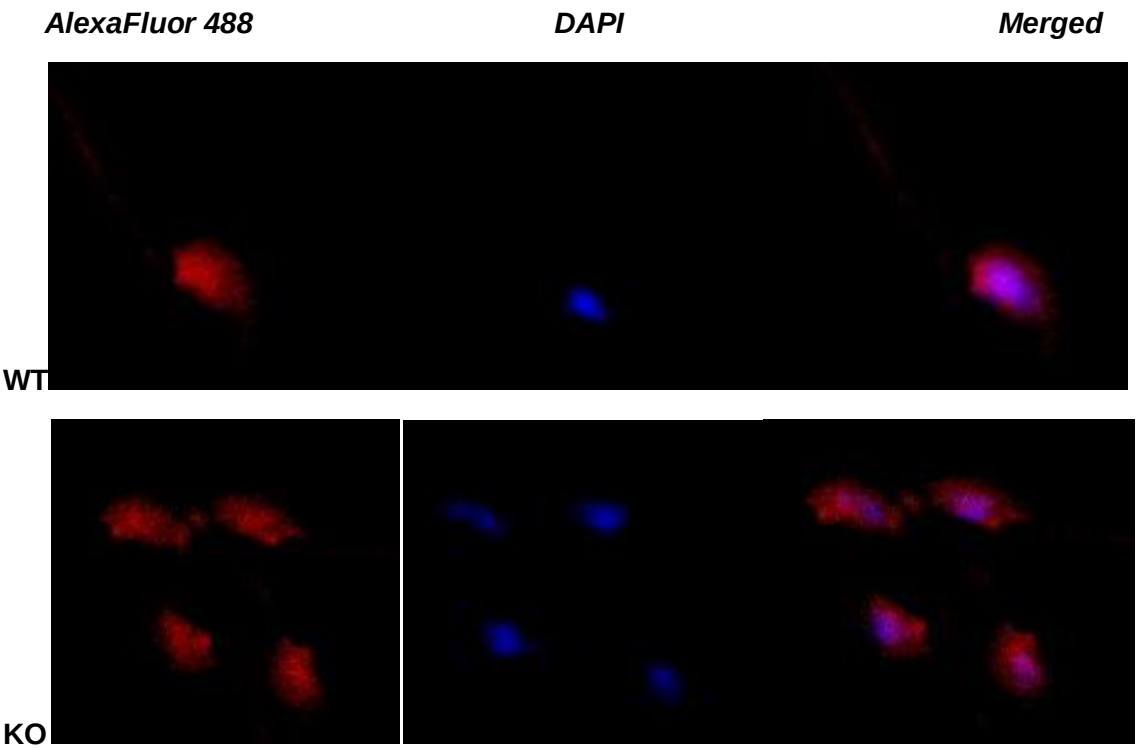


Figure 18 PLCβ1 expression in wild type and PLCζKOsperm head region.

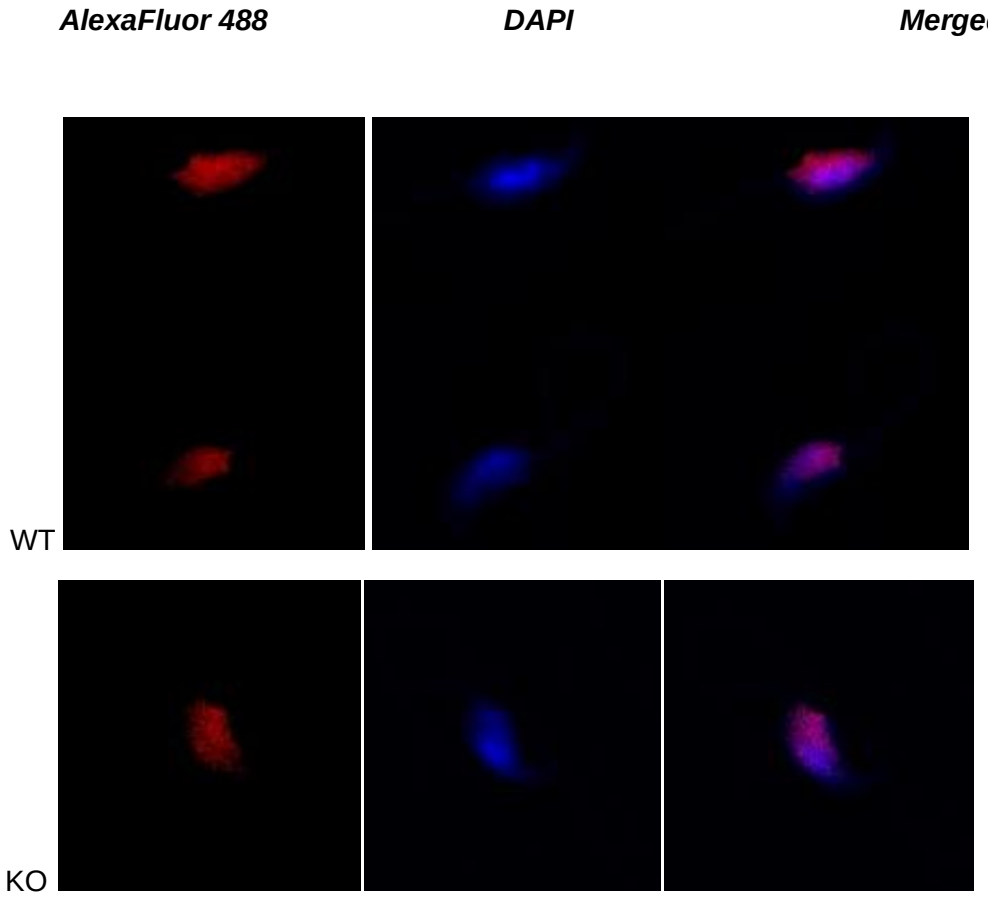


Figure 19 PLCβ2 expression in wild type and PLCζ KO sperm head region.

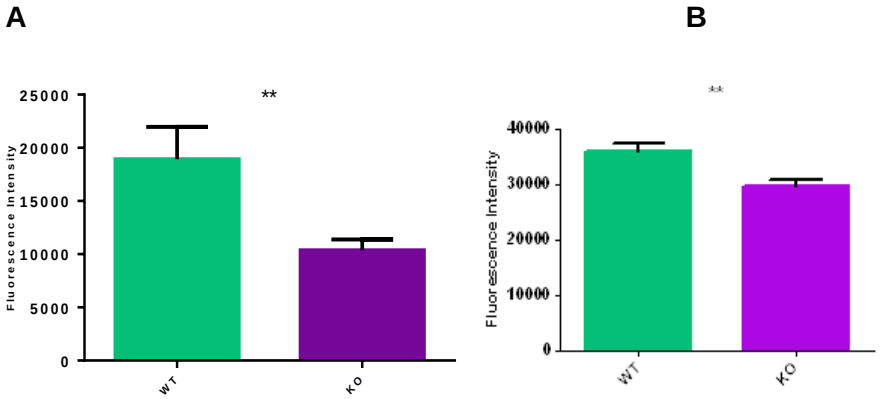
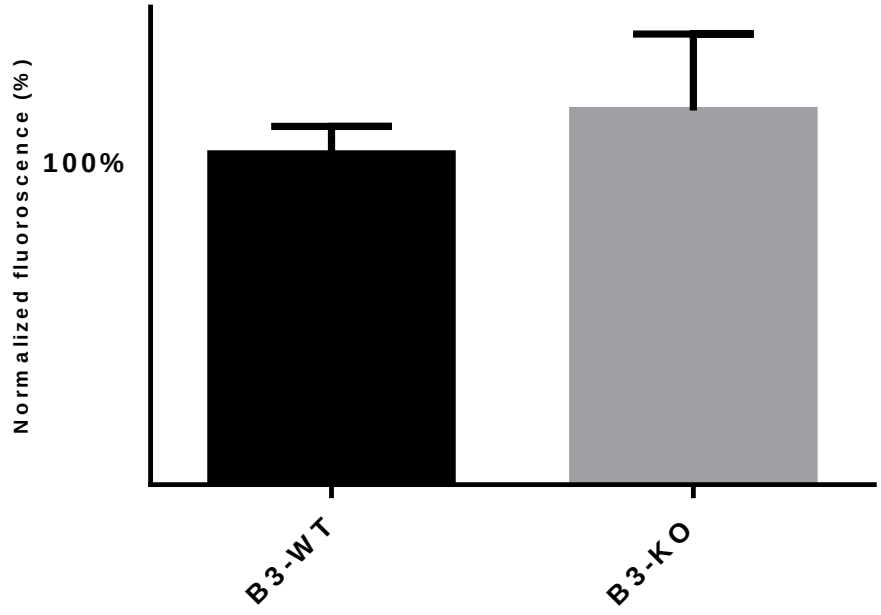


Figure 20 Protein expression of A. PLCβ1 and B. PLCβ2, in WT and PLCζ KO sperm cells

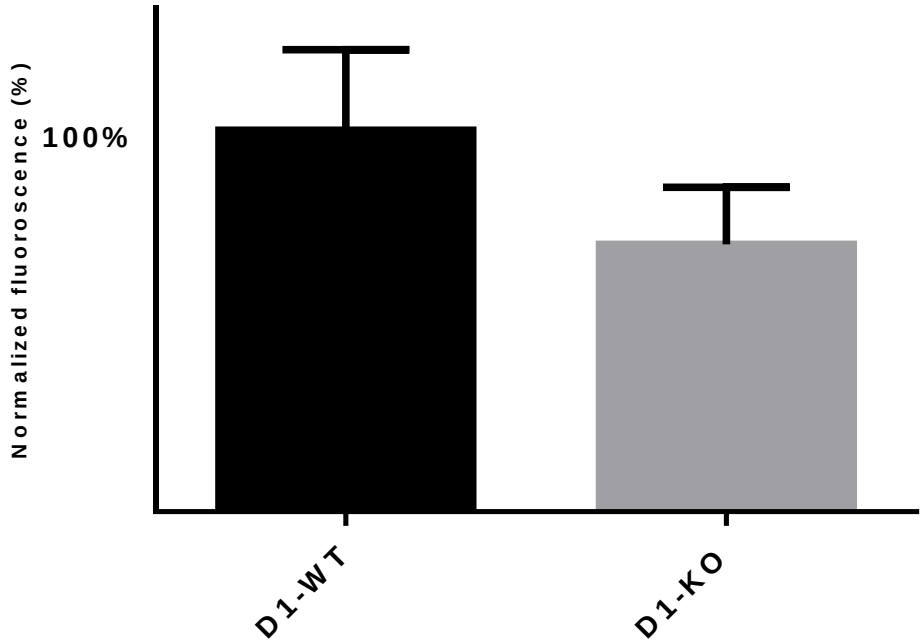
Intensities of fluorescence were measured and plotted on a non-logarithmic scale considering the level of significance for at least three cells per image. Figure 20 presents the intensities of fluorescence in PLCβ1 and PLCβ2 in wild type and PLCζ

knockout sperm head regions. Immunofluorescence analysis of other detected PLC isoforms was also performed and the comparative intensities are shown in Figure 21.

A



B



C

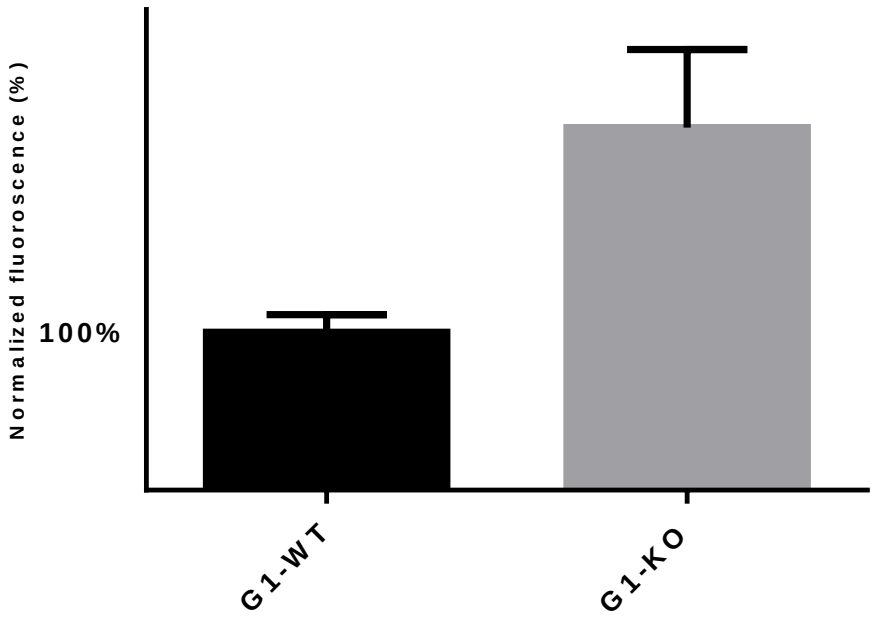


Figure 21 Protein expression of A. PLCβ3, B. PLCδ1, C. Plcɣ1 in WT and PLCζ KO sperm cells

The expression of PLCβ3 protein was found to be higher in PLCζ knockout sperm which are in line with the immunoblot and mRNA expression findings. Moreover, PLCɣ1 showed an approximately 2-fold higher protein expression in PLCζ knockout sperm cells.

3.3 Discussion

PLC isoforms are widely expressed in most mammalian tissues and responsible for regulating an enormous number of physiological processes including gamete maturation, acrosome reaction and embryo development. They work by hydrolysing PIP_2 into IP_3 upon stimulation which triggers Ca^{2+} signals via the IP_3R,

and diacylglycerol (DAG) which activates protein kinase C (PKC) (Bill & Vines, 2020). Expression of PLCs in gametes suggests their potential involvement in Ca^{2+} release in several biological processes related to reproduction and development including oocyte activation. Insufficient evidence relating to their expression in oocytes and sperm constitutes a huge gap in identifying potential secondary factors that might activate the oocyte in the absence of PLCζ. As such, a comparative transcriptomic and protein expression analysis of PLC expression in wild type compared to PLCζ knockout sperm could provide clues about the identity of secondary factors of oocyte activation.

3.3.1 Gene expression and histone modification

The sperm cell is known to deliver several classes of RNAs to the oocyte post gamete-fusion which have been confirmed in bovines and humans (Selvaraju et al., 2017). These transcripts are mostly related to spermatogenesis, sperm functions, and seem to play play important roles in phenotypes of offspring epigenetically (Jodar et al., 2013; Ostermeier et al., 2004). Some review literature reported pathological changes in placenta and congenital abnormalities in foetus and offspring generated by somatic nuclear transfer which lacks sperm specific RNAs (Lalancette et al., 2008; J. Xu & Yang, 2003).

A whole transcriptomic approach to identify the PLC mRNA levels of expression at a certain stage of development in sperm and oocytes would give a rough idea of their expression. DBTMEE was used to extract publically available information relating to mRNA expression of PLC isoforms in oocytes, sperm, zygotes, and 2-call embryos. Individual genes and their levels of transcript expression were identified. At the transcriptional level, mature sperm cells produce almost all PLC

isoforms. PLCζ was found to be expressed at the highest level among all isoforms followed by PLCβ3 which was the second-highest expressed isoform in sperm. Transcripts of other PLC isoforms were also detected in sperm.

Transcriptomic studies done by Selvaraju et al., demonstrated that bovine spermatozoa are enriched in the PLCβ1 isoform transcript, and also contain the PLCζ transcript (Selvaraju et al., 2017). These PLC isoforms may be delivered to the oocyte in both transcript and protein forms. Considering the fact that oocytes can translate foreign RNAs (Saunders et al., 2002a), their high abundance suggest their possible translation in oocytes once delivered by the sperm.

Transcriptomic profiling of MII mature mouse oocytes before fertilisation suggests a low abundance of PLC isoform transcripts except for PLCɣ1 and PLCɣ2, in such oocytes. PLCɣ1 was found to be the most highly expressed isoform. One possible reason for this general low abundance could be that the dormancy of physiological processes in mature MII arrested oocytes causes a fall in abundance of PLC isoforms and other gene transcripts. As this stage, oocytes may need to keep PLCs in a less expressed state to prevent Ca^{2+} release within them which might activate the oocyte parthenogenetically (R. M. Schultz et al., 2018; Su et al., 2007).

Previous studies have looked at the maternal mRNA biochemical status in oocytes and revealed that MII oocytes contain masked/dormant mRNAs that do not become translated before cell cycle resumption. Their acquire dormancy due to the lack of a poly-A tail which inhibits their translation. During fertilisation, dormant RNAs undergo polyadenylation that facilitate their recruitment to polysomes to initiate translation (Huarte et al., 1985; Mendez & Richter, 2001).

It has been well documented that during maturation from the GV to the MII state, oocytes change their transcriptional profile through a disruption of the transcription of most genes and arrest at MII clearly justifies this state of low-abundant transcripts. However, some components of signalling pathways are retained and do not became degraded, for example protein kinase pathways. Receptor tyrosine kinase which activates PLCɣ1 and Protein kinases transcripts were found to be stable (Paynton et al., 1988; Su et al., 2007). The high expression of PLCɣ1 in current study suggests that it sustains maintenance of such a signalling pathway through PIP_2 hydrolysis and activation of kinase protein through DAG throughout the arrested state. (Paynton et al., 1988; Su et al., 2007).

Oocytes showed generally unaltered expression of PLC isoforms transcripts immediately after fertilisation. The only difference detected was a slight rise in levels of PLCβ1 and PLCβ3 and a slight fall in PLCɣ2 and PLCε1 levels. The presence of PLCζ in the zygote was also confirmed at transcript level which is potentially delivered by the sperm. The transcriptional status of PLC isoforms in the zygote also suggests a similar pattern of PLC expression as in the unfertilised oocyte which is supported by whole-cell transcriptomic studies (Paynton et al., 1988; R. M. Schultz et al., 2018; Su et al., 2007). At the two-cell stage, PLCβ1, PLCβ3, PLCδ1 and PLCδ3 are expressed as transcripts to a greater extent and a low-abundance of PLCɣ1 was found in the same stage indicating that transcription of several PLC isoforms rise at the two-cell embryo stage. This suggests a transformation of the whole transcriptome of the embryo from the zygote to the two-cell embryo stage of development. An important clue can be drawn from this finding that sperm fusion does not affect the oocyte transcriptome to a significant level and oocyte PLC isoforms are very steady in their transcription levels from the

beginning to the end of fertilisation. To some extent, it can be hypothesised that activating factors should not be active in the oocyte as this requires stimulation from the sperm/external environment (Paffoni et al., 2008).

In the current project, gene expression of PLC isoforms was analysed in sperm cells in comparison to testis and ovary. In RT-PCR , almost all isoforms were detected qualitatively in sperm cells except PLCxd1. In testis samples, a similar trend was observed whereas in ovary all isoforms were detected. Due to the presence of several cell types in the gonads the variation was expected. Brain and testes RNAs were included to serve as a positive control for the maximum number of PLC isoforms. RT-PCR findings confirmed the qualitative expression of PLC mRNAs in sperm cells which provides an opportunity to shortlist potential candidates to see how these might be affected by PLCζ knockout.

Another indicator of PLC isoform expression that was checked was their histone enrichment as this can help to identify genes facilitated for mRNA transcription. Due to the densely packed genome of sperm the expression, histone modification and enrichment sequences can provide clues about the likely expression of specific genes (Champroux et al., 2018; Jodar et al., 2013; Yoshida et al., 2018). Considering the evidence showing that a sperm factor causes oocyte activation, such information about chromatin structure could give an insight into histone modification enrichment. This would help to identify PLC subtypes which are most likely to be expressed in sperm whereby it could identify potential sperm derived oocyte activation factors. As indicated in Figure 10, PLCδ1, PLCδ3, PLCδ4, PLCε1 and PLCζ genes show modification of the 4th lysine residue of histone H3 at a high level (PLCζ is highest among all PLC isoforms). Genes that are rich in this H3K4me3 modification are generally highly accessible to factors that facilitate

transcription. Another modification, H3K27me3, which targets 27th lysine of histone 3 protein, was used for comparison purposes as this is associated with reduced mRNA expression. In this study, the PLC isoforms PLCβ3, PLCɣ1, PLCxd1, PLCxd2 and PLCζ showed greatest levels of H3K4me3 enrichment and no H3K27me3 while the other isoforms showed equal or similar levels of H3K27me3 compared to H3K4me3. This suggests that the PLC genes with greater values of H3K4me3 have higher possibilities of expression elevation. However, the role of this modification has been studied in mouse oocytes and it seems to have no impact on fertilisation but has a significant impact after 2-cell embryo stage development (C. Yu et al., 2017) suggesting that the fertilised egg starts re-organisation of transcript expression at this two-cell stage.

Overall transcription and histone enrichment analysis of PLC isoforms in sperm cells indicates that PLCβ3, PLCɣ1, PLCxd1, PLCζ, and PLCε1 are prominently expressed and have rich histone modification profiles. PLCβ2, PLCδ1, and PLCδ4 are the same in their expression levels but their histone enrichment score at 27th lysine methylation is also like the 4th lysine methylation which is not enough to exclude any of the latter candidates for a functional role.

The importance of histone modifications has been studied and found to be linked to male infertility. Denomme et al., investigated the importance of the sperm histone related epigenome and demonstrated that it is linked to male factor infertility and poor embryo development. They found 1634 CpG sites in histone CpG island regions with significant difference in good and poor male groups, with less methylation in the poor group, suggesting a role in infertility and poor embryo development (Denomme et al., 2017). This provides a potential scope to study histone modification in relation to sperm derived oocyte activation factors.

3.3.2 Comparative gene expression- WT vs ζ1 KO

Comparing the expression profiles of PLC isoforms in wild type and PLCζ knockout sperm was thought to be a good way to look at how such isoforms might undergo differential expression in the PLCζ knockout model and thus could be the potential factors managing secondary Ca^{2+} transients. Quantitative analysis of PLC isoforms in wild type and KO sperm cells was performed to see if there is any differential expression of any such isoforms between wild type and PLCζ knockout sperm. Testis and ovary samples were also included to achieve consistency in expression analysis, identify the expansion of differential expression, if any, at the tissue level, and provide positive controls for comparison.

The results of the RT-qPCR analysis suggest that in sperm cells, all isoforms in wild type are expressed at higher levels than in the PLCζ knockout except PLCβ1, β2 and PLCε1.

In the sperm samples, levels of RNA were assessed at two levels, first, relative to wild type expression in wild type sperm and second, by analysing fold change among isoforms in wild type sperm compared to those from the PLCζ knock out model. The results of this analysis suggest that a significant number of isoforms underwent differential expression at the transcription level. Ultimately, studies on protein expression will be needed to confirm differential expression of PLC isoforms in PLCζ knockout sperm.

3.3.3 Protein expression

Expression of PLC isoforms in many different somatic cell types is well investigated but less so in germ cells. Some PLC gene knockout studies have been performed but no direct influence on oocyte activation has been reported apart from impact

on embryo development and ability to produce offspring. A study of PLCβ1 knockdown in oocytes suggests its potential role as a modulator of Ca^{2+} oscillations and oocyte activation (Igarashi et al., 2007). To better understand the presence and role of PLC subtypes at activation, it is important to confirm their protein expression. A further comparison of PLC subtypes in wild type and PLCζ knockout sperm will strengthen the hypotheses made about the transcription levels. Some proteomic studies on gametes confirmed PLC subtype expression (Table 11).

Table 11 Phospholipase C isoforms and related genes detected in proteomics studies

Isoform/Associated protein	Gene	Protein name	References
Sperm	PLCδ4	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-4	(Bayram et al., 2016; Vicens et al., 2017; G. Wang et al., 2013)
	PLCε1	1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Eta-1	
	PLCζ	1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Zeta-1	
	PLCɣ2*	1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Gamma-2	
Oocyte	PLCβ1	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-1	(Igarashi et al., 2007; Shufang Wang et al., 2010)
	PLCδ1	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1	
	PLCε1	1-Phosphatidylinositol 4,5-Bisphosphate Phosphodiesterase Eta-1	

*in human only

Expression of the PLCβ1 isoform is well documented in oocytes, particularly during maturation from the GV to the MII state. PLCβ1 plays important role in oocyte maturation and works via translocation from the membrane to the nucleus and then back to the membrane (N Avazeri et al., 2000; Nathalie Avazeri et al., 2003;

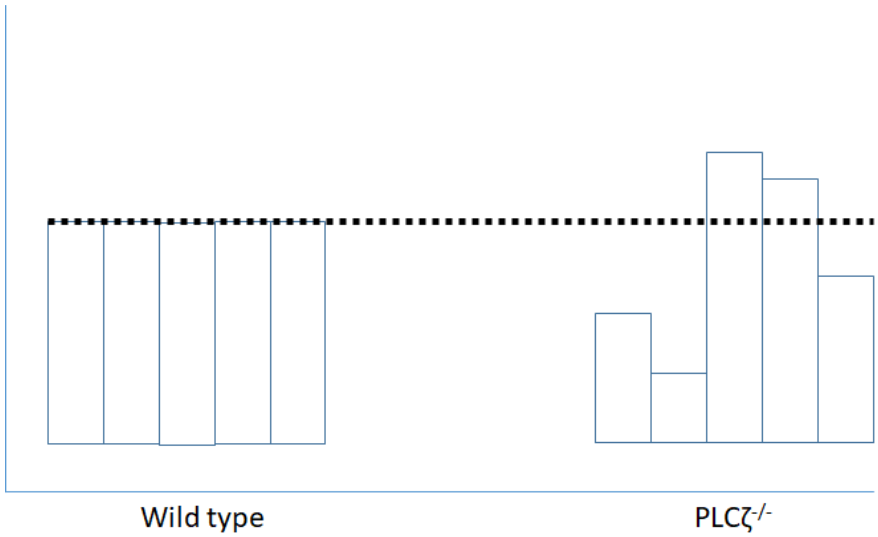
Igarashi et al., 2007; Lefèvre et al., 2007). PLCβ1 has been shown to be implicated in modulating Ca^{2+} oscillations (Igarashi et al., 2007), but this movement property was not actually reported during oocyte activation and could be an important way for PLCβ1 to act. In sperm, PLCβ1 was specifically linked to the process of meiosis during spermatogenesis (Pandey et al., 2019) and has been shown to be located in the acrosomal membrane of mature sperm cells (Walensky & Snyder, 1995). Its importance in sperm during the acrosome reaction, fertilization and embryo development have also been reported as essential (Choi et al., 2001). But the question of whether sperm derived PLCβ1 plays a role during oocyte activation is not clear so far.

PLCβ2 has been studied in the context of spermatogenesis which indicates its important role in male germ cell development (F. Li & Zhou, 2012). The PLCβ3 isoform was confirmed to be expressed in oocytes and shown to be lethal in knockout homozygous animals suggesting a role in embryo development (Shu Wang et al., 1998). No expression has been confirmed in sperm cells and no studies have been reported about a role during oocyte activation. In the context of fertilisation and oocyte activation, the role of the PLCβ4 isoform in mammalian germ cells is poorly understood. The same scenario can be seen for PLCɣ1, ɣ2, δ1, δ3, δ4 , xd1 and other isoforms, particularly in mammalian models.

Comparative analysis highlights that a single PLC isoform, PLCζ, when knocked out, affected the expression of other isoforms which suggests that the overall release of IP_3, either by receptor activation or by transfer of sperm soluble factor in the oocyte, may have been altered. It is worth adding here that PLCζ knockout sperm cells with an altered PLC isoform profile might give a different pattern of Ca^{2+} transients in the oocyte (if sperm PLCs other than PLCζ are involved) which

have been reported in PLCζ knockout studies (Hachem et al., 2017; Nozawa et al., 2018).

The results of the current chapter support the hypothesis that sperm expresses a number of PLC isoforms. Transcriptomic evidence suggests their high abundance, and of PLCβ1 and PLCζ in particular. Histone-modification status suggests that PLCβ and PLCδ isoforms have an increased level of expression. mRNA and protein expression studies confirmed several PLC isoforms in sperm and oocytes. Gene knockout of PLCζ has an impact on the expression of other PLC members especially in sperm cells which might alter the action of potential secondary factors in knockout sperm. Increased protein expression of PLCβ3 provides a clue that it might be a potential secondary factor. Attempts have been made in subsequent chapters to investigate potential factors and confirm their role.



Chapter 4: Sperm derived non-PLCζ oocyte activating factors- insights from PLCζ KO sperm proteomic analysis.

4.1 Introduction

Successful fertilisation requires a sperm to carry out a series of essential interrelated biological pre-fertilisation processes and then after gamete fusion release its contents into the oocyte to wake the latter from its arrested phase. This series of activities have to happen in a short period of time and involves processes including a change in Ca^{2+} concentration, a range of protein modifications, and a block to other sperm cells fertilising the oocyte (Yeste et al., 2017). Protein modifications play an important role in fertilisation and are involved at every step of this process. Such protein modifications are becoming important as markers of fertilisation to assess potential reasons for infertility. Unsuccessful oocyte activation in one of the factors that could lead to fertilisation failure. To keep the oocyte in the arrested state it is important for it not to undergo any signalling events that would trigger activation until gamete fusion which then allows the sperm to

initiate a rise of Ca^{2+} in the oocyte. In recent years PLCζ has been reported to cause the oscillatory release of Ca^{2+} in the oocytes which in turn activates the oocyte. It is worth noting that this oscillatory Ca^{2+} signal lasts for two to three hours which is important for blocking polyspermy and allowing the oocyte to exit its state of meiotic arrest. However, gene knockout of PLCζ does not abolish the ability of the sperm to activate the oocyte, although it now does so inefficiently and with a delay. *In vitro* studies of this process has shown increased polyspermy in some of the oocytes fertilised by PLCζ knockout sperm which could be due to insufficient Ca^{2+} transients. An *in vivo* study showed offspring could be conceived by PLCζ knockout males although litters are smaller in number, and such males also produce less offspring in total over a set period of uninhibited mating, which may be due to polyspermy in the fertilised oocytes. Such findings suggest the presence of a secondary factor(s) in either sperm cells or oocytes that can trigger oocyte activation in the absence of PLCζ. In chapter 3, expression studies were described that indicate that PLCζ knockout sperm cells show a differential expression of other PLC isoforms compared to wild type controls. This differential expression of PLC isoforms itself suggests that the expression profile of this class of proteins is reorganised in PLCζ knockout sperm and suggests that it may be beneficial to study the whole proteomes of PLCζ knockout sperm to investigate whether there are changes in other proteins such as those that are involved in other aspects of cell signalling in the sperm, or during the fertilisation process.

4.1.1 Proteomics – a possible approach for identifying secondary factors of oocyte activation

It is important to note that the dynamics of global proteome change in cells following knockout of a specific gene can be traced using proteomic analysis, and

this approach could be very useful in studying the global effect of such a gene knockout on the fertilisation process. Proteomic analysis might provide new insights relating to the expression, regulation, and modifications of candidate proteins associated with infertility (Kolialexi et al., 2008). Such an approach might also help to identify the molecular basis of normal physiological processes, for instance spermatogenesis, oogenesis, gamete maturation, fertilisation, and embryo development. Proteomic analysis of mammalian gametes has revealed the presence and functional roles of several post-translational modifications (PTMs), glycosylation being an important one that plays important roles in fusion, sperm-oocyte interactions and paternal chromatin transfer (Baker et al., 2007; Shetty et al., 2001; Yoshii et al., 2005). Similarly, causes of infertility in sperm have also been identified with the help of such an approach (Baker et al., 2007; De Mateo et al., 2007; Pixton et al., 2004; Shetty et al., 1999). Sperm motility and morphology have also been fully characterised with identification of proteins responsible for these aspects of sperm biology. Any defects in these proteins may cause infertility due to asthenozoospermatozoa (Chan et al., 2009).

As the literature suggests, proteomic studies can be important for identifying the normal and pathological state of sperm cells and in the identification of factors that underlie an abnormal state in specific types of cells for a particular physiological process. Considering the great potential application of the proteomic approach in identifying such factors this approach was used in the current project to understand better how PLCζ knockout sperm can still trigger oocyte activation. This approach has the potential to identify factors that might be upregulated in compensation for PLCζ deficiency in addition to identifying secondary factors that are involved in parallel to PLCζ in WT sperm. This proteomic study was limited to sperm cells to

provide a comprehensive analysis of potential sperm borne oocyte activating factors.

4.2 Results

The proteomes of wild type and PLCζ knockout sperm were compared. A workflow of data normalisation and analysis is presented in Figure 22. Briefly, raw Label-free quantification (LFQ) intensities were transformed to normalised values followed by subtraction of the mean range to show normal distribution of data. Samples that did not show detection of a particular protein in terms of LFQ intensity were replaced using the normal distribution/imputation tool in the software so that they had a normal distribution of intensities and could be further analysed.

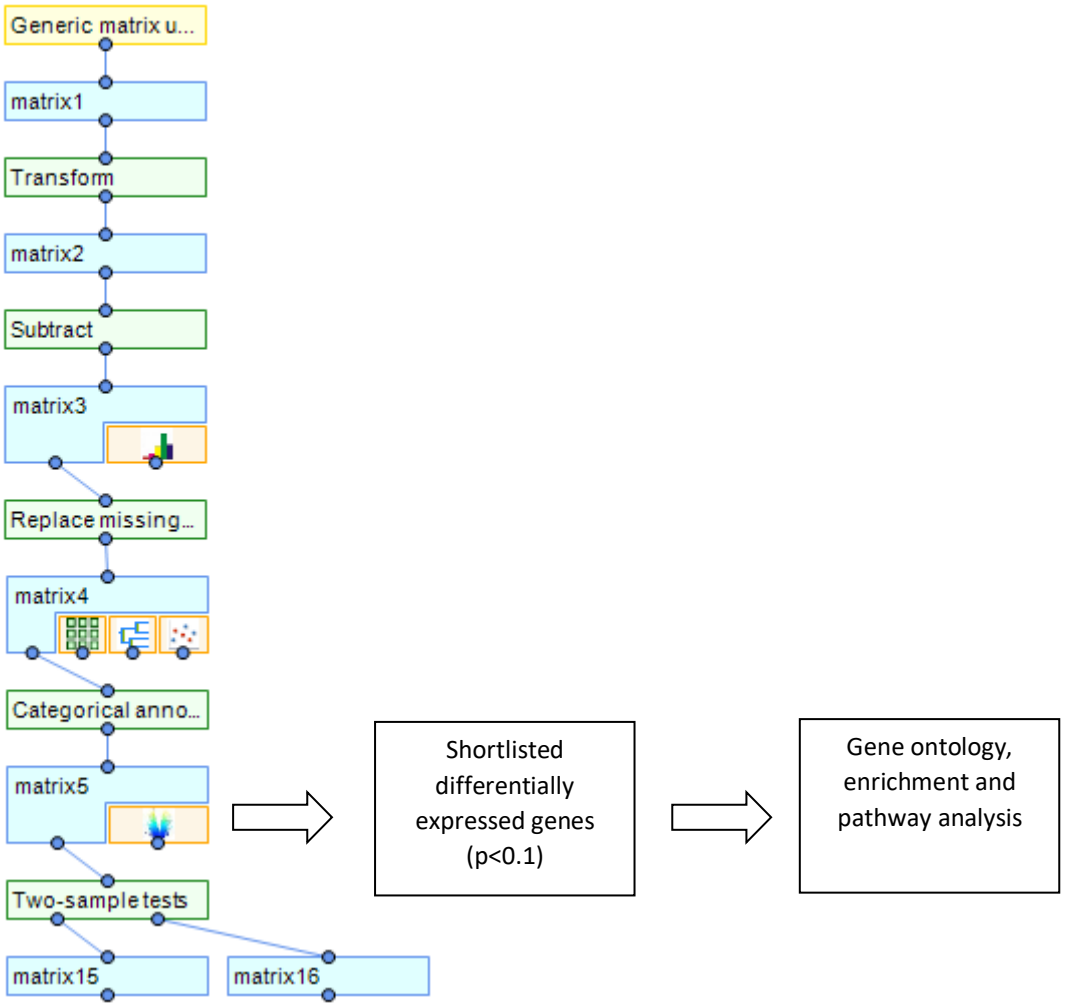


Figure 22 Workflow of proteomics data analysis.

LFQ intensities were used to analyse the data to determine the relative amount of protein in two sets of samples that had three samples in each set. LFQ intensities were normalised using the transform feature and presented as a normal distribution using the method of taking off the median. Figure 23 presents the normal distribution of data presented for each sample in two sets.

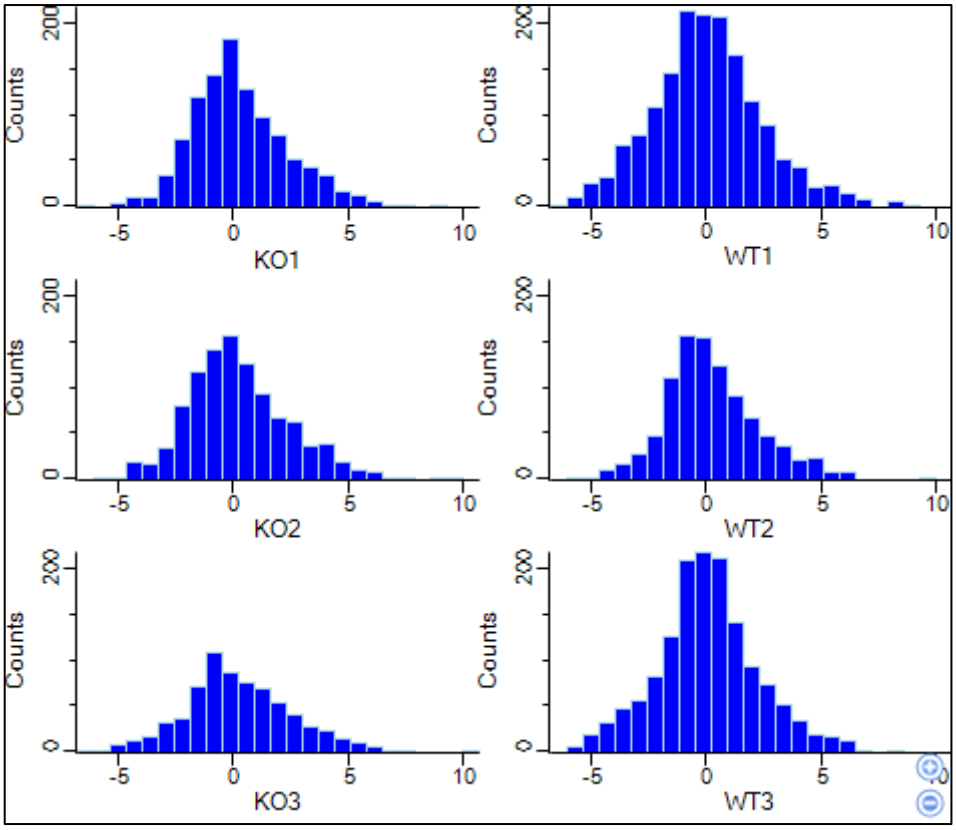


Figure 23 Normalised histogram of samples

Histograms of each sample suggests a consistency of peptide counts in all samples except KO3 in which the intensities of proteins were relatively low. A quality check of the data was performed using multi-scatter analysis and component analysis functions to prepare the data for further analysis having passed initial parameters. Multi-scatter plot analysis suggests that within the set the samples have a good correlation as presented in Figure 24. A positive correlation was observed in all samples and the trendline appearance from bottom left to upper right established confidence about the data to be analysed further. The graphs show a linear relationship among the groups.

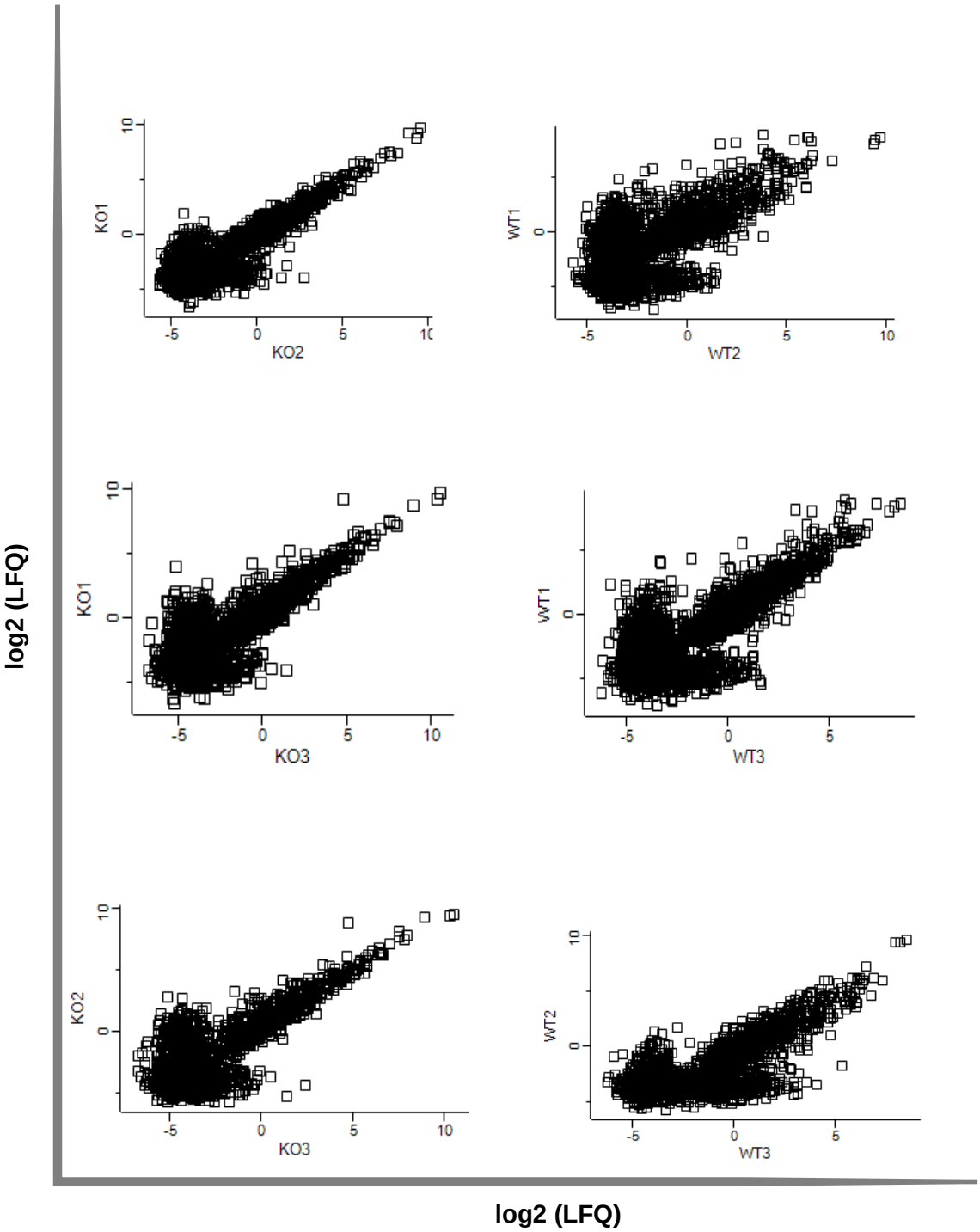


Figure 24 Scatter plot analysis of intra-group samples.

An overall view of scattering of pairs of all possible combinations is shown in Figure 25. Variable scatter plots show a linear correlation in most of the pairs except WT1.

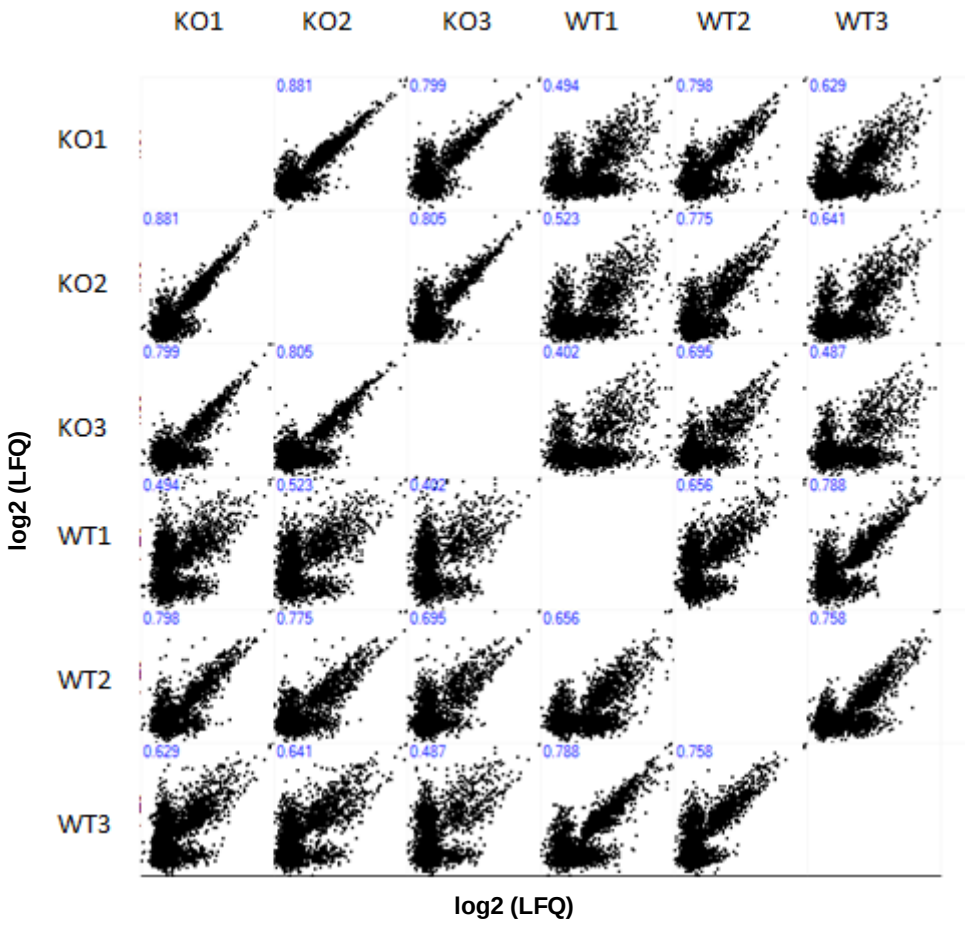


Figure 25 Scatter plot analysis of inter-group samples

A further principal component analysis (PCA) is another check that confirms the suitability of samples for further analysis. PCA is a statistical procedure that converts the correlated variables into linearly uncorrelated components which provides the distance and relation between the two groups (wild type and PLCζ knockout) in this study. This analysis helps to select the samples within the set that are in close proximity (statistically, these being similar in terms of their gene expression). Figure 26 presents the distance between the samples within the set groups.

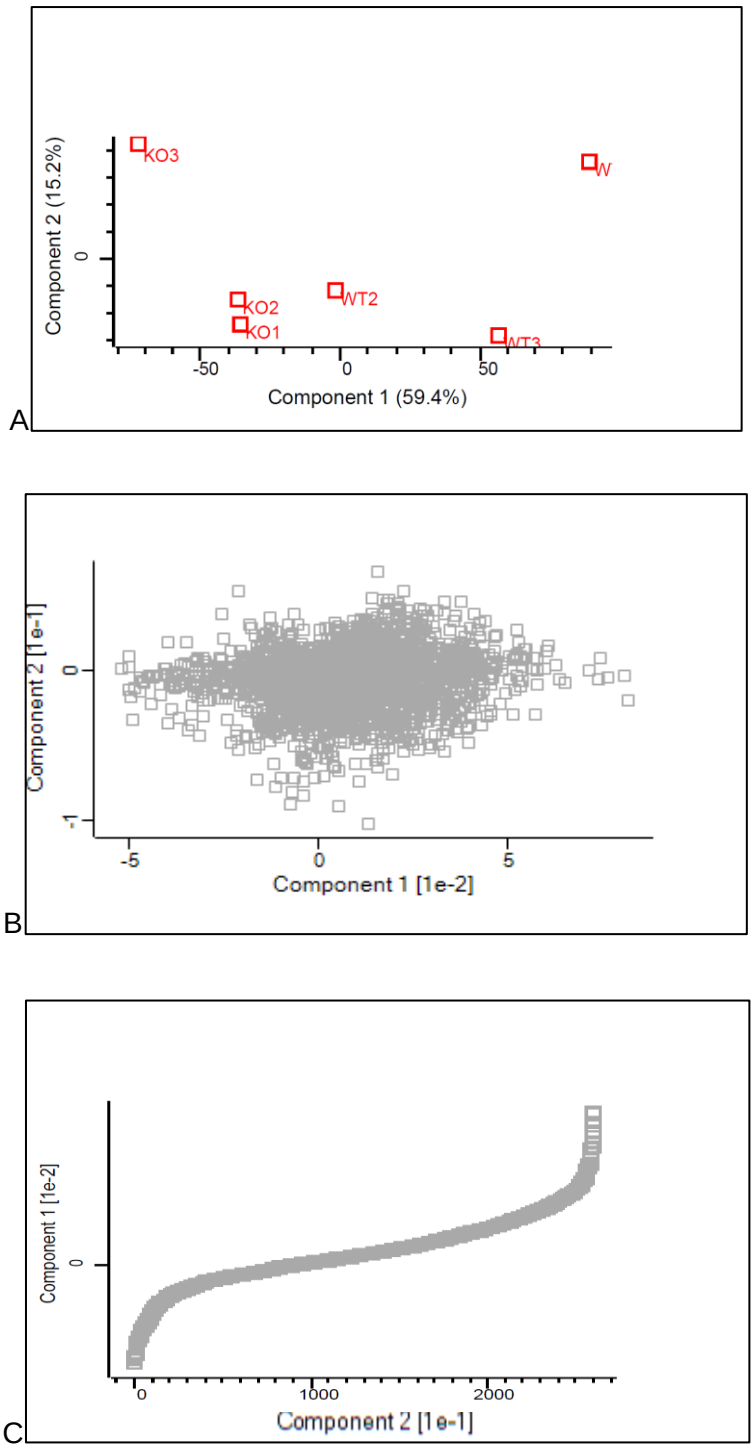


Figure 26 Principal component analysis of WT and KO samples group. A. 2D plot of component 1 and 2; B. Overall distribution of points between component 1 and 2; C. 1D linear trend of samples (horizontal region represents common regions between two components).

PCA analysis suggests that enforcing the condition of a first two-component of variables leads to less non-linearity among the samples. To group the samples in each set, PCA analysis suggested that for the KO samples, KO1 and KO2 are closest to each other and thus grouping KO1 and KO2 is the best fit for comparative analysis. In the case of wild type samples, WT2 and WT 3 are closest to each other. This correlation can also be tested in a scatter plot where WT1 was found to be an outlier. Using a combination of scatter plot analysis and PCA analysis, the groups found to be the best fit for analysis were thus KO1 and KO2 from the knockout set and WT2 and WT3 from the wild type set.

Hierarchical cluster plotting of intensities of each group was found to be similar to a great extent which also supports the fact that the proteome analysis detected a high percentage of the same proteins in all the samples (Figure 27).

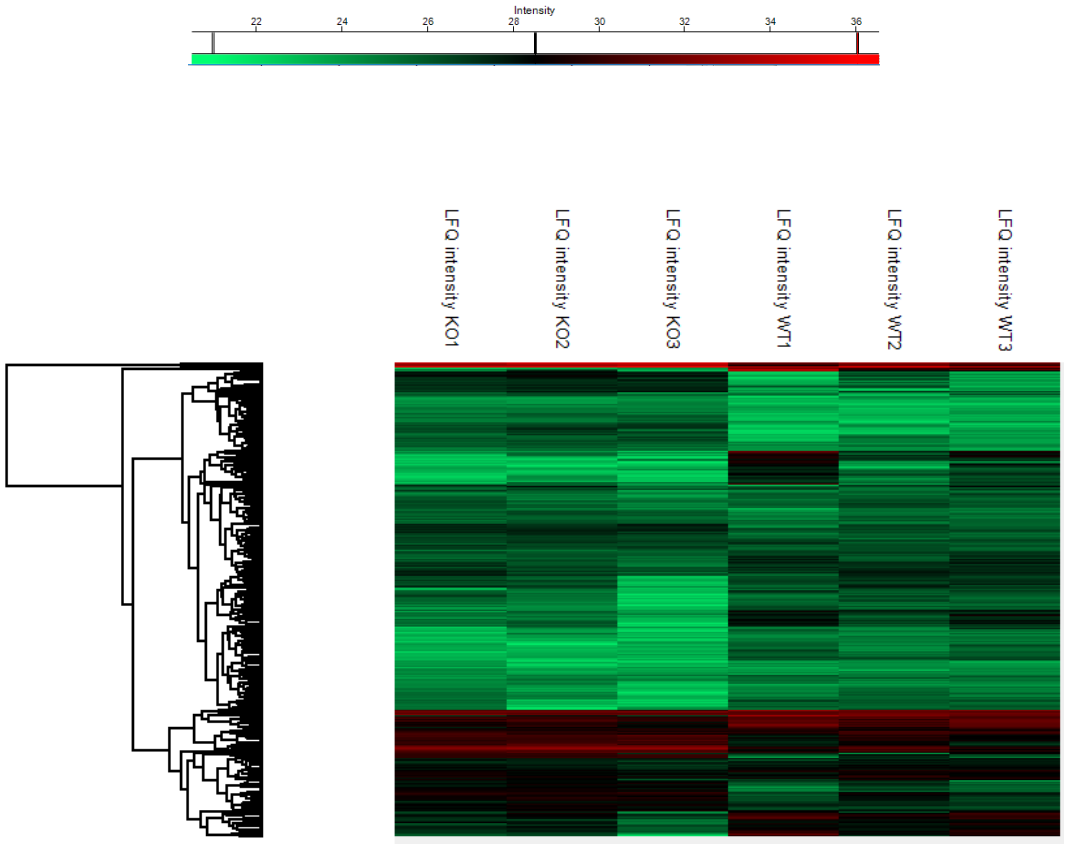


Figure 27 Hierarchical clustering of LFQ intensities of each of the samples.

Another level of check analysis was attempted to identify the best samples for differential expression analysis using paired tests and a volcano plot. Student's t-test was performed to compare WT and KO groups with 95% confidence criteria. A numbers of genes came up with significant values (Figure 28). Interestingly, shortening the group size from three sample to two did not lead to any significant change in values.

A B

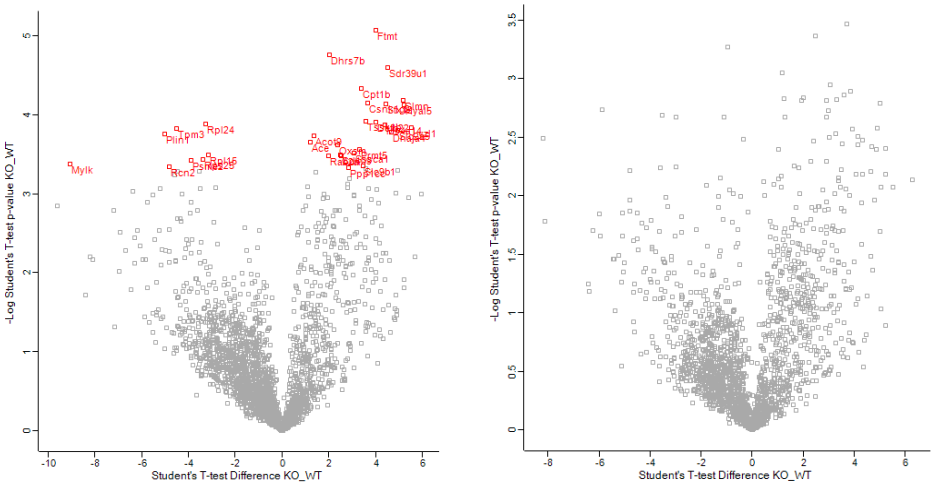


Figure 28 Student t-test of difference of WT and KO datasets with a confidence interval of greater than 95% (p<0.05) in A. all samples in the respective groups, B. two-variable samples from each group.

Student t-test outcome was further analysed on a volcano plot. This analysis was performed to check the extent of disturbance in the analysis that an outlying sample brings to the expression analysis. Among knockout samples only KO1 - KO2 were selected, and wild type groups were made up of WT1 - WT2 and WT2 - WT3. A simple volcano function was used to plot the difference between these pairs of samples. To maintain the uniform confidence criteria, p>0.05 was used in the analysis. The resulting plots are shown in Figure 29.

A B

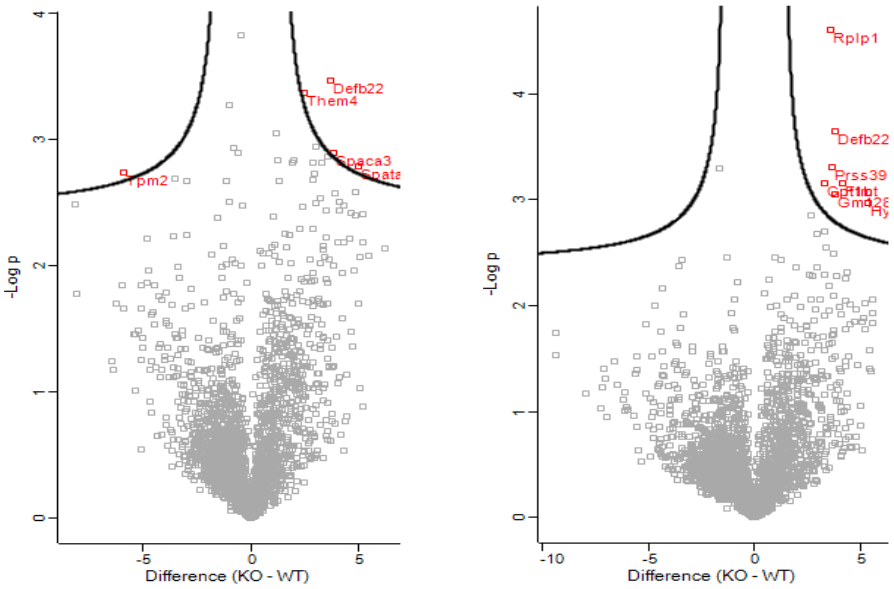


Figure 29 Volcano plots of groups having two samples in each A. represents KO1-KO2 and WT2 and WT3 (groups that were found to be more closed in principal component analysis), B. represents KO1-KO2 and WT1 and WT2

To take all the sample groups together for analysis, it is important to have similar differential expression. The analysis shown above suggests a great level of variation among the groups that have been included in the proteomic analysis. Only one gene (*Defb22*) was found to be co-existent in this re-grouping exercise.

Volcano analysis is a unique function of the pair test (mainly t-test) on a certain FDR parameter (variable). It is used to understand the interactors in pairs. A volcano scatter plot of all groups containing all samples can also be plotted to understand the overall differential expression among groups. This analysis was used to look at individual genes that have not shown statistically significant differences in expression. Points in between the cut-off curves on the graph suggest a similar expression (not-differential). On the other hand, differentially

expressed genes are outside of the curves. In the current approach, two different false discovery rate (FDR) values, that is 0.01 and 0.5, were used to determine the change in significance expression difference. The results suggested that 242 and 66 genes were differentially expressed at FDR 0.1 and DR 0.05 with difference ranges from -9.6 to 5.9 times fold change as shown in Figure 30.

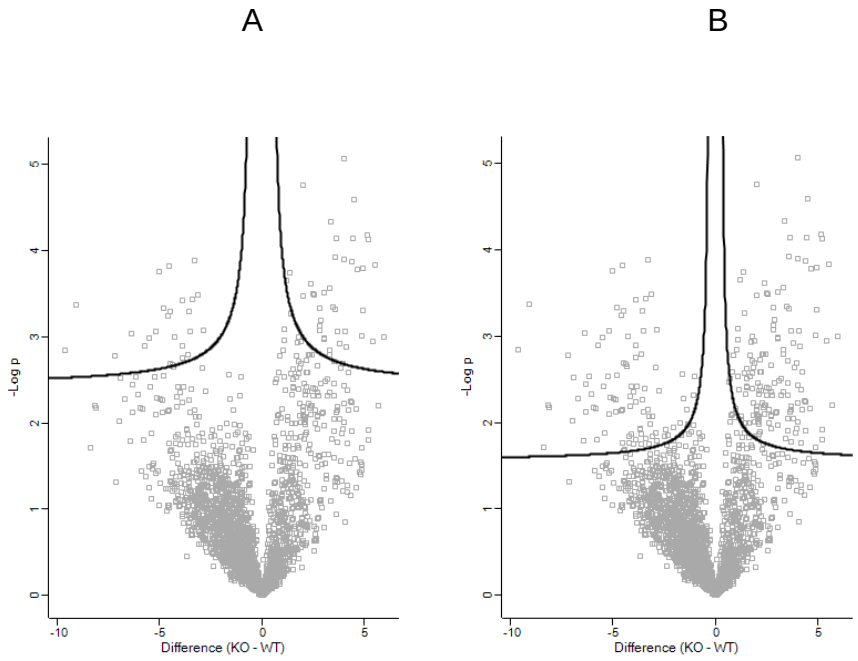


Figure 30 Volcano scatter plot showing the difference between KO and WT plotted against log p values when A. False Discovery Rate = 0.05, B. False Discovery Rate = 0.1

Profile plotting was performed to determine the trend of expression in individual samples. This makes it possible to identify any significant intra-group variation in a simpler visual manner. The data used was normalised and missing intensities were auto-filled using a normal distribution (on the Perseus platform). Looking at the detected PLC isoform profile in the data set, as shown in Figure 31, the WT1 sample showed the greatest level of variation for PLCδ1 and PLCβ3. It justifies the

exclusion of WT1 in the volcano scatter analysis, as shown in Figure 29A. The same level of variation can also be seen with multi-scatter plot analysis.

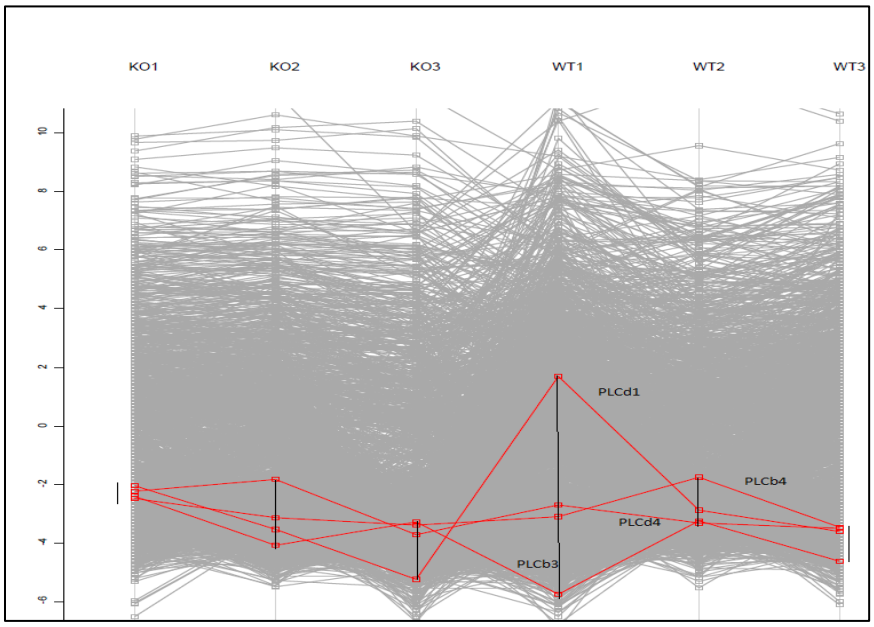


Figure 31 Profile plot of normalised intensities of all genes detected in the analysis.

Data analysis of the proteomic findings indicate that a great number of genes are differentially expressed between the wild type and PLCζ knockout sperm. Although PLC isoforms did not show a significant level of change, the suggested change in expression of PLCβ3 and PLCδ1 is consistent with that shown by western blot analysis. The fold change of selected PLC isoforms, as shown in the proteomic data highlighted in Table 12, cannot be quantitatively compared with western fluorescence intensity but suggest a similar trend of change in protein expression. These findings confirm that the proteomic data is reliable in terms of looking at expression change of genes, irrespective of their significance as identified by the analysis.

Table 12 Fold change of selected PLC isoforms

-log (p)	Gene	Fold changed (measured at difference)
0.66	PLCβ3	1.10
0.15	PLCβ4	-0.20
0.46	PLCδ1	-1.74
0.40	PLCδ4	0.51

Another important aspect of proteomic analysis is performing gene ontology enrichment analysis using the reference databases. This part of the analysis can be performed with shortlisted genes to see if they are related to the fertilisation process and Ca^{2+} signalling pathways. STRING v.11 and ShinyGO v0.61 platforms were used to perform the pathway enrichment analysis. Genes shortlisted with cut-off FDR, p-value 0.1 were used in ontology related analysis.

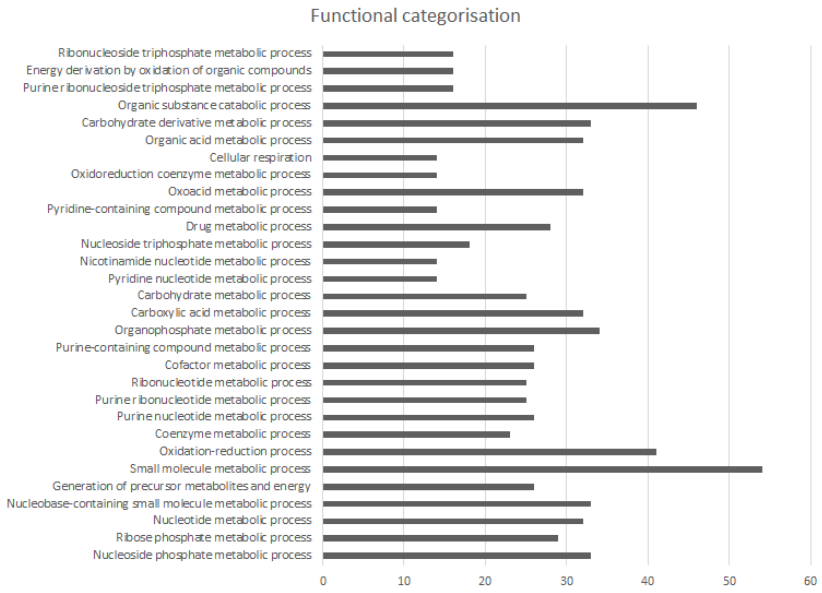


Figure 32 Biological process categorisation of differentially expressed genes

In the biological process mapping of differentially expressed genes, a great number of shortlisted genes are related to organic substances, drug molecules, metabolism, and nucleic acid and phosphate metabolism as shown in Figure 32. Hierarchical clustering tree analysis was performed to correlate important

pathways related to metabolic processes. Clustering was done for three categories, namely biological processes, cellular component, and molecular function. Pathways with many shared genes are clustered together. A P-value cut-off (FDR) of 0.1 was used in all clustering analysis. Pathways highlighted with dots were found to be more significant by the analysis, as shown in Figure 33.



Figure 33 Hierarchical clustering of identified gene pathways at three levels. A. Biological process, B. Cellular component, and C. Molecular function

Within the category of biological processes, processes related to small molecules, redox reactions, ribose metabolism and nucleic acid metabolism all showed significant changes in expression. In the cellular component category, sperm cells were predominantly identified. Proteins localised in acrosomal vesicles, flagella, plasma membrane, and cytosol were identified. Sperm mitochondria and sperm proteins were also significantly expressed. Other proteins involved in cytoskeletal structure, microtubules, and actin structure were also clearly identified. A significant change of expression of proteins involved in secretory vesicles is intriguing and it will be important to study their potential role in Ca^{2+} signalling pathways. Molecular function clustering suggests changes in several enzyme functional groups which do not seem clearly to relate to fertilisation, oocyte activation and Ca^{2+} signalling pathways.

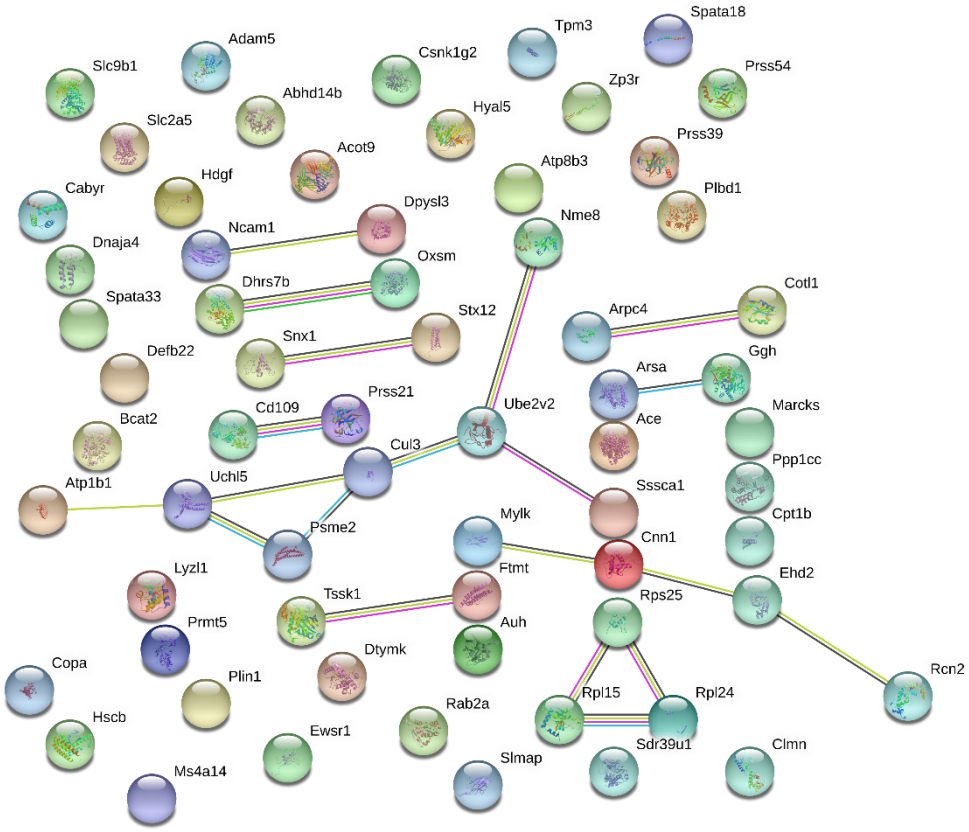


Figure 34 Interactive plot of shortlisted genes based on FDR cut off p<0.05. Connection nodes are shown between pathways if they share more than 20% of genes

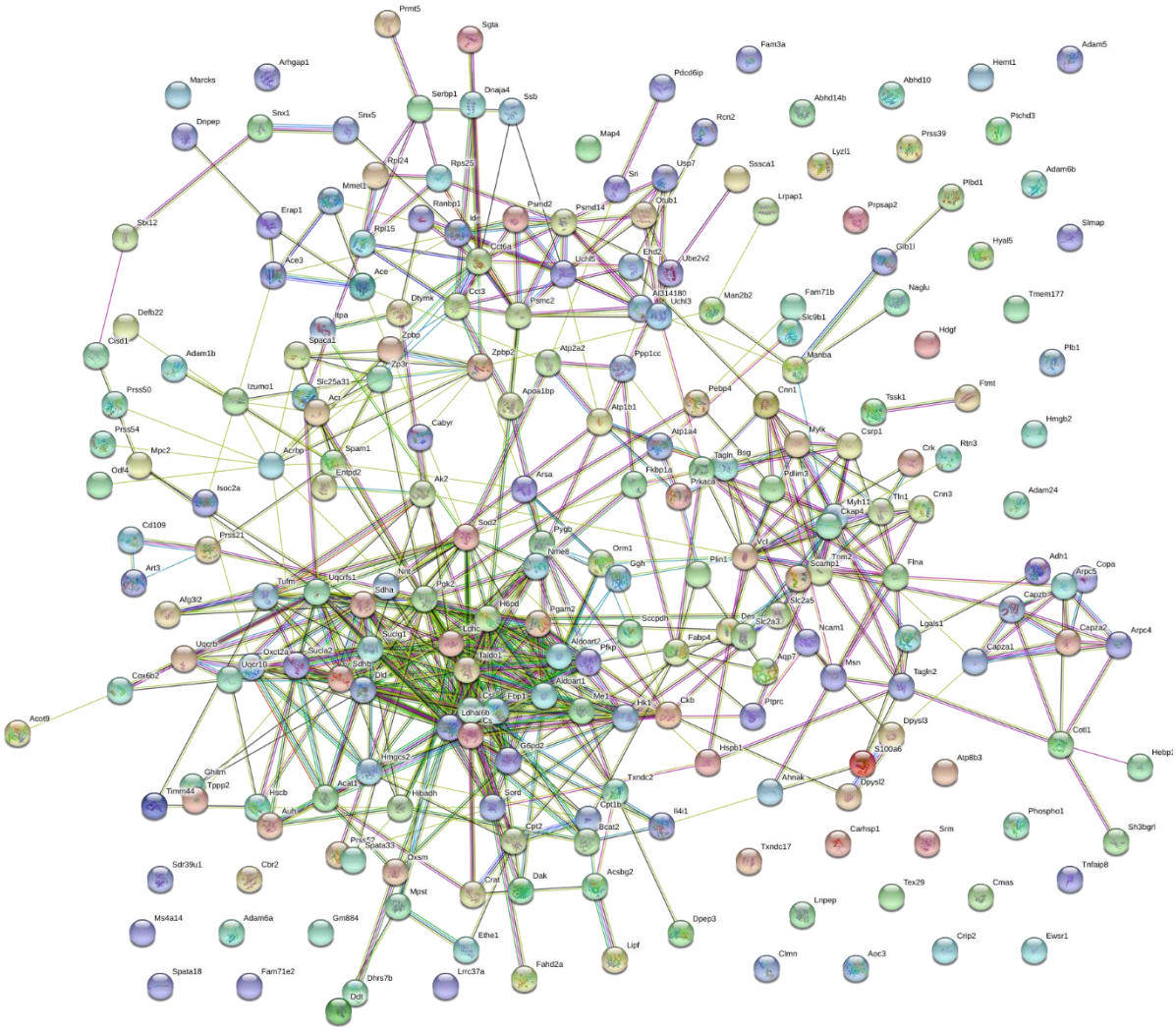


Figure 35 Interactive plot of shortlisted genes based on FDR cut off p<0.1. Connection nodes are shown between pathways if they share more than 20% of genes.

Interactive plotting of shortlisted genes was performed to understand potential associations between proteins connected through the type of analysis conducted above. Similar to the clustering trees, the interactive plot gives an overall view of all protein interactions. A high level of interactions was observed among genes. Genes that have no connections also presented at the outer regions with no nodes, as shown in Figure 35.

4.3 Discussion

Proteomic studies have played an important role in the life sciences in identifying major biomarkers in normal physiology and disease biology. In particular, such studies have identified numerous pathways and proteins involved in reproductive biology including fertilisation and embryo development. Germ cell proteomics have always been complicated to understand clearly because of the fast pace of transformations within the developing sperm cell proteome (Upadhyay et al., 2013).

In this proteomic study capacitated sperm cells were used to profile the proteome of sperm cells for peptide detection and quantification. Capacitation of sperm cells changes a significant number of proteins which have a direct role in the acrosome reaction and sperm-oocyte interactions (Castillo et al., 2019). So as not to miss the opportunity to detect the expression of oocyte activating factors after capacitation, cells were capacitated before quantification experiments. Sample preparation and fragmentation were performed as per the procedures described in subsections 2.16.3 and 2.16.4 and analysed at TDI Mass Spectrometry Laboratory of Nuffield Department of Medicine, University of Oxford. Post-fragmentation, peptides were quantified through label-free quantification on LC-MS/MS iBAQ platform at concentration 60 fractions. LFQ intensities of unique peptides were used for analysis on Perseus software and resulted genes were further analysed for enrichment on STRING and ShinyGO online gene ontology enrichment platforms. Raw data and enrichment analysis were done in-house. To broaden our coverage of gene expression changes during the analysis, a lower significant threshold was used. One main reason to set a high threshold was to look at individual target gene

expression as well as at other associated and interacting genes which may not fall under the criteria of higher significance. There were a few genes that were not detected in knockout sperm and that were detected in wild type and vice versa. Individual gene mapping of such genes was done before wild type vs PLCζ knockout comparative analysis. Among those that were undetected the majority of the proteins were cytoskeleton and actin-related.

4.3.1 PLCζ knockout sperm enriched in acrosome regulators

Primary data analysis indicated the variation among the samples which were removed mid-way in the expression analysis. At a significant FDR cut-off p>0.1, a total of 242 genes showed differential expression. Increasing the confidence level FDR cut off p>0.05 this list was shortened to 66 genes. These figures were obtained when no variation was considered among the samples. Removing the samples with high variation reduced the differentially expressed genes to 5. The latter analysis identified *Defb22, Them4, Spaca3, Spata5, Tpm2*, listed in Table 13. Analysis with the variable sample resulted in *Defb22* being in common but the former list was considered a good representation of differentially expressed genes at this stage.

Table 13 Differentially expressed genes in fewer variation samples group

Gene name	**Name**	**Functional class**	↑↓
Spaca3	Sperm acrosome membrane-associated protein 3	Sperm adhesion to oocyte surface	↑
Spata5	Spermatogenesis-associated protein 5	Membrane fusion	↑
Defb22	Beta-defensin 22	Motility of sperm cells	↑
Them4	Acyl-coenzyme A thioesterase	Fatty acids metabolism	↑
Tpm2	Tropomyosin beta chain	Actin and myosin interaction	↓

Table 13 shows upregulation of proteins related to oocyte-sperm fusion and gamete interaction which might possibly lead to stronger sperm-oocyte binding/fusion that could result in increased polyspermy due to insufficient release of enough cortical granules at the time of oocyte activation by PLCζ KO sperm (Nozawa et al., 2018). Spaca3 serves as an acrosome marker in sperm cells (Ito & Toshimori, 2016) and interacts with OMC32. Positive interaction of OMC32 and Spaca3 regulates the release of digestive acrosomal hydrolases during the acrosome reaction. Upregulation of Spaca3 could enhance acrosin release which could increase the digestion potential of sperm cells allowing sperm cells to penetrate the zona pellucida better and therefore come into increased contact with the oocyte (Nagdas et al., 2016). Spata5 is a fusion facilitator which helps sperm cells to fuse with the oocyte surface membrane and acts as a co-factor for Spaca3 (Nagdas et al., 2016). Expression of *Them4* was also detected in another study and found to be involved in metabolising week fatty acid chains and to be localised in the acrosomal membrane of the sperm head (Dorus et al., 2010). Its direct role along with the Tpm2 gene in fertilisation could not be established due to lack of studies but they were found to be expressed at high levels as transcripts in sperm cells and oocytes in publicly available database used in this study such as DBTMEE.

Differentially expressed genes detected in the current study therefore may have an indirect relationship to the high level of polyspermy seen after fertilisation with PLCζ knockout sperm. Essentially, PLCζ knockout sperm may have increased acrosomal function and sperm-oocyte binding/fusion due to the upregulation of certain genes. In the PLCζ knockout model the genes discussed above seem not very significantly related to any role in regulating Ca2+ levels in the oocyte. Also,

during the enrichment analysis of these genes, none of them found directly involved in signalling pathways of oocyte activation, for instance IP3-DAG, CamKII pathways etc.

4.3.2 PLCζ inactivation linked to over binding-fusion capacity

In terms of expansion of our understanding of involved genes, excluded samples were considered during the analysis with two levels of significance. This flexibility of inclusion resulted in several genes being identified as having an expression pattern that was different in knockout samples. This analysis resulted in genes being identified that are involved in several gamete binding, fusion and interaction processes, as listed in Table 14.

Table 14 Differentially expressed genes at FRD cut off P<0.05

Gene name	**Name**	↑↓
Ace	Angiotensin-converting enzyme	↑
Atp8b3	Phospholipid-transporting ATPase IK	↑
Auh	Phospholipid-transporting ATPase IK	↑
Prss39	Inactive serine protease 39	↑
Defb22	Beta-defensin 22	↑
Tssk1	Testis-specific serine/threonine-protein kinase 1	↑
Zp3r	Zona pellucida sperm-binding protein 3 receptor	↑
Spata33	Spermatogenesis-associated protein 33	↑
Slc9b1	Sodium/hydrogen exchanger 9B1	↑
Nme8	Thioredoxin domain-containing protein 3	↑
Adam5	Disintegrin and metalloproteinase domain-containing protein 5	↑

Ace has glycosylation activity and releases GPI anchored proteins which are crucial for the oocyte-binding ability of the sperm. *Atp8b3* helps with the distribution of PIP_2 and is involved in the acrosome reaction. *Atp8b3* knockout sperm have

been shown to have disturbed fertilisation *in vitro* and this protein to be directly involved in the acrosome content release process (L. Wang et al., 2004). Prss39, Auh, Zp3r, Adam5 are involved in sperm-oocyte binding and fusion interactions which might be involved in polyspermy facilitation. PLCζPLCζOn the other hand, inappropriate Ca^{2+}Ca2+ transient frequency in the oocyte resulting in alterations in the rate of release of the cortical granules and in consequence, the polyspermy is occurring. The latter is the most obvious reason of polyspermy in case of PLCζ knockout sperm.PLCζ. The differentially expressed proteins reported here possibly causing a gain in acrosome action which needs further validation.

Enrichment analysis of differentially expressed genes was carried out to determine gene enrichment significance. Genes shortlisted based on FDR $p<0.1$ were used for enrichment and classification analysis. The PANTHER online platform was used to perform the classification. In the molecular function class coverage of binding and fusion proteins was 29.4%. It suggests that a huge number of genes related to gamete binding functions were differentially expressed. Recent studies have indicated that loss of expression of a particular gene can influence the expression of other genes via effects at the transcriptional level, especially on closely related genes (El-Brolosy & Stainier, 2017). Such changes might have happened in the PLCζ knockout sperm which would be interesting in future studies to investigate whether such a mechanism of gene compensation might be responsible for changes in the expression of other sperm proteins in PLCζ knockout sperm. The catalytic class of protein covered 53% of total genes with differential expression. Other categories include structural molecule and transporter activity. The biological process class contained 29% of genes belonging to a metabolic activity function of which 6.5% were related to embryo

development and fertilisation processes. Cell component analysis suggests surface membrane and extracellular region as 8% of the total, and the remainder lies in the cell, organelle and cell junction protein classes. Enzymatic classification revealed a majority of these classes possessing hydrolase and transferase activity. A small fraction, 4.4%, are signalling proteins or involved in signalling pathways.

4.3.3 PLCζ deletion overexpresses other cytosolic PLC isoforms

Expression analysis of less significant genes was another approach to find out the correlation between proteomic analysis and direct protein expression findings. Going back to the fold change of detected PLC isoforms, as shown in Table 12, an important finding that came out of this analysis was that the expression change of PLC isoforms is similar what was found in immunolabelling experiments. Interestingly, PLCβ3 and PLCδ4 showed upregulation in proteomics.

PLCδ4 has been reported to be essential for the ligand-induced acrosome reaction (K Fukami et al., 2001). In terms of requirement, if PLCδ4 is compared to PLCζ, the common element between their action is that they produce IP_3 and fertilisation is not completely blocked in their absence but they are needed to do some essential function for natural fertilisation such as PLCδ4 in acrosome reaction (K Fukami et al., 2001), and PLCζ ininducing Ca^{2+} Ca^{2+} oscillations and cortical granule release (Hachem et al., 2017; Nozawa et al., 2018). Expression wise PLCδ4 and PLCζ have been detected in several mouse and human sperm proteomic analyses to compare species and infertility (Urizar-Arenaza et al., 2019; Vicens et al., 2017; G. Wang et al., 2013). But they were not reported to be among significantly expressed proteins. In the current proteomic findings, PLCβ3 showed increased expression in PLCζ knockout sperm which supports the hypothesis of

its involvement in IP_3 induced Ca^{2+} release in the absence of PLCζ. As discussed before, this change in other PLCs' expression could be a result of knockout gene impact on closely related genes. The missing component is the mechanism of activation of PLCβ3 in the oocyte. PLCβ4 has slightly reduced its expression by a 0.5-fold change in PLCζ knockout sperm which seems not very significant. PLCδ1 showed down-regulation by a 1.4-fold change.

It is interesting that my proteomic analysis shows that of a few genes that show changes in expression in PLCζ knockout sperm compared to WT sperm, one of these is PLCβ3; furthermore, immunoblot and immunofluorescence analysis confirmed that PLCβ3 protein expression is significantly enhanced in PLCζ knockout sperm. This is interesting partly because it suggests that this isoform may have enhanced activity in PLCζ knockout sperm, but also because it could mean that the ability to trigger the Ca^{2+} transients that are independent of PLCζ action, may be enhanced in the PLCζ knockout sperm. In addition to PLCβ3, my proteomic analysis also indicated enhanced expression of PLCδ4, which has previously been shown to play a role in the acrosome reaction (Fukami et al., 2001), in such sperm. Another gene that shows significantly enhanced expression at the protein level in PLCζ knockout sperm was Spaca3, which has been shown to play a role in the acrosome reaction (Ito and Toshimori, 2016; Nagdas et al., 2016). Although previous studies did not indicate any obvious differences in the capacity of PLCζ knockout sperm to undergo the acrosome reaction, they are associated with a higher degree of polyspermy (Hachem et al., 2017; Nozawa et al., 2018). Therefore, it would be interesting in future studies to investigate whether this higher degree of polyspermy is purely a consequence of the reduced capacity of PLCζ knockout sperm to generate the normal pattern of Ca^{2+} oscillations in the egg, or

whether it also reflects subtle changes in the acrosome reaction and sperm-egg interactions. A further gene expressed at higher levels in the PLCζ knockout sperm is Spata33; this has been suggested to be involved in the meiotic process during spermatogenesis (Hengling Chen et al., 2013). In contrast, the levels of Tpm2, a protein involved in actin-myosin interaction (Avrova et al., 2018; Borovikov et al., 2017), so potentially playing a role in spermiogenesis or sperm motility (X. Xiao & Yang, 2007), are substantially lower in PLCζ knockout sperm. Such changes are interesting given that previous studies of PLCζ knockout male mice did not identify any obvious differences in spermatogenesis, or in sperm viability or motility (Hachem et al., 2017; Nozawa et al., 2018). Further studies would be useful to study whether there might be subtle differences in these parameters in the PLCζ knockout sperm. Another intriguing question raised by our proteomic findings is why loss of expression of PLCζ in the sperm, a protein thought to only play a role in the oocyte, is leading to such substantial changes in the expression of these other sperm proteins. Intriguingly, recent studies have indicated that loss of expression of a particular gene can influence the expression of other genes via effects at the transcriptional level (El-Brolosy & Stainier, 2017).

Chapter 5: PLCβ isoforms: collaborators with PLCζ at fertilisation

5.1 Introduction

Hypotheses about the mechanism of oocyte activation have been diverse. The majority of them identify Ca^{2+} release as the primary trigger of oocyte activation in all species which is achieved by several different mechanisms. In mammals, the IP_3 pathway, a primary component of which is PLCs, is the predominant one operating during oocyte activation (Horner & Wolfner, 2008). PLCζ lacks a PH domain and stays in the cytoplasm where it interacts with a vesicular source of its substrate PIP_2. Moreover, knockout of PLCζ led to a significant fall in Ca^{2+} transient number but fertilisation remains active both *in vitro* and *in vivo*. This raises the question of whether another type of PLC can trigger such Ca^{2+} transients in the absence of PLCζ. Previous research using micro-injection of semi-purified recombinant PLC isoforms, i.e. PLCβ1, γ1,γ2 and δ1 resulted in no significant rise in Ca^{2+} when compared to the response to sperm extract micro-injection in intact mouse oocytes. However these PLCs may need to be activated by some mechanism, for instance by ligand-activated G-proteins ($G\alpha_{q/11}$) through a series of GPCR activation steps. Before their action on PIP_2 they undergo several regulatory and stimulatory steps. Hence, regulation of PLCβ isoforms is specifically

regulated and cannot necessarily be mimicked just by increasing their intracellular concentration in cells. For instance, protein kinase C (PRKC) phosphorylates and inhibits PLCβ1 indirectly. PLCβ2 is regulated by PRKA through inhibition of cAMP of Gβγ. PRKA also phosphorylates PLCβ3 on Serine1105 resulting in Gαq- and Gβγ inactivity (Zhong et al., 2008). Secondly, PLCβ2, β3 and β4 have not been investigated properly in mouse oocytes to reveal their roles in oocyte activation. Such regulation may be absent for PLCζ and this allows it to hydrolyse PIP_2 without prior modifications (Shah et al., 2013). But the most recent curiosity about other PLC isoforms is more related to the secondary factor(s) that are involved in inducing Ca^{2+}release in the oocytes at fertilisation in the absence of PLCζ. Revising the literature on PLCs reveals a huge gap in their study in mammalian oocytes. Table 7 shows that the expression of not all PLC isoforms have been studied in sperm cells. It is worth mentioning that several PLC isoforms including PLCβ1, 2, 3,4 are also localised in the cell cytoplasm, like PLCζ and carry the ability to hydrolyse PIP_2 at the oocyte nuclear envelope and cytoplasmic vesicles (Di Raimo et al., 2016). In Chapter 1 I characterised the expression of PLCβ isoforms in sperm cells, strengthening our understanding of PLC isoforms' potential role in sperm.

A close look at potential upstream regulators of the Ca^{2+} releasing IP_3 pathway suggests beginning with activation of GPCRs, then G-proteins, thereby activating PLCβ isoforms. In the context of oocyte activation, the Ca^{2+} rise is observed as oscillations in the oocyte. The noticeable point is that decades ago it has been confirmed that similar oscillatory responses can be seen in other types of cells from a single external stimulus/ligand action on GPCR/G-proteins (Woods et al., 1986). The main difference is that these oscillations do not sustain for hours in non-oocyte

cells. In hepatocytes, oscillatory Ca^{2+} transients can be observed in a dose-dependent manner when cells are treated with angiotensin/phenylephrine (Thurley et al., 2014; Woods et al., 1986). Undoubtedly, the response of Gq proteins mediates Ca^{2+} release through PLCβ activation. A cross-coupling model best describes the machinery that also fits in terms of understanding oocyte Ca^{2+} oscillations i.e. as a repetitive response. Therein, Gq activation potentiates PLCβ isoforms' activation in a positive feedback fashion which cleaves PIP_2 to IP_3. Binding of IP_3 to IP_3 receptors releases Ca^{2+} from ER stores and creates a transient. This increased cytosolic Ca^{2+} desensitises IP_3 receptors in a negative feedback mechanism and stops further Ca^{2+} release. This is the reason why an IP_3 mediated Ca^{2+} rises appear as transients and not as curves on traces. Subsequently, the ER becomes ready to refill Ca^{2+} stores via Ca^{2+} influx through SOCE enhanced by a TRPM and Ca V3.2 mechanism (Bernhardt et al., 2015, 2018) and ER Ca^{2+} pumps. Re-sensitisation of IP_3 happens at low cytosolic Ca^{2+} levels which prepares IP_3 receptors to do their job again (Harootunian et al., 1991; Meyer & Stryer, 1991). Now the next question arises, how do single stimuli induced Ca^{2+} transients transform to repetitions and form oscillations? Release of IP_3 is completely independent of the availability of IP_3 receptors and depends on potency and concentration of agonist. Excess of IP_3 could be present in the cytosol due to ligand action but as discussed before IP_3 receptors get desensitised on elevated cytosolic Ca^{2+} levels (during the first action) and desensitised at low levels of Ca^{2+}. The remaining IP_3 can resume acting on IP_3 receptors now which resumes the cycle of Ca^{2+} release and appear as oscillations on traces. Additionally, the amplitude of the Ca^{2+} peak is dependent not only on IP_3 but is also affected by PLCβ promotion. Increased Ca^{2+} in the cytosol has been reported to promote the

action of PLCβ isoforms which adds synergistic action of PLCβ through CICR (Grundmann & Kostenis, 2017).

Gαq G-protein is ubiquitously expressed, including in mature MII arrested oocytes. Gαq activates through replacement of bound GDP with GTP through guanine nucleotide exchange factor by action of a stimulus. When bound to GDP, Gaq is an inactive trimeric structure with β and γ subunits, and when with GTP is free to bind with PLCβ isoforms (Gilman, 1987). Gq and PLCβ isoforms have been confirmed to be present in mature oocytes and sperm. On treatment with Gq agonists and Ca^{2+} ionophores, they respond well and lead to increased cytoplasmic free Ca^{2+}. Immuno-inhibition of Gq protein does not seem to affect oocyte activation (Williams et al., 1998) yet partial reduction of PLCβ1 protein expression through a transgenic approach reduced the amplitude of Ca^{2+} oscillations and the fertilisation rate (Igarashi et al., 2007). Not enough literature has been cited on the action of other β subtypes such as β2, β3 and β4 in oocyte activation. However, the disruption and knocking out of these subtypes have major impact in fertility, viability and development. Homozygous null mutations in PLC β1 and PLCβ4 genes caused reduced fertility and litter size, and low viability (PLCβ1: <20% in 5th week) after birth (D. Kim et al., 1997). Ballester et al., reported that a homozygous gene mutation in PLCβ1 in a β-lactoglobulin transgenic mice makes it infertile, if both male and female have the mutation. Moreover, homozygous males and females have fertility rates of 18% and 10% when mated with non-transgenic mice (Ballester et al., 2004). Another study indicated reduced fertility in older animals lacking PLCβ1 (Böhm et al., 2002). Choi et al., reported that PLCβ1 null sperm showed a reduced acrosome reaction and fertilisation rate (Choi et al., 2001). In oocytes, PLCβ1 has been shown to be implicated in maturation (N

Avazeri et al., 2000). PLCβ3 null mice also showed a reduced survival rate (reduced by 50%) (W. Xiao et al., 2009) likewise PLCβ1 and PLCβ4. This information suggests that PLCβ subtypes play an important role in the events occurring around fertilisation and development. Study models lacking PLCβ subtypes have thus been shown to have several complications which indicates they may be implicated in oocyte activation but this needs to be further investigated. Moreover, which gamete (sperm or oocyte) is contributing to oocyte activation (considering the sperm delivers the activating factor in the oocyte) remains unanswered. Non-clarity of findings and fuzzy bases of exclusion of potential isoforms have limited our borders of investigation. It is an important point that mechanistic investigation of both gametes needs to be done and also important to identify the responsible factor.

Before the presentation of results from PLCζ knockout mice, several approaches have been investigated to understand the importance of PLCζ. Immuno-depletion of PLCζ from hamster sperm extract resulted in no Ca^{2+} inducing activity (Saunders et al., 2002a) but PLCζ knockout sperm can trigger a few Ca^{2+} transients in mouse oocytes (Hachem et al., 2017; Nozawa et al., 2018). RNAi inhibition of PLCζ resulted in a lower number of Ca^{2+} transients with a similar amplitude as in the wild type, and this finding should be considered along with the PLCζ knockout studies (Hachem et al., 2017). The results show that Ca^{2+} transients are less but not absent in PLCζ knockout mouse models which suggests the potential involvement of other PLC isoforms. PLCβ isoforms are activated by Gq as well as through a slight increase in resting Ca^{2+} levels at 100 nM Ca^{2+} (Kouchi et al., 2004). Also, a transgenic approach that reduced PLCβ1 levels by 30% in oocytes reduced the amplitude of Ca^{2+} oscillations by 20% which opens the possibility of β1 being

involved in oocyte activation. However, this slight fall in PLCβ1 had no impact on late stages of fertilisation and development up to blastocyst which is slightly different than PLCζ developmental indicators (Hachem et al., 2017) where small litter sizes were observed. In any case PLCβ1 depletion by transgenic approach could maybe be compensated by PLCβ2, β3 or β4.

A direct approach to investigate this hypothesis would be to inhibit PLCβ isoforms in PLCζ knockout models at fertilisation. This chapter is an attempt to study the role of PLCβ isoforms, their upstream regulators in gametes and to clearly understand the underlying mechanisms.

5.2 Results

5.2.1 Effect of PLC isoform inhibition on fertilisation

U73122, an aminosteroid PLC inhibitor, was used to assess the requirement for PLC isoforms during fertilisation. An *in vitro* fertilisation study using different concentrations of U73122 resulted in no oocyte activation occurring, as assessed by lack of PN formation and the first cell cleavage. U73343, an inactive form of U73122, was used as a negative control. The results, as shown in Figure 36, indicated no fertilisation at 20μM and a significant inhibition was observed at 5μM which confirms the potency of U73122 as a PLC inhibitor and its application in inhibiting PLCs in germ cells.

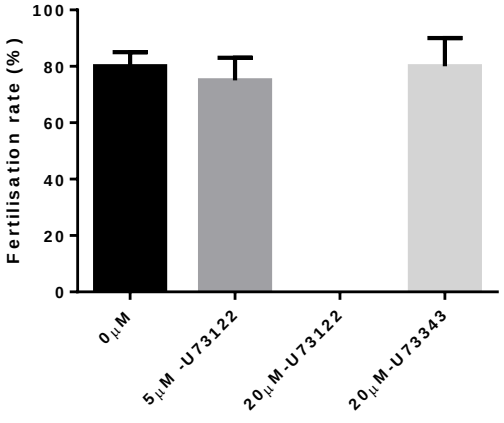


Figure 36 Fertilisation rate after treatment of oocytes with U73122 and U73343 (inactive analogue) to check essentiality of PLC isoforms in fertilisation/oocyte activation

This confirms that PLCs are essential enzymes during fertilisation.

5.2.2 U73122 effect on PLC isoform function

To study U73122 specificity towards different PLC isoforms, PLC enzymatic activity *in vitro* assays were performed in the presence of U73122. To study PLC activity, a fluorescent substrate analogue of PIP_2 (PLCGlow) was used. When exposed to PLC enzyme, PLCGlow breaks down into IP_3 and a florescent side product, 6-aminoquinoline, proportional to the substrate concentration. A Ca^{2+} free control was used to optimise the PLC activity prior to U73122 treatment. Figure 37 shows the activity profile of different PLC isoforms treated with different concentrations of U73122. Interestingly, the PLCβ subclass (β1, β2, β3) showed a significant block to their activity whereas other isoform classes showed effectively no inhibition of their activity in the presence of U73122. Thus PLCδ1, PLCɣ1 and ɣ2 showed no significant change in their activity with only a slight disturbance within the treatment groups, while PLCɣ1 showed a slight increase in activity at higher concentrations of U73122.

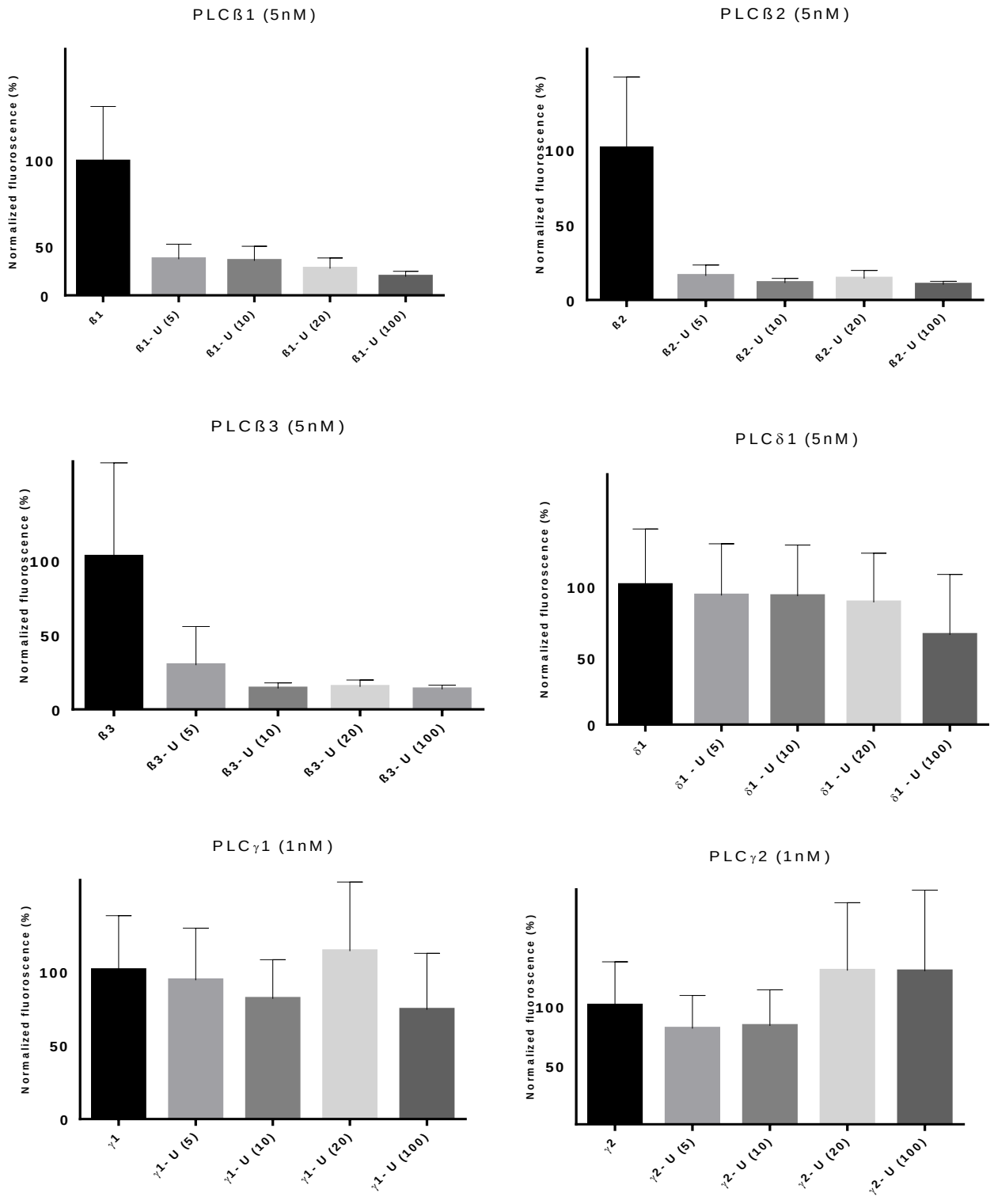


Figure 37 Recombinant PLC isoform activity in presence of U73122 (ranging from 5-100 µM.Untreated controls are shown as first bar on the graphs and used as standard. Intensities are plotted as maximum normalised fluorescence intensity (n=3).

5.2.3 Gαq inhibition effect on fertilisation & development

FR900359 (FR), a plant-derived depsipeptide, is a Gaq-specific potent inhibitor (highly efficient between 300nM to 1µM) (Schrage et al., 2015) which was used in this project in a series of experiments to understand the potential downstream effects of Gaq/PLCβ signalling on fertilisation and related events. PLCζ knockout and wild type mice were used in the experiments. Effects of inhibition of PLCβ isoforms were studied individually in sperm and oocytes and also in both gametes to find the effects of global Gaq inhibition on fertilisation.

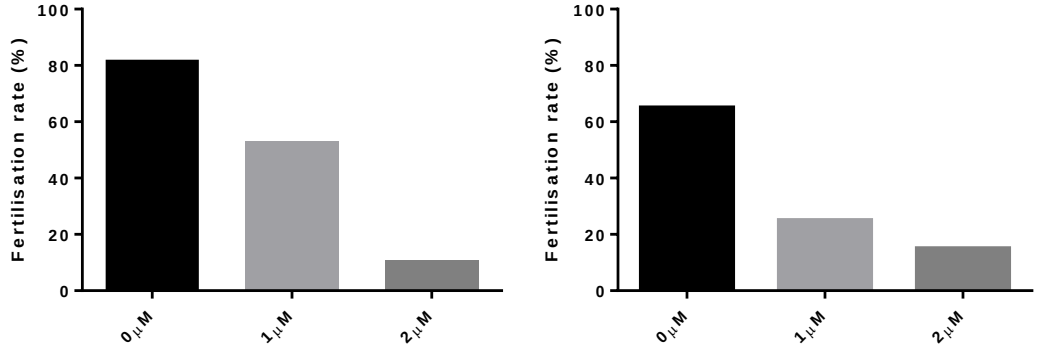


Figure 38 Fertilisation rate at different concentration of FR in wild type oocytes and A. using WT sperm and B. using PLCζ1 KO sperm. Sperm cells were not treated with FR

Wild type and PLCζ knockout sperm were used to inseminate wild type mature oocytes to study the effect of Gaq inhibition on oocytes. Oocytes were pre-incubated in FR, while sperm cells were left untreated (Figure 38). This led to a significant reduction in fertilisation which was FR concentration dependant. With wild type sperm, the fertilisation rate was over 80% whereas with PLCζ knockout sperm the rate was around 65%. These findings suggest Gaq proteins in the oocytes are involved in oocyte activation.

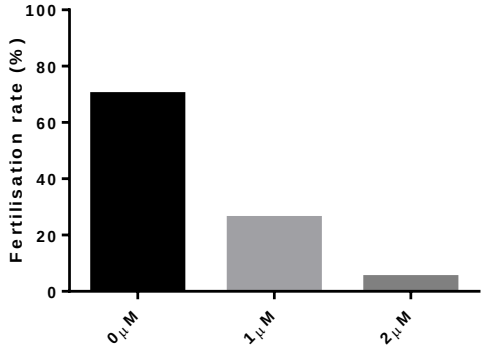


Figure 39 Fertilisation rate of FR treated PLCζ1 knockout oocytes inseminated with wild type sperm

The results suggest a similar pattern of dose dependent fall of fertilisation rate in PLCζ knockout oocytes which suggests a direct role for Gaq/PLCβ during fertilisation (Figure 39). PN formation was observed 6 hours after insemination confirming that the sperm-oocyte fusion occurred.

Table 15 Effect of Gq-protein inhibition on fertilisation & development

Group	FR Conc.	n=	2 PN Formation (n)	Fertilization Rate (%)	Two Cell Stage reached by (%)	Morula formed (%)	Blastocyst (%)
KO x KO	-	11	4	36	36	36	27
	1 μM/ml	12	1	8	8	0	0
	2 μM/ml	12	2	16	0	0	0
WT x KO	-	11	7	63	55	55	55
	1 μM/ml	15	4	26	13 (delayed division)	0	0
	2 μM/ml	22	3	14	0	0	0
WT x WT	-	17	14	82	82	82	82
	1 μM/ml	15	8	53	53	33	13
	2 μM/ml	10	2	20	20 (delayed division)	0	0

PN formation was also observed 6 hours after insemination and is depicted in Table 15. PN formation and polyspermy were observed in treated oocytes which suggest that the entry of subsequent sperm was not properly restricted and is related to improper Ca^{2+} release in the treated oocytes. Considering the level of Ca^{2+} release, which seems compromised in FR treated oocytes, possibly cortical

granule mobilisation was not enough to block polyspermy. A reduced amount of embryo cleavage can be correlated with less oocytes leaving the arrested phase due to inactivity of CamKII caused by an insufficient rise in Ca^{2+} levels.

A use of a similar strategy was employed for the next set of experiments but in this case sperm derived Gaq was inhibited by treating only sperm cells with FR.

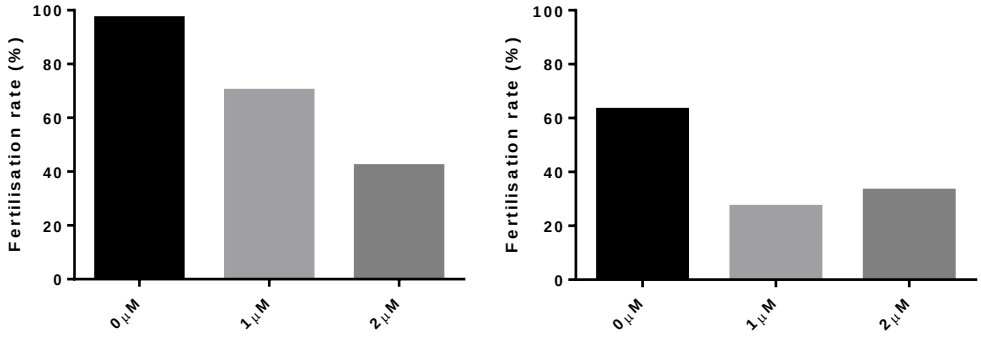


Figure 40 Fertilisation rate in presence of FR treated sperm cells. A. Wild type sperm, B. PLCζ knockout sperm

Sperm cells were diluted three times to diminish the concentration of FR to non-effective levels before insemination. Figure 40 shows inhibition of sperm derived Gaq proteins reduces the sperm's ability to fertilise oocytes resulting in a reduced fertilisation rate. PN formation after fertilisation with the FR treated sperm was unaffected suggesting that the genetic material was transferred to the oocytes with both wild type and PLCζ knockout sperm.

To study the mutual contribution of Gaq from both male and female gametes in oocyte activation fertilisation was performed after both sperm and oocytes were pre-incubated with FR. Both cells were pre-incubated with FR to inhibit the Gaq/PLCβ component of the IP_3 pathway and the fertilisation rate was observed.

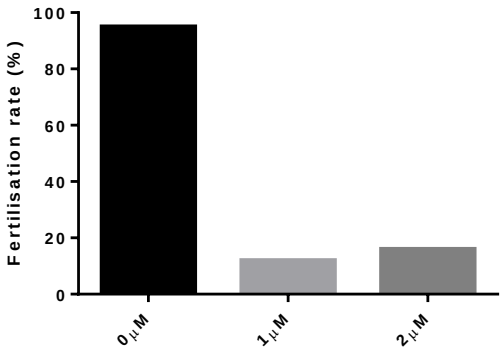


Figure 41 Fertilisation rate at different concentration of FR in fertilisation media

Interestingly, there was a much greater reduction in oocyte activation (Figure 41) when both sperm and oocytes were treated with FR, even at low concentrations, suggesting blockage of Gaq signalling in both gametes have a more significant impact on fertilisation rate. In contrast, the block in previous experiments was partial (one-sided) and the fertilisation rate showed a dose-dependency (Figure 39 and 40).

5.2.4 Gαq inhibition effects on sperm activity

As a control in these studies, it was important to check whether inhibition of Gaq had any effect on pre-fusion events such as the acrosome reaction and sperm-oocyte binding/fusion. To check that the acrosome reaction, sperm cell viability, PN formation and parthenogenetic activation were unaffected in the presence of FR, these parameters were assessed.

A ***PI*** **B**

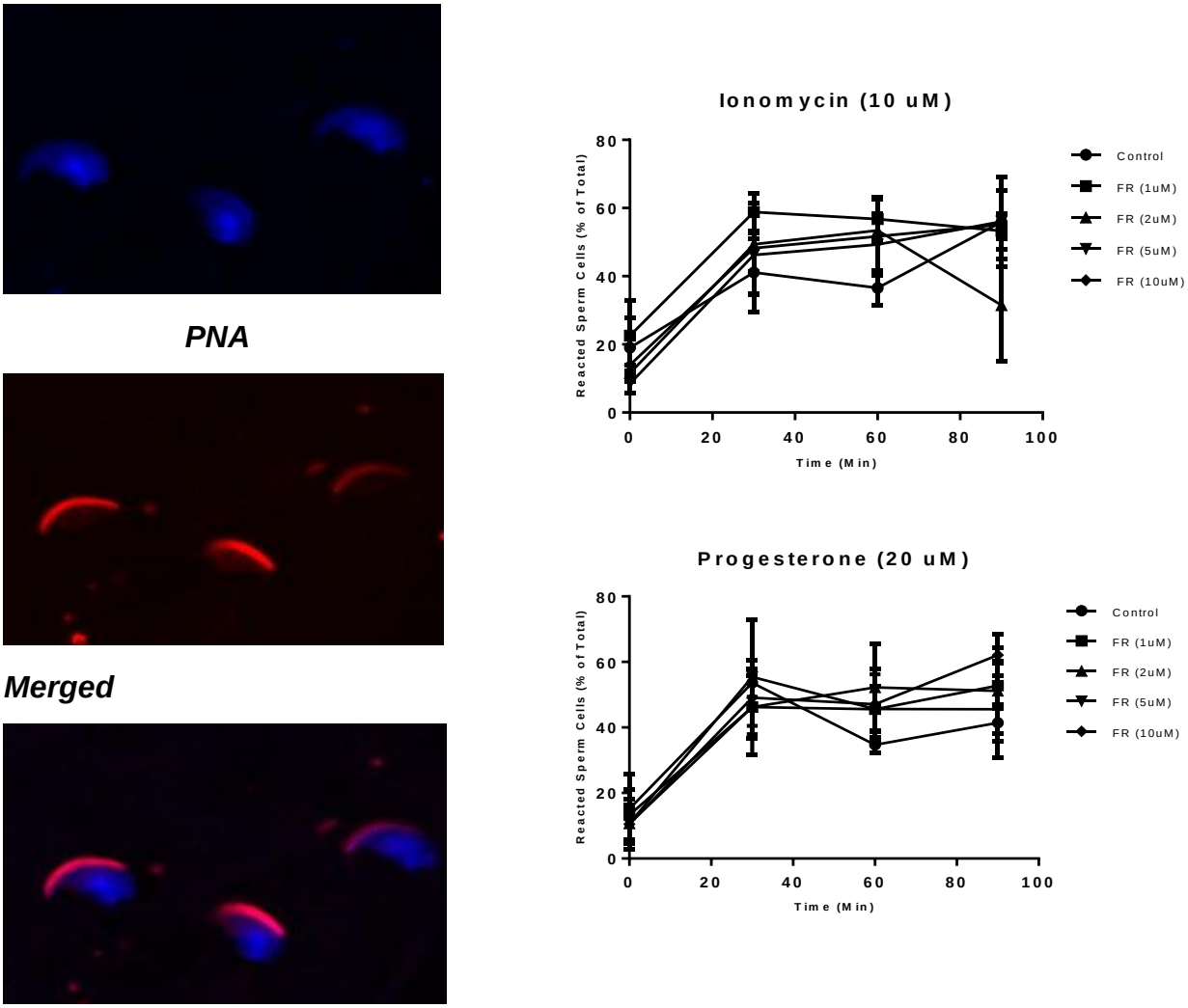


Figure 42 Acrosome reaction in the presence of FR. A. PNA staining images of the unreacted acrosome; B. Time-dependent progression of progesterone and ionomycin induced acrosome reaction in the presence of FR (DMSO was used as negative control)

Figure 42 illustrates the acrosome reaction in the presence of the FR Gaq inhibitor. Ionomycin and progesterone were used to induce the acrosome reaction. No significant change in acrosome progression was observed even at higher concentrations of FR. FR has no impact on the acrosome reaction or on gamete fusion. This suggests that the acrosome reaction does not involve Gaq in mouse spermatozoa and the Ca^{2+} signal in such sperm involves other mechanisms. Furthermore, sperm-oocyte binding and sperm cell viability were assessed in the

presence of FR. The results suggest that FR has no impact on the binding of sperm and oocyte, and sperm viability, as shown in Figure 43.

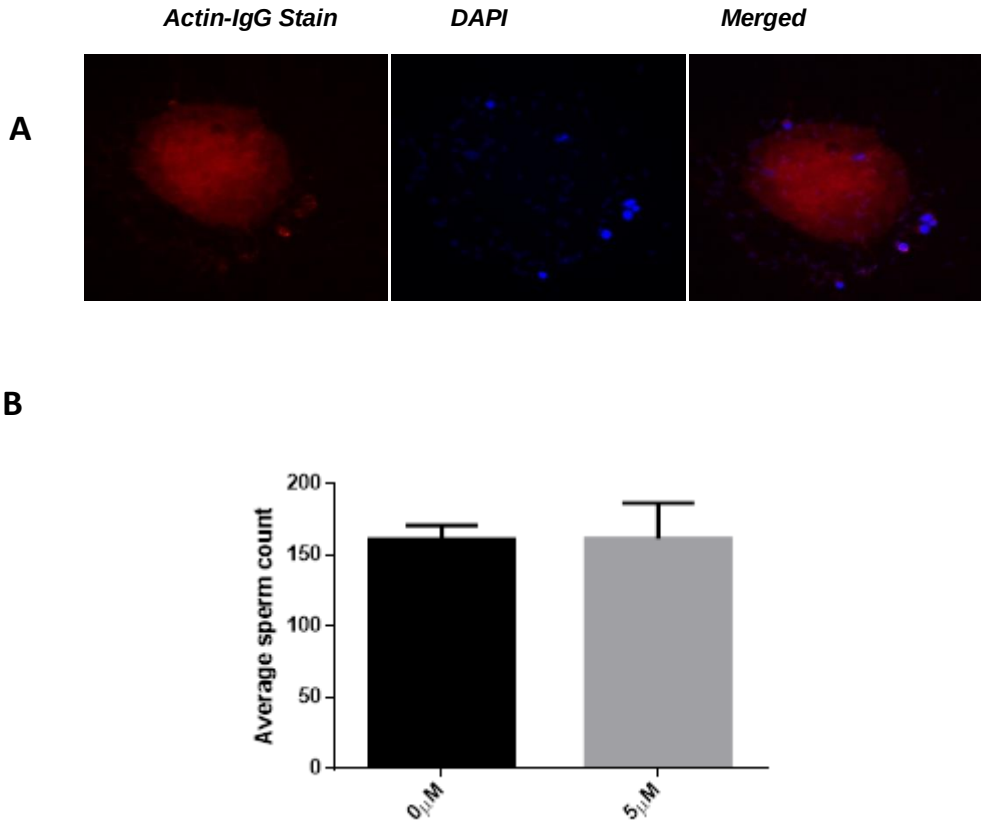


Figure 43 Sperm-oocyte binding in the presence of FR. A. Images of Actin stained oocyte and DNA stained sperm cells; B. sperm count in presence and absence of FR, incubated for 3 hours post insemination.

The results suggest that there is no significant change in an average number of sperm binding to oocytes in the presence of FR. Also, pronuclear formation is not affected by FR treatment, as shown in Table 15.

5.2.5 Gαq inhibition effect on Ca2+ release

To study the effect of the Gaq inhibitor on Ca^{2+} release at fertilisation, live oocytes were imaged as discussed in section 2.12. In terms of optimisation and calibration, mature oocytes were treated with Ca^{2+} ionophore (ionomycin) and Ca^{2+} chelator (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) at different

concentrations. Cells responded to these Ca^{2+} release modulators and imaging settings were optimised accordingly and used throughout the Ca^{2+} imaging experiments (Figure 44). Ca^{2+} oscillations were observed in IVF experiments performed under similar conditions (Figure 45).

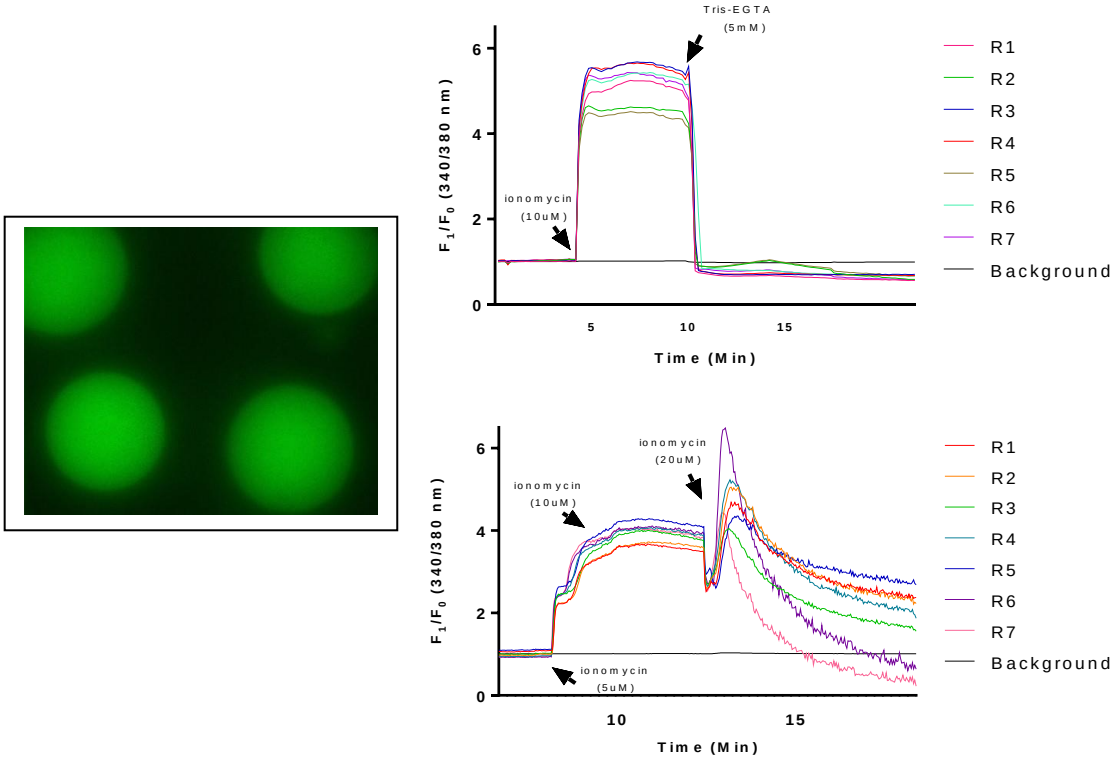


Figure 44 Effect of Ionomycin (Ca2+ ionophore) and Tris-EGTA (Ca2+ chelator) on global Ca2+ release. Oocytes were loaded with Fura-2-AM Ca2+ dye F_1/F_0 represents normalised intensities

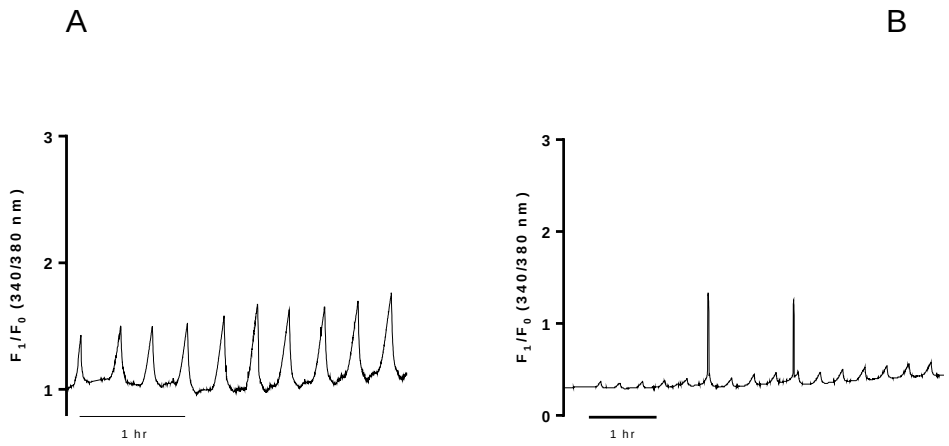


Figure 45 Sperm induced Ca^{2+} oscillations during *in vitro* fertilisation. A. Wild type sperm; B. PLCζ1 KO sperm

Gαq inhibitors were used to test the effect on sperm induced Ca^{2+} release during IVF with both wild type and PLCζ1 knockout sperm. Results showed reduced amplitude and frequency at lower concentrations of the Gαq specific inhibitor, FR, as shown in Figure 46. An increased concentration of FR caused a significant delay in Ca^{2+} release and affected its normal oscillatory pattern with a disturbed and atypical release. A parallel study was performed using another Gαq inhibitor, YM-254890.

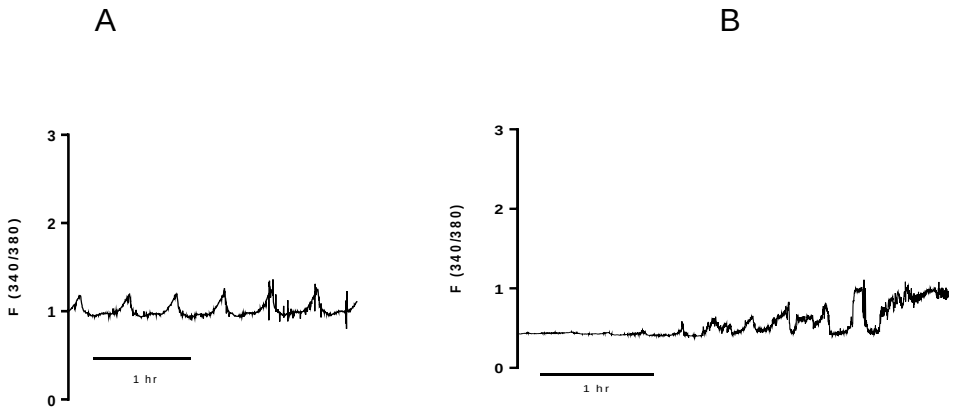


Figure 46 Sperm (WT) induced Ca^{2+} oscillations during *in vitro* fertilisation. A. Oocytes treated with 1μM FR; B. Oocytes treated with 5μM FR

Inhibition of Gaq using YM showed a similar effect on Ca^{2+} release at fertilisation as with FR. YM treated oocytes showed delayed sperm induced Ca^{2+} release with a few transients which gained high amplitudes after a few hours (Figure 47).

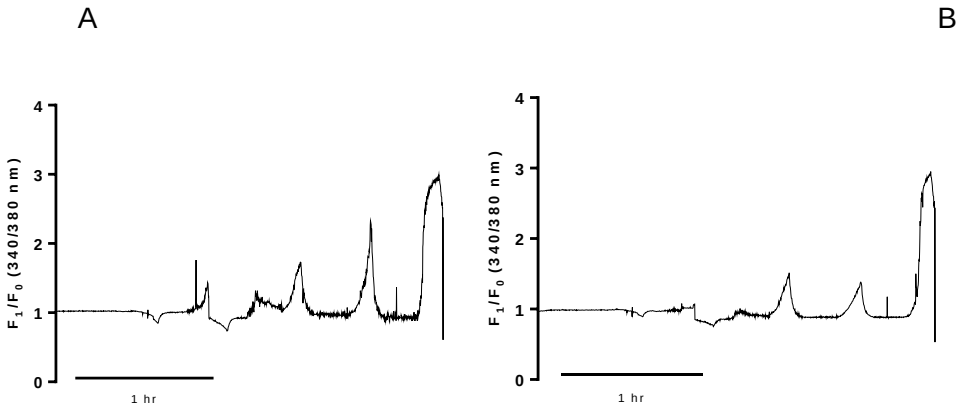


Figure 47 Sperm (WT) induced Ca^{2+} oscillations during *in vitro* fertilisation. Oocytes treated with A, YM- 50µM; B. YM- 100µM

To study if the delayed Ca^{2+} transients in oocytes after IVF with PLCζ knockout sperm are Gaq -PLCß mediated, FR treated oocytes were fertilised with PLCζ knockout sperm and Ca^{2+} release was measured. No Ca^{2+} transients were observed in Gaq inhibited oocytes fertilised with PLCζ knockout sperm which clearly suggests Gaq/PLCß mediated Ca^{2+} release at fertilisation in the absence of PLCζ expression (Figure 48).

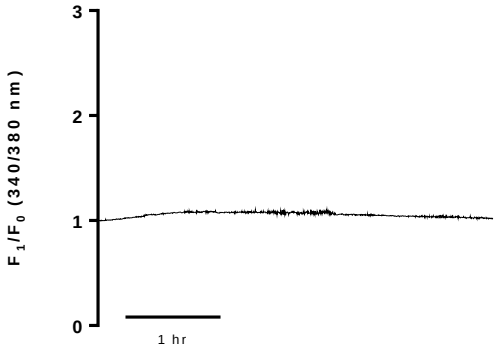


Figure 48 Sperm (PLCζ1 KO) induced Ca^{2+} oscillations. Oocytes treated with 5μM FR.

5.2.6 Effect of mRNA translation inhibitors on sperm induced Ca2+ release & development

To investigate if the oocyte activating factor after IVF with PLCζ knockout sperm is RNA in nature, protein translation inhibitors were used. No Ca^{2+} transients were observed for several hours in the oocytes treated with 5μM and 10μM cycloheximide (Figure 49). Similar findings were observed in oocytes treated with 5μM and 10μM harringtonine (an alternative translation inhibitor).

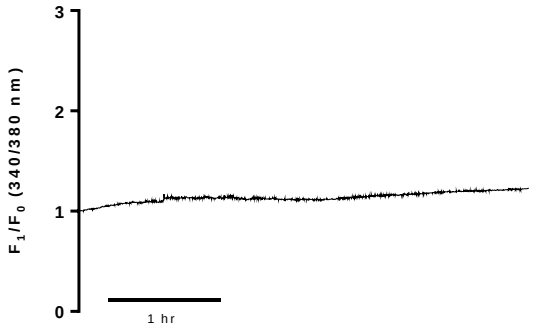
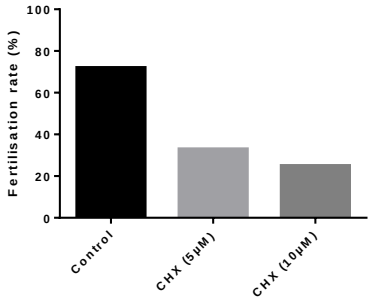


Figure 49 Sperm (WT) induced Ca^{2+} oscillations. Oocytes treated with translation inhibitor Cycloheximide (5μM and 10μM)

Looking at PN formation and embryo development data, cycloheximide seems to be activating oocytes in a way that does not involve Ca^{2+} release. First cleavage was observed in around 50% of cases for all treatments. Polyspermy was also observed in several oocytes which is potentially due to lack of Ca^{2+} release and cortical granule exocytosis.



Oocytes treatment	*n=*	*1PN*	*2PN*	*Poly-PN*	*Two-cell stage*
Control	***22***	***1***	***16***	***0***	***11***
CHX (5μM)	**30**	8	10	9	**22**
CHX (10μM)	**35**	15	9	11	**20**

Figure 50 Effect of translation inhibitor, Cyclohexamide, on fertilisation

5.3 Discussion

It has been well documented that PLCs are an important class of enzymes that play a role in oocyte activation, fertilisation and further development. Their expression changes post fertilisation suggesting a re-organisation of PLC isoform

role in the developing embryo. At the stage of oocyte activation and fertilisation, oocytes do not show any drastic change in PLC expression and this remains the case until the 1-cell embryo stage. But it is worth noting that PLCβ1, β2, β3 and β4 are among the most highly expressed PLC isoforms in oocytes, and in sperm cells. U73122 was thought to inhibit PLCs as a whole and does not allow PLCs to generate IP_3 so it would also be intriguing to identify which PLCs other than PLCζ might be involved in Ca^{2+} release at oocyte activation (Genevieve Dupont et al., 1996; Nicolson et al., 2002).

The PLC inhibition capacity of U73122 has been proven by several previous pieces of evidence. Jin et al demonstrated that U73122 inhibits PLC-mediated Ca^{2+} release in both differentiated and undifferentiated NG108-15 cells and dorsal root ganglion neurons in a concentration dependent manner and provided evidence that U73122 was therefore a useful tool for studying IP_3-mediated Ca^{2+} mobilization (Jin et al., 1994).

Another confirmation of U73122's ability to inhibit PLC activity was made by Wu et al., who studied U73122's effect on sperm extract's ability to trigger Ca^{2+} oscillations in oocytes. Pre-incubation of sperm extracts with U73122 completely blocked their ability to trigger Ca^{2+} release in oocytes, and hence their capacity to activate the oocyte (H. Wu et al., 2001).

5.3.1 PLCs are essential for oocyte activation

The importance of PLC isoforms has been reported in a number of studies of fertilisation. Several studies have used the PLC inhibitor, U73122, to inhibit IP_3 generation (Dupont et al., 1996; Neacsu et al., 2019; Yule & Williams, 1992) and block fertilisation and late embryo development. After treatment with U73122

neither PN formation nor cell division occurred, thus fertilisation was completely blocked in the presence of U73122.

5.3.2 U73122 inhibits PLCβ isoforms, not others, at fertilisation

At the beginning of this chapter, an attempt was made to reinvestigate the effect of the block of specific PLCs by U73122. In fact the specificity of U73122 has never been systematically examined although the inhibitor has been shown to inhibit G-protein mediated PLC enzymes (Yule & Williams, 1992) which are PLCβ isoforms. In the present study, the use of U73122 resulted in neither PN formation nor cell division suggesting that fertilisation was completely blocked when carried out in the presence of this PLC inhibitor. Vasco et al., studied the specificity and inhibitory effects of U73122 on individual PLC isoforms by studying the long term growth of MG-63 osteosarcoma cells. Culturing cells with U73122 reduced cell viability significantly. To understand better how PLC expression in MG-63 cells has been changed, they studied expression of individual isoforms and linked it to the specificity of U73122 activity. They found that this inhibitor reduced protein levels of PLCβ1, PLCβ2 and PLCβ4 whereas PLCδ1, PLCδ4 and PLCγ1 showed no significant change in treated groups (Lo Vasco et al., 2016). It indirectly suggests that U73122 mediated inhibition of PLCβ subtypes resulted in reduced viability. Similar findings were observed in the current study (Figure 37). Klein et al used mass spectrometric analysis to study the binding ability of U73122 and its molecular interaction with PLCs and inhibitory potential. They found that U73122 activates PLCδ isoform activity. LC/MS/MS peptide sequencing suggested that U73122 requires covalent modification of cysteines for binding (Klein et al., 2011).

Collectively, current study findings, backed by previous studies, show that U73122 specifically inhibits PLCβ subtypes.

5.3.3 Gαq – PLCβ implicated in oocyte activation but not in sperm activity

Gαq activation specifically activates PLCβ isoforms and not PLCγ and PLCδ isoforms (Harden et al., 2011). G-proteins, including Gq members, are widely expressed in mouse oocytes (Williams et al., 1996) and sperm (Garty et al., 1988). Not enough evidence is available in support of a role for Gαq proteins in individual mouse gametes at fertilisation but *in vitro* activation of the same resulted in fertilisation (Williams et al., 1992). There has been a lack of studies on PLCβ isoforms during fertilisation and this study showed a potential role for Gαq activation at fusion and stimulation of PLCβ isoforms.

As discussed before, PLCβ members are primarily activated through Gαq protein. The ligand-mediated activation property of Gαq can thus be controlled through its inhibition using a Gαq specific inhibitor which will also inhibit PLCβ isoforms without affecting other PLC subclasses. FR was employed to execute this task, this being a plant-derived potent Gαq specific inhibitor characterised for its potential in a range of cell lines (Schrage et al., 2015). Effects of the use of FR on oocytes and the results of fertilisation indicators were interesting in that they were dose-dependent and a similar trend of fall in fertilisation rate was observed with both wild type and PLCζ1 knockout sperm and oocytes. These findings clearly indicate that oocyte activation in the absence of PLCζ is linked to Gαq mediated pathways. An intriguing aspect of the findings was that similar results were observed when Gαq was blocked in sperm cells suggesting a role for sperm Gαq at fertilisation. Gαq/PLCβ inhibition and observations on its effects on fertilisation clearly suggest

that a PLCβ is involved in fertilisation and works through Gαq activation and is also supported by some previous evidence to some extent (Moore et al., 1994; Williams et al., 1992).

Expression of PLCβ isoforms in mouse sperm cells have been confirmed both at RNA and protein levels. Assuming that the secondary oocyte activating factor resides in sperm and relating it to the fact that PLCβ is expressed in sperm cells, as confirmed in Chapter 3, it is important to confirm whether sperm derived PLCβ isoforms have any role(s) in oocyte activation. FR induced inhibition of Gαq caused a reduction in fertilisation rate as observed in oocytes which indicates direct involvement of sperm as well as oocyte-derived Gαq/PLCβ isoforms in oocyte activation.

5.3.4 Gαq modulation affects sperm induced Ca2+ transients in mouse oocytes

Repetitive Ca^{2+} release is an important indicator at fertilisation and is an essential requirement for oocyte activation *in vivo*. These Ca^{2+} transients are the consequence of release of Ca^{2+} from the oocyte's internal stores mediated by IP_3. The pattern of Ca^{2+} release is oscillatory in nature. The study of Ca^{2+} oscillations is an important way to investigate the role of factors causing its release and ultimately their effect on oocyte activation as well as fertilisation (Karl Swann & Lai, 2016). Using the approach of inhibiting the upstream stimulator of PLCβ, Gαq was inhibited to see the effect on Ca^{2+} release. Oocytes were treated with FR and YM inhibitors and inseminated with wild type or PLCζ knockout sperm cells for Ca^{2+} imaging studies. The results showed a significant reduction in amplitude and frequency at lower inhibitor concentrations, followed by a delay and an atypical

pattern of Ca^{2+} release in wild type sperm. Interestingly, Gαq blocked oocytes showed no Ca^{2+} transients after fertilisation with PLCζ knockout sperm (Figure 49). This presents strong evidence that Gαq/PLCβ mediated Ca^{2+} release occurs at fertilisation in the absence of PLCζ induced Ca^{2+} release. At higher concentrations of such inhibitors Ca^{2+} release was affected more significantly.

Previously, Igarashi et al. studied the effect of transgenic depletion of PLCβ1 expression in oocytes on Ca^{2+} release in oocytes. Reducing oocyte PLCβ1 expression led to a decrease in Ca^{2+} transient amplitude. On other hand, PLCβ1 overexpression disturbed the frequency of Ca^{2+} transients. These findings indicate the importance of PLCβ1 stimulation during oocyte activation which occurs through the Gαq pathway (Igarashi et al., 2007). Igarashi's findings support a role for PLCβ1 at fertilisation and the importance of investigating other PLCβ isoforms in oocytes. Hence the current project was designed to target all PLCβ isoforms.

Collectively, current findings provide strong evidence of a potential role for Gαq-PLCβ signalling in sperm induced Ca^{2+} release required for oocyte activation. Specific targeting of PLCβ isoforms by U73122 and the pattern of Ca^{2+} release using Gαq inhibitors identifies PLCβ isoforms as potential secondary factors at oocyte activation. The mechanism of PLCβ isoforms involvement at oocyte activation is parallel and independent of the primary factor, PLCζ (Figure 51) and this explains the dual effects of U73122 on this process (Figure 52).

5.3.5 Sperm RNA may not be the secondary factor

A further idea has been put forward which is that Ca^{2+} transients can be triggered in the absence of PLCζ by a sperm-derived RNA. This proposed factor is believed to be translated once it reaches the oolemma environment and thereby causes

Ca^{2+} release. This hypothesis is consistent with studies in which PLCζ cRNA microinjection in oocytes caused Ca^{2+} oscillations (Jones, 2018; Saunders et al., 2002b). To test this hypothesis, protein translation inhibitor, cycloheximide,was used to inhibit RNA translation in oocytes including the RNA delivered by sperm. Cycloheximide is known as an oocyte activating molecule without causing Ca^{2+} release. Its effect on sperm induced Ca^{2+} release in oocytes was investigated in the current study. Results of this part of the study showed that cycloheximide eliminated sperm induced Ca^{2+} oscillations in oocytes but had no effect on PN formation and cleavage. An explanation of this finding could be that cycloheximide interfered with PIP_2 synthesis and hydrolysis. The presence of a translation inhibitor has been reported to cause a decrease in cellular levels of the IP_3 substrate, PIP_2, which in turn is probably causing a significant fall in IP_3 turnover. In cardiomyocytes, cycloheximide has been reported to inhibit norepinephrine- and angiotensin II-induced increases in PIP_2 levels (J. X. Xu et al., 2014). This indicates that cycloheximide has adverse effects on IP_3 production and thereby on IP_3-mediated Ca^{2+} signalling. Cycloheximide has also been found to be a phospholipid metabolism inhibitor (Farese, 1969; Farese et al., 1969; Glinsmann et al., 1969). Possibly, cycloheximide is interfering with the IP_3 release pathway and disturbing IP_3 mediated Ca^{2+} release which may be Gαq-PLCβ induced Ca^{2+} release. Another possibility indicated by this finding is that the proposed RNA factor is not being translated due to the inhibitors, and hence leads to no rise in intracellular Ca^{2+} in the treated oocytes.

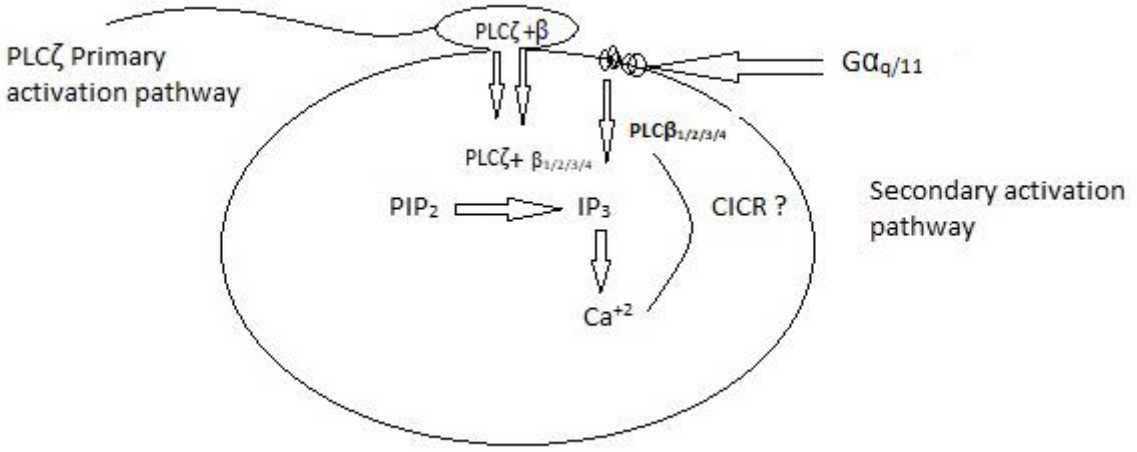


Figure 51 Proposed mechanisms of action secondary factors at oocyte activation

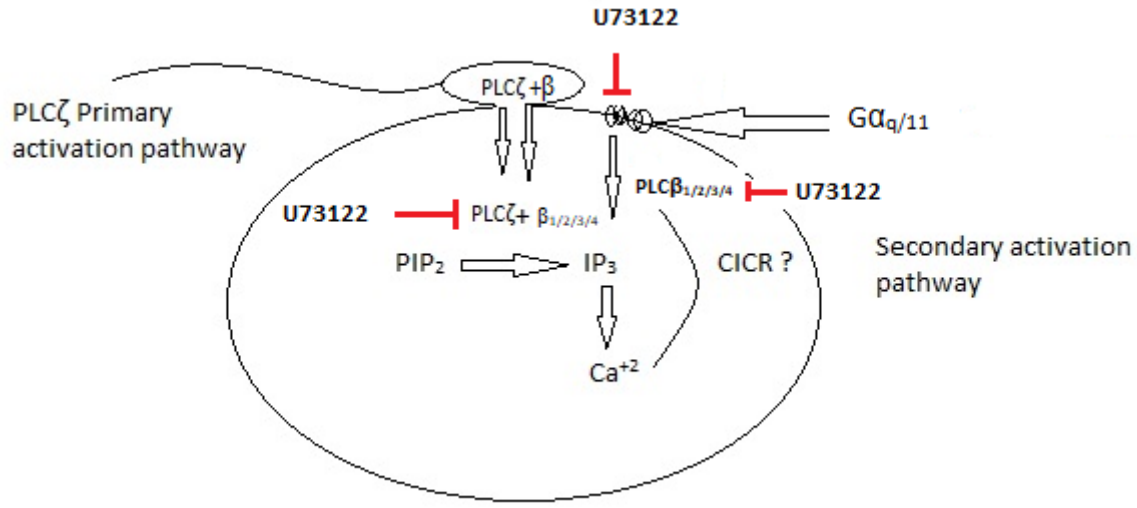


Figure 52 Possible mechanisms of inhibitory effect of U73122 on activating PLC isoforms in oocytes.

Chapter 6: General Discussion

6.1 General Discussion

Oocyte activation relies on cell signalling mediated by specific factors, right from the change in surface properties of the fertilised egg to the pronuclei formation that begins the process that leads to an embryo. These factors are delivered, in part, by sperm and others are already present in the oocyte to create a nourishing environment for the development of an embryo. Some of these factors make a change in an unfertilised oocyte to prepare it for cell cycle resumption and set it along the path of embryo development, along with the genetic materials delivered by the sperm cell, others are involved in other aspects of oocyte activation. A controversial point relating to oocyte activating factors has been whether these factors come from the sperm or are induced within the oocyte. Ca^{2+} transients in a mature oocyte are something that occur only after sperm fusion and are considered as a hallmark of the beginning of oocyte activation. Knocking out PLCζ, the primary mediator of Ca^{2+} oscillations at fertilisation, led to the surprising finding that a few Ca^{2+} spikes can still take place after IVF with PLCζ knockout sperm and oocyte activation and fertilisation still occurs with generally normal development,

albeit with increased polyspermy overall (Hachem et al., 2017; Nozawa et al., 2018).

Exocytosis of cortical granules and release of the oocyte from the arrested state are simultaneous processes and triggered by the first Ca^{2+} transients. Several theories have been put forward as to the source of such transients such as receptor activation on the oocyte surface, delivery of Ca^{2+} releasing factor by the sperm, CICR sensitisation (Dupont et al., 1996; Igarashi et al., 2007; Z. Macháty et al., 2005; S. Miyazaki, 1991; Moore et al., 1993; Tavalaee & Nasr-Esfahani, 2016; J. Tesarik, 2002; Williams et al., 1992). Research on sperm and oocyte-derived factors also suggests that there may be a mutual contribution of both cells to trigger Ca^{2+} signals in the oocyte (Aarabi et al., 2014; Hachem et al., 2017; Igarashi et al., 2007; Claudio Sette et al., 2002; Yuan et al., 2016). One focus for such studies is to investigate receptors on the oocyte that may be involved in oocyte activation. A few studies of the mouse model have focused on guanine nucleotide-binding proteins (G-protein) coupled receptors (GPCRs) which might lead to an oocyte's activation, formation of pronuclei, recruitment of maternal mRNAs, DNA synthesis and cleavage (Moore et al., 1993). The mechanisms of activation of muscarinic receptors have been established and they involve PLCβ isoforms. Serotonin receptors have also been reported in the context of oocyte activation (Miyazaki et al., 1990). In echinoderms, sperm *bindin* mediated global phosphorylation of proteins on the oocyte or egg surface via a receptor-mediated action is essential for zona fusion and might also be directing several downstream functions simultaneously in sea urchins (Abassi & Foltz, 1994; Glabe & Vacquier, 1978).

Essential roles of surface receptors at fertilisation, triggering global changes in oocytes, supports the hypothesis that Gαq-PLC signalling stimulation is a part of

sperm mediated receptors stimulations on oocytes which in turn release secondary Ca^{2+} transients. In addition to the primary IP_3 pathway, triggered by primary factor PLCζ (Hachem et al., 2017; Nozawa et al., 2018) and secondary factors PLCβ (the current study), a few other Ca^{2+} release pathways have been studied in the context of oocyte activation such as the cyclic ADP ribose (cADPR)/ryanodine receptor pathway and CICR pathways (Fluck et al., 1999; Petr et al., 2002; Karl Swann, 1991; Jan Tesarik & Sousa, 1996). Some non-protein, RNA factors have also been suggested (Jones, 2018) which might have minor effects on secondary Ca^{2+} transients.

6.1.1 PLCζ is the primary oocyte activating factor but there are secondary factors as well

The literature so far suggests several gaps in studies of mammalian oocyte activation. Considering the fact that mammalian oocyte activation requires a cytosolic free Ca^{2+} rise after sperm fusion, it is important to investigate PLCs in oocytes and sperm cells, including in the PLCζ knockout models, to understand PLC expression in mammalian gametes and potential genetic compensation in PLC knockout models followed by an in-depth proteomic analysis of PLCζ knockout sperm. The current study was undertaken to execute these objectives and the findings reported strengthen our understanding of oocyte activation.

This is the first study that provides a comprehensive analysis of PLC isoforms in mouse sperm cells. In addition to that, comparative expression analysis was done in PLCζ knockout sperm to identify the differential expression of PLC isoforms in such sperm to provide clues about potential genetic compensation. The results from these studies revealed that mouse sperm expresses a range of PLC isoforms

including all major subclasses of PLCβ, PLCɣ, and PLCδ isoforms. Qualitative mRNA expression analysis revealed that sperm cells express RNAs of all major PLCs whereas at protein levels PLC B1, β4, δ1, y1, y2, l, xd1 were detected. Their protein expression was confirmed by western blot and immunofluorescence analysis. PLCβ isoforms were found to be expressed in the head as well as the tail region whereas the delta and gamma isoforms are present mainly in the head region with traces in the tail. PLCxd1 was present in the head region. The expression of PLCβ1 in sperm was not well characterised before but its presence in the acrosome region with Gq protein was confirmed (Walensky & Snyder, 1995), however, its importance in fertilisation has been reported. The fertilisation rate declined by over 20-25% after a PLCβ1 deficit and a similar trend was observed for the sperm acrosome reaction (Choi et al., 2001). The current study confirms the location of PLCβ1 in mouse sperm in the head and tail regions. PLCβ2 was confirmed in testis, spermatogenesis stages but not in mature sperm cells (F. Li & Zhou, 2012). Current work confirms that sperm cells transcribe PLCβ2 and it is highly possible that it also expresses as protein. PLCβ3 expressing RNA has been identified and PLCβ 4 has been confirmed at both RNA as well as protein levels in sperm cells. PLCɣ1 and ɣ2, and PLCδ1 isoforms were also detected by western blot analysis. These findings confirmed that mature sperm cells express a range of PLC isoforms and their roles have also been confirmed in the acrosome reaction and sperm-oocyte binding (Choi et al., 2001; Walensky & Snyder, 1995). In the context of oocytes, all major PLC isoforms have been reported to be expressed and need not be re-investigated (Dupont et al., 1996; Igarashi et al., 2007; Y. L. Miao & Williams, 2012; Saunders et al., 2002a).

Knowing the fact that oocyte activation is triggered by an IP_3 induced Ca^{2+} release it is important to look at the transcripts of such PLCs in the sperm, oocyte and fertilised egg to understand if there are any re-organisation and change in expression of these isoforms as fertilisation proceeds. Sperm cells have transcripts of all isoforms with PLCβ3 and PLCζ being most highly expressed whereas oocytes have most PLCs but less expression which may be because the oocyte needs to be less sensitive towards Ca^{2+} release before fertilisation. PLCɣ1 is highly expressed in the unfertilised mature oocyte and requires src-family kinases to activate it which is less possible in the unfertilised oocyte (Runft et al., 2004; Talmor-Cohen et al., 2002). The changes in maternal RNA and protein degradation is an important process at fertilisation which seems not to affect the expression of PLCs significantly (Krauchunas & Wolfner, 2013; R. M. Schultz et al., 2018). H3K4me3 methylation of chromatin in sperm cells indicated that sperm cells have positive values for histone 3rd lysine residue methylation for most PLCs, with PLCζ1 highest, which facilitates their transcription and allows protein expression of several PLCs (Park et al., 2015). These findings about the expression of PLC isoforms in sperm cells indicates the expression of several PLCs with positive histone enrichment and modification state. The localisation of PLCs has been confirmed in cytosolic and membrane regions suggesting sperm's ability to release the PLCs in the oocyte cytoplasm where they can target PIP_2 on the membrane as well as cytoplasmic vesicles. MII oocytes express a range of PLCs transcripts but comparatively less than sperm cells and their high H3K27me3 state do not allow any increase in their expression to prevent pre-mature activation and meiotic resumption. In conclusion, sperm express several PLCs which opens the possibility of sperm delivering PLC isoforms to the oocyte that may potentially be

involved in Ca^{2+} release. Stimulation of these PLCs is possibly occurring by a surface receptor activation pathway.

Since the outcome of PLCζ knockout revealed the presence of a secondary oocyte activation factor, fertilisation and oocyte activation research has taken a new direction of looking at other factors with one focus being on other PLCs. PLCζ knockout mice are sub-fertile and oocyte activation can still occur with successful cleavage. This is essentially due to a reduced number of delayed Ca^{2+} transients which affects litter size *in vivo*. A search for secondary factors working in line with PLCζ1 or through another mechanism was the next question that needed to be investigated. In Chapter 3 the characterisation of PLCs in germ cells suggested plenty of suitable candidates for a secondary factor with a possibility that a PLCβ subclass might play such a role as PLCβ1 has been reported as an important candidate in both sperm and oocyte that has a direct influence on fertilisation (Choi et al., 2001; Igarashi et al., 2007). Comparative expression analysis was done to understand the expression reorganisation of PLCs in PLCζ1 knockout sperm. At the mRNA transcription level, the PLCζ1 knockout sperm showed no significant change but a slight increase in PLCδ1 and δ3 and a significant fall in PLCxd1 and PLCε isoforms. Down-regulated PLCδ1 and PLCδ3 genes in the knockout sperm are less likely to have impact on oocyte activation as they have been reported to work mainly during the acrosome reaction and not fertilisation and unaffected PLCδ4 has been reported also to be involved in the acrosome reaction (K Fukami et al., 2001; Kiyoko Fukami et al., 2003). Protein expression was partly consistent with transcript expression. It was demonstrated that PLCβ1 and β2 showed down-regulation where PLCβ3 and β4 were upregulated along with PLCɣ1 with a slight fall in PLCδ1. PLCδ1 knockout mice are fertile *in vivo* (Nakamura et al., 2005). The

PLCɣ subclass showed down-regulation in PLCζ knockout sperm and has been demonstrated to not be involved in mammalian oocyte activation (Mehlmann et al., 1998). Due to different activation pathways of the PLCɣ subclass, they remain controversial for their involvement in inducing Ca^{2+} oscillations during oocyte activation (Runft et al., 2004; Claudio Sette et al., 1998, 2002). To strengthen the differential expression of genes in the knockout model, a further attempt was made to explore the proteome profile of the PLCζ knockout sperm. Proteomic analysis of PLCζ knockout sperm suggests differential expression of detected PLCs. PLCβ3 was up-regulated whereas δ1 was down-regulated. No changes were observed in β4 and δ4. PLCβ3 expression changes are consistent with immunofluorescence findings and support the hypothesis of the involvement of the PLCβ subclass in oocyte activation which releases Ca^{2+} parallel to PLCζ1 through a similar or different pathway.

6.1.2 PLCβ isoforms are potential secondary factors, others are not

The findings of the current project and Igarashi et al., so far indicate a potential role for PLCβ isoforms in Ca^{2+} release and oocyte activation. The next question was to look at the mechanism of stimulation of the PLCβ subclass and the source of this among germ cells. The approach was adopted of investigating the contribution of individual cells and the mechanisms of activation of this subclass at fertilisation. It is worth mentioning that it has been found previously that the PLCβ subclass plays an important role at fertilisation (Choi et al., 2001; Igarashi et al., 2007). To investigate what triggers PLCβ class activation and how its inhibition affects the fertilisation, the approach of pharmacological targeting of upstream regulators of PLCβ pathway was adopted.

PLCβ isoforms are stimulated via the GPCR-Gq pathway on the action of stimuli. It is likely that such stimuli are either transferred from sperm or stimulated via the oocyte surface as a result of gamete binding/fusion. To confirm whether this pathway plays a role at fertilisation, pharmacological inhibitors of upstream were used. FR is a Gαq-specific inhibitor which has been characterised for its potency and efficacy. It was demonstrated that FR induced Gαq-inhibition affects fertilisation significantly. Interestingly, it showed inhibitory effects when used separately in sperm and oocyte and also with both gametes. To further investigate the effect, PLCζ knockout cells were employed to reproduce the effects and the results were consistent. Cross-check of findings was done using a general PLC inhibitor. Several reports suggested that U73122 inhibits several subclasses of PLCs but the specificity was not clear. In the current project, it was demonstrated that, in a study of the beta, gamma, and delta PLC isoforms, U73122 is a PLCβ-subclass specific inhibitor that inhibits primarily PLCβ1, PLCβ2 and PLCβ3. This property of U73122 was used to cross-check the effect of FR.

Wu et al., did microinjections of chromatographic sperm fractions precipitates to confirm which PLCs are involved in Ca^{2+} release that are delivered by sperm. The results suggest that PLCγ and δ isoform fractions do not cause intracellular Ca^{2+} release and exclude PLCγ1, γ2 and δ4 isoforms from the picture which strengthen our findings regarding a role for PLCβ (H. Wu et al., 2001).

Overall, the observations collectively suggests that the PLCβ subclass, from both sperm and oocyte, operates at fertilisation to promote Ca^{2+} release through the IP_3 pathway at fertilisation and assist PLCζ to sustain Ca^{2+} transients.

6.1.3 Cryopreservation affects sperm proteomics- indication of inactivation of secondary factors

The finding that no Ca^{2+} transients were induced in oocytes after IVF with thawed cryopreserved PLCζ knockout sperm can further be explained by the findings of Ortiz-Rodriguez, et al. They demonstrated, in a horse model, that cryopreservation of sperm downregulates several essential genes in embryos which are implicated in pathways related to oxidative phosphorylation, DNA binding & replication (Ortiz-Rodriguez et al., 2019). These findings indicate cryopreservation of sperm changes its overall transcriptomic/proteomic profile which could eliminate even delayed Ca^{2+} transients induced in oocytes by PLCζ knockout sperm. Moving further to proteomic analysis of cryopreserved sperm, a study by Li et al., reported that in humans, cryopreserved sperm are 50-60% less motile with compromised curvilinear, straight line and path velocities than fresh sperm. They also showed that capacitation or acrosome reaction associated proteins are downregulated in cryopreserved sperm cells (S. Li et al., 2019). Several other proteomics studies also suggest that cryostress decreases protein abundance and impairs sperm quality which directly affects fertilisation and development (Perez-Patiño et al., 2019; S. J. Yoon et al., 2016). Within sperm cells, cryopreservation reduces the effect of Ca^{2+} modulators and acrosome inducers such as progesterone (J. C. Kim et al., 2008).

6.2 Conclusion

Oocyte activation comprises a series of physiological processes encompassing Ca^{2+} transients, cortical granule release and resumption of meiotic progression of the cell cycle. Initiators of this essential process and their origin are still partially unclear. The current study was undertaken to investigate those oocyte activating factors and explore the possibility that they work through the IP_3 pathway and reside in one or both gamete cells. An in-depth proteomic analysis was also performed to strengthen and validate preliminary findings. The findings relating to PLC expression in sperm cells suggests that they express a range of PLC isoforms including the PLCβ subclass which are known to be essential for sperm binding, sperm-oocyte fusion, and the acrosome reaction. Comparative expression analysis revealed reorganisation of the PLCβ subclass expression in PLCζ knockout sperm which could possibly act as a substitute for PLCζ function. These findings were further confirmed and supported by proteomic analysis of PLCζ knockout sperm. Proteomic studies revealed differential expression including up-regulation of PLCβ3 and a range of acrosome and fusion proteins that might explain the enhanced polyspermy following IVF with PLCζ knockout sperm. To validate the possibility of PLCβ subclass involvement in oocyte activation, Gαq-protein modulation with suitable inhibitors was performed which revealed its important role in fertilisation and development. Inhibiting Gαq in sperm as well as oocytes resulted in similar patterns of a reduction in fertilisation rates. These findings clearly suggest that the PLCβ subclass of isoforms are involved in oocyte activation and development in mammals.

6.3 Future Prospects

The current study showed that PLCζ and PLCβ isoforms, play a significant role in oocyte activation through receptor and sperm soluble factor pathways which are implicated in releasing IP_3 mediated Ca^{2+} release and eventually oocyte activation. This project also showed that a Gαq /PLCβ pathway plays an important role in oocyte activation. Further studies of sperm ligands that bind to the oocyte's G-proteins would be an important next step which would help to decipher the complete mechanism of receptor activation on the oocyte membrane that triggers the Gq pathway. Identification of the oocyte's G-proteins and sperm ligands as well as the signalling network would increase our understanding of mammalian oocyte activation. Probably, Gαq/11 stimulation is affected by cryopreservation, due to the downregulation of binding and fusion proteins, which results in no Ca^{2+} transients in IVF with thawed cryopreserved PLCζ knockout sperm sperm. Studies on the effect of cryopreservation on Ca^{2+} transients triggered by a Gαq pathway and PLCζ induced Ca^{2+} oscillations and associated proteins would improve our understanding of this process further.

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