Mitochondrial heterogeneity and sorting in mammalian oocytes and pre-implantation embryos

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Abstract

Mitochondria are the dominant organelle in mammalian oocytes and embryos. More than 200,000 copies of mitochondria can be found in a single oocyte or early embryo. During mammalian early development, mitochondria will be aliquoted to different daughter cells via steps of meiosis and mitosis. The experiments presented in this thesis aim to examine mitochondrial heterogeneity in their activity in mouse oocytes and early embryos and investigate the links between mtDNA mutation load and aging.

Firstly, TMRM/mito-Dendra based ratiometric analysis using confocal imaging is developed for accurate measurement of MMP in oocytes. This ratiometric approach normalizes for the mass of mitochondria thereby allowing direct comparison of inter- and intra-cellular MMP. With this ratiometric analysis, a positive correlation between MMP and oocyte *in vitro* competence was then found. MMP is also spatially distributed in *in vitro* grown oocytes such that high MMP is found in specific sub-cellular compartments, which is not observed in *in vivo* grown oocytes and this spatial regulation of mitochondrial activity is important for oocyte *in vitro* maturation.

Secondly, by utilizing Next-Generation Sequencing of mtDNA from single or pooled oocytes, the correlation between mitochondria dysfunction and mtDNA mutation was studied. The results show that oocytes from oocyte-specific Drp1 KO mice and oocyte-specific Tfam KO mice are not prone to accumulate mtDNA

mutations. In contrast, oocytes from reproductively aged mice and PolG$^{mut/mut}$ 'mutator' mice showed more mtDNA mutations and distinct mutation spectrums.

Finally, a few methods have been attempted to investigate the heterogeneity in mitochondrial activity in blastocyst, but no conclusion can be drawn from current results due to limitations from each method. However, a negative correlation between mitochondrial activity and embryo developmental competence *in vitro* was found, where embryos that can grow to blastocyst stage normally display lower mitochondrial activity than those abnormal embryos.

In all, mitochondrial membrane potential and its heterogeneity are proved to play a role in establish oocyte competence *in vitro*. Oocytes from mice with advanced age are prone to increased mtDNA mutation, but it is not likely caused by a failure of mitochondrial fission, decreased mtDNA copy number, or disrupted mtDNA proofreading. Still the heterogeneity in mitochondrial membrane potential at blastocyst stage needs more investigation, and the correlation between mitochondrial activity and embryo *in vitro* competence can be a meaningful aspect to look at.

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Conference Proceedings

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Thesis declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Jun Liu	Date May 20th, 2021

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Professor John Carroll	Date May 20th, 2021

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Chapter 1. Introduction

As the powerhouse of cells, mitochondria produce energy in forms of ATP for many cellular processes. During oocyte maturation and early embryo development, the quality of mitochondria is an important determinant of developmental competence of both oocyte and embryo.

1.1 Oocytes and early embryonic development

Oocyte maturation refers to the process from germinal vesicle breakdown (GVBD) to meiosis-II (MII), which is crucial for the generation of a fertilizable egg and healthy offspring. Briefly, after GVBD, chromosomes condense and microtubules form a bipolar spindle around them [1]. Driven by consecutive actin-based pushing forces [2], the spindle moves to the cortex along its axis. Once the spindle reaches the cortical domain of the oocyte, anaphase and first polar body extrusion are initiated. After the emission of first polar body, a mature egg is generated and ready for fertilization.

Figure 1.1. Stages of oocyte maturation and fertilization [3]

1.1.1 Nuclear maturation

Maturation of oocyte requires both nuclear and cytoplasm to be matured. *In vivo* oocytes are maintained in a state of meiotic arrest until the preovulatory LH surge [4]. Extracted oocytes undergo spontaneous meiotic resumption, which indicates that nuclear maturation is controlled by meiosis-inhibiting factors from other cellular compartments of the follicle [5-7]. It was observed that supplementation of culture media with dibutyryl cyclic adenosine 3', 5' monophosphate (cAMP), a synthetic cAMP analogue, extended the duration of meiotic arrest in culture, which proved the core role of cAMP in the maintenance of meiotic arrest [8]. This central role of cAMP in meiotic arrest is further evidenced by the finding that, stimulation of cAMP production, or inhibition of cAMP hydrolysis via phosphodiesterase enzyme inhibitors can maintain high cAMP levels and result in the delay in meiotic resumption [7, 9, 10]. The level of cAMP in oocytes is regulated by a combination of cAMP production, cAMP hydrolysis and the regulation of both activities through mediators, such as cyclic guanosine 3', 5' monophosphate; cGMP, and natriuretic peptide C; NPC. cGMP that is produced in the granulosa cells diffuses through gap junctions into the oocyte and inhibits the cyclic nucleotide phosphodiesterase [11, 12], which maintaining a high level of cAMP in the oocyte. The activation of LH receptor can decrease follicle cGMP levels by altering the balance between cGMP synthesis and hydrolysis, which results in the degradation of cAMP by up regulated level of phosphodiesterases. Oocytes that undergo in vitro maturation without granulosa cells mature

spontaneously as no cGMP from granulosa cell available for the inhibition of phosphodiesterases [13].

1.1.2 Cytoplasmic maturation

Cytoplasmic maturation is the gross structural changes, including the re-arrangement of cellular organelles (mitochondria, endoplasmic reticulum (ER), cortical granules, ribosome), and the completion of metabolic processes that lead to the accumulation of mRNA, protein and substrates to support fertilization and early embryo development [14, 15]. Nuclear and cytoplasmic maturation is asynchronous *in vitro*, in which oocytes from antral follicles undergo nuclear maturation without completed cytoplasmic maturation. To achieve a successful oocyte meiosis, proper chromosomal segregation (nuclear maturation) and proper distribution of organelles (cytoplasmic maturation) are required [15, 16]. They are two different processes but they take place simultaneously and corelate with each other. Intracellular events during oocyte cytoplasmic maturation contributes to the completion of nuclear maturation, oocyte capacity to be fertilized, and also early embryonic development.

1.1.3 Gap junction

Oocyte development relies on the bidirectional communication between the oocyte, granulosa cells (GCs) and cumulus cells (CCs). This communication involves gap junctions between the oocyte and GC and CC, as well as the production of paracrine factors, such as the oocyte-secreted factors (OSF), bone

morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9). This promotes the development and maturation of both the oocyte and the somatic cells, responses to metabolic requirements and external signals such as gonadotropins. During oocyte nuclear maturation, gap junctions between CCs and oocytes allow a quick transport of small metabolites and molecules from the CCs into the oocyte [17]. CCs and oocyte-cumulus cell gap junctions are important to oocyte maturation, fertilization and also early embryonic development [18]. Moreover, the presence of CCs during IVM elevates oocyte ATP levels, which suggests CCs and the gap junctions also contribute to mitochondrial function [19].

1.1.4 Early embryo development

After fertilization, a zygote containing genetic material of sperm and egg is formed. Then the process of cleavage begins, during which the zygote cleaves rapidly and produces a cluster of cells without growing to a larger size. Usually one day after fertilization, the first division happens, and the next divisions take place every 12 to 24 hours. After around 4 cell division cycles, a compact mass of 16 or more cells forms, which is called a morula. The morula is the first stage where blastomeres can be classified as internal or external. As the cells continue to divide to 64 cells stage, the first cell-fate decisions of mammalian development take place, which leads to the derivation of two distinct cell lineages, the trophectoderm (TE) and inner cell mass (ICM). The inner group of cells are called

inner cell mass (ICM), which will become the embryo. The outer cells, called trophoblast cells, will eventually develop into the chorion, which is the embryonic portion of placenta. Typically, this process has been considered as occurring in two independent cell-fate decision. The first decision involves the physical separation of outer TE progenitor cells from ICM during 8- to 16-cell and 16- to 32-cell cleavages, through 'asymmetric/differentiative' cell-divisions, which are distinct from 'symmetric/conservative' divisions producing two outer-cells [20, 21]. The second decision involves gene expression refinements and active cell sorting with the ICM which results in epiblast cells [22-25]. A number of key transcription-factor genes have been identified to be involved in these two cell-fate decisions, for example, Tead3 and Cdx2 in the TE and Nanog in epiblast are required to regulate the necessary gene expression patterns to support these emerging cell types [26-29].

1.1.5 In vitro maturation (IVM)

IVM of oocytes is a widely used reproductive technique. The specific techniques used for IVM differs among clinics and the outcomes can be extremely variable due to differences in protocols [30-36]. IVM of oocytes have some advantages over traditional in vitro fertilization (IVF): reduced psychological impact, lower cost of the treatment, and no hormonal stimulation before oocyte retrieval. However, much work is needed if it is to become a routine procedure in ART. A number of key problems exist, including a low rate of development in many

programs, epigenetic modifications have been reported as a result of IVM [37] and chromosomal abnormality increases after IVM [38]. There is the need for future efforts to be focussed on improving IVM conditions a=so as to increase oocyte quality and increase developmental potential.

In summary, early embryonic development is a process of continuous mitotic divisions, coupled with many other cellular events, and this highly dynamic process needs an adequate supply of energy, which is primarily provided in forms of ATP produced by mitochondria.

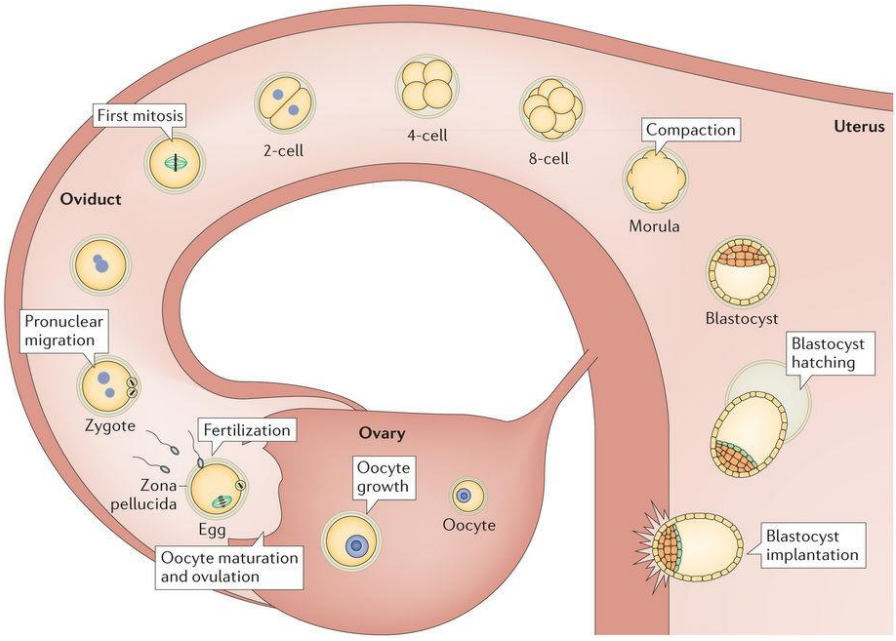

Figure 1.2. Overview of pre-implantation development [3]

1.2 Mitochondrial structure and function

1.2.1 Mitochondrial structure

Mitochondrion is a double membrane-bound organelle found in all eukaryotic organisms. Structurally, it consists of five distinct parts, the outer membrane, intermembrane space (the space between the outer and inner membranes), inner membrane, the cristae space (formed by infoldings of the inner membrane), and the matrix (space within the inner membrane) (Figure 1.3). There are large numbers of integral membrane proteins called porins on the outer membrane, which allow molecules of 5000 daltons or less in molecular weight to go through. There are also enzymes in the outer membrane involved in the elongation of fatty acids, oxidation of epinephrine, and the degradation of tryptophan, including monoamine oxidase, kynurenine hydroxylase, rotenone-insensitive NADH-cytochrome c-reductase, and fatty acid Co-A ligase. Between the outer and inner membrane is the intermembrane space. The concentrations of small molecules like ions and sugars in the intermembrane space is the same as in the cytosol due to porins. But large proteins like cytochrome c, which needs a specific signaling sequence for transportation across the outer membrane, is exclusively localized to the intermembrane space [39]. The inner mitochondrial membrane contains diverse proteins which play different roles in multiple processes, including redox reactions of oxidative phosphorylation, ATP synthesis, metabolite transportation, mitochondrial fusion and fission. What's more, there is a membrane potential

over the inner membrane, formed by electrons transference through the electron transport chain (ETC). To enhance its ability for ATP production, inner membrane folds into numerous cristae to expand the surface area. The space enclosed by the inner membrane is mitochondrial matrix, which contains around 2/3 of the total protein in the mitochondrion [40]. It also contains a number of enzymes, ribosomes, tRNA and mitochondrial DNA (mtDNA). Those enzymes are involved in the oxidation of pyruvate and fatty acid, as well as citric acid cycle.

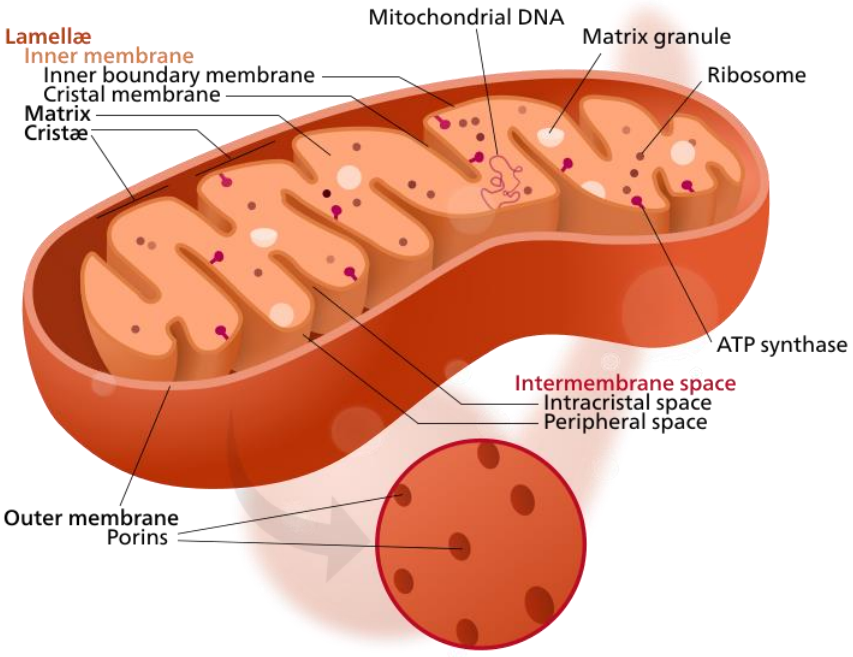

Figure 1.3. Mitochondrial structure [Wikipedia]

1.2.2 Mitochondrial genome

Except nucleus, mitochondrion is the only organelle that contains its own genetic material, known as mitochondrial DNA (mtDNA). Mitochondrial DNA is a double stranded circular DNA, located in mitochondrial matrix. In mammals, each mtDNA molecule consist of 15,000 to 17,000 base pairs, depending on

species [39]. Human mtDNA is 16569 bp (Figure 1.4). It encodes for 37 genes [41]; 13 of them are for enzymes that are involved in oxidative phosphorylation; 22 are for transfer RNA (tRNA) and 2 are for ribosome RNA (rRNA). Importantly, some proteins such as complexes I, III, IV and V of the oxidative phosphorylation system are composed of both nuclear DNA (nDNA)-encoded and mtDNA-encoded subunits, which are required to ensure mitochondrial function [42, 43].

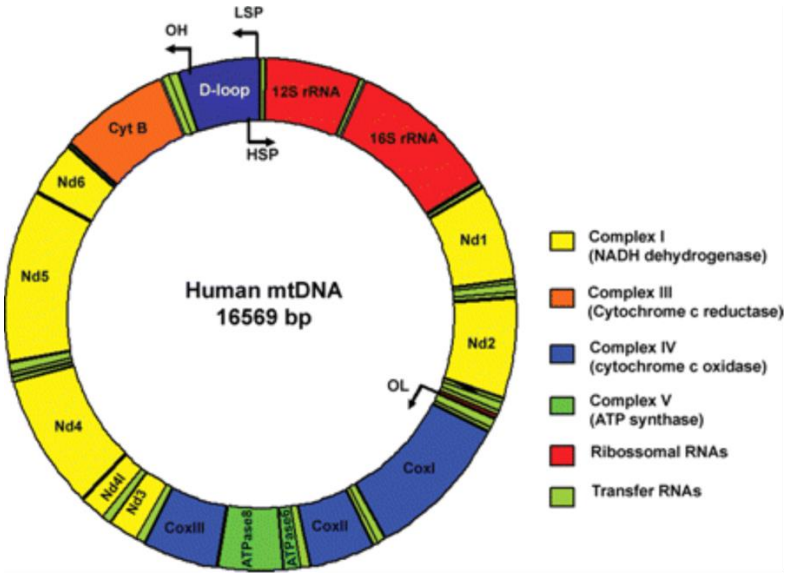

Figure 1.4. The human mtDNA genome [44]

1.2.3 Mitochondrial ATP production

Mitochondrial ATP production relies on the citric acid cycle (CAC, also called tricarboxylic acid (TCA) cycle) in the matrix and the ETC located on the inner membrane. Metabolites like pyruvate, fatty acids and amino acids are consumed during CAC, and after a series of chemical reactions, certain amount of energy in forms of ATP is produced. CAC also generates some intermediates (e.g., citrate,

iso-citrate, succinate, malate, etc.), reduces NAD^+ to NADH and FAD^{2+} to $FADH_2$, and produces carbon dioxide as a waste byproduct. Then NADH and $FADH_2$ are passed to ETC to transport electrons in the form of hydride ions (H^+). The ETC consists of 5 complexes, from I to V: NADH dehydrogenase, succinate dehydrogenase, ubiquinol cytochrome c reductase, cytochrome c oxidase and ATP synthase, respectively. Protons (H^+) are pumped into intermembrane space through complex I, III and IV, which results in a strong electrochemical gradient across the inner membrane. Then protons are diffused back and facilitated by complex V, and the potential energy triggers the synthesis of ATP from ADP (Figure 1.5).

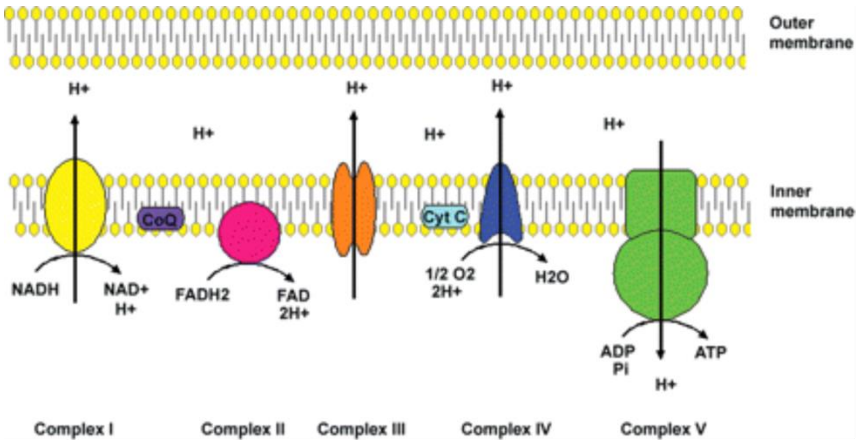

Figure 1.5. The ETC [44]

1.2.4 Mitochondria and ROS

In most mammalian cells, mitochondria are important sources and targets of ROS [45], which is the consequence of superoxide anions, produced by incomplete transfer of electrons to oxygen [46]. There are at least 9 sites in the mitochondrion which can produce ROS [45], which can thereby induce intracellular oxidative

stress. Normally, the generation of ROS is balanced by antioxidant defence mechanism [47], but when this balance is disrupted by excessive ROS production, mitochondrial disorders occur, resulting in a variety of pathologies [48]. Importantly, due to the proximity of mtDNA to where ROS are generated and its lack of histones, mtDNA is particularly vulnerable to ROS-induced DNA damage [49, 50]. The ROS-accelerated accumulation of mtDNA damage results in a progressive decline in respiratory function over time and finally leads to mitochondrial dysfunction, which is thought to be a vital factor of aging [51-53].

1.3 Mitochondria in oocyte and early embryo

Mitochondria in oocytes and embryos have some special characteristics. During oocyte maturation and early embryonic development, mitochondria undergo a series of changes, including morphology, distribution, abundance and activity.

1.3.1 Metabolism of oocyte and early embryo

Mitochondria in MII oocytes are round, with a dense matrix and few cristae, and remain unchanged in structure during 2-16-cell embryos, while at blastocyst stage, they become elongated and less electron dense, with increased numbers of cristae [54], which might reflect increased cellular activity and changed metabolism. During oogenesis, immature oocytes obtain the substrates such as pyruvate from follicle cells. However, after ovulation and during early embryonic development, this communication is lost. Oocytes and early embryos utilise pyruvate as the metabolic substrate (Figure 1.6), and early development from GV oocyte to 2-

cell stage *in vitro* requires medium supplied with pyruvate but not glucose or lactate [55]. Recently, it has been demonstrated that exogenously supplied pyruvate is essential for zygotic genome activation (ZGA), which might be the cause of further cell-cycle block beyond 2-cell stage in the absence of pyruvate [56, 57]. Lactate starts to function since 2-cell stage, while glucose starts from 8-cell stage [58]. Glutamine is another important energy source through TCA cycle during all stages of pre-implantation mouse development after zygote stage [59]. Thus, pyruvate and glutamine are the main ATP-generating substrates before 8-cell stage and subsequently in morula and blastocyst, glucose and glutamine become the primary substrates [55, 58-62] (Figure 1.7).

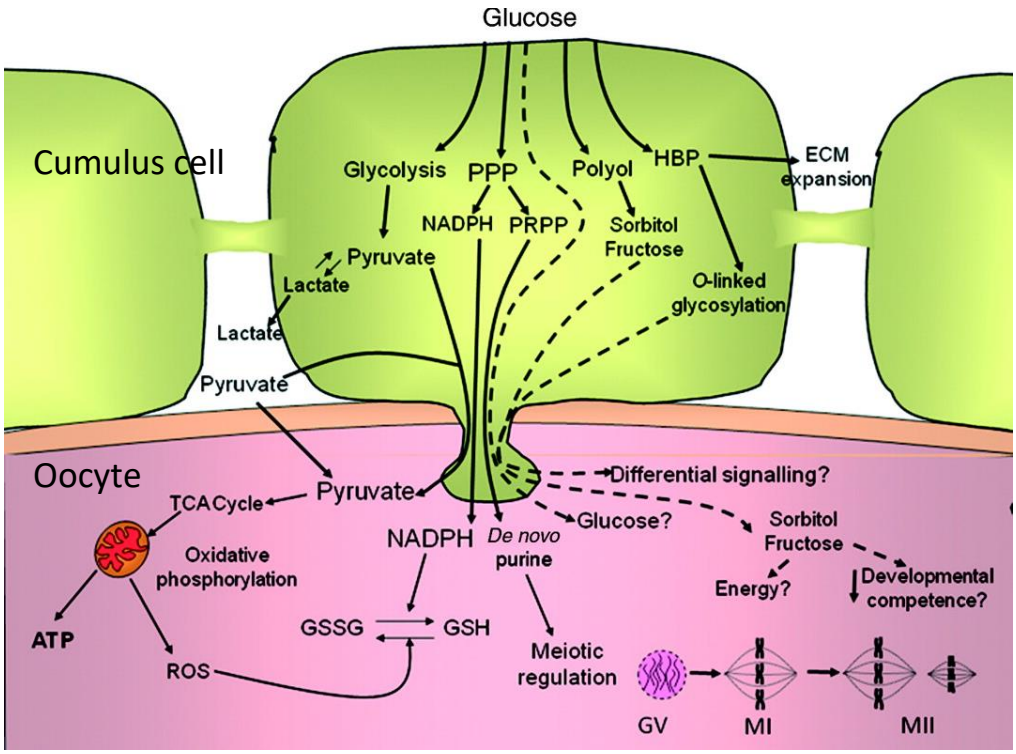

Figure 1.6. Metabolism of COC [63].

During oocyte maturation and the first few cell cycles of embryo development, glycolysis is supressed, which suggests that oxidative phosphorylation in mitochondria is the exclusive energy resource during this period[64]. Actually, during *in vitro* culture of the embryo after fertilisation, the presence of glucose and phosphate is pernicious and can block the embryo at 2-cell stage [65]. Lactate is abundant in the oviductal fluids and *in vitro* culture medium that allows efficient transportation and metabolism in oocytes [64, 66, 67], but intracellular ATP measurement in mouse oocytes and zygotes showed that lactate is oxidised to pyruvate but not directly for ATP generation [64]. In summary, ATP production during early development is stage-specific, which switches from exclusive mitochondrial oxidative phosphorylation to a coordination between glycolysis and oxidative phosphorylation.

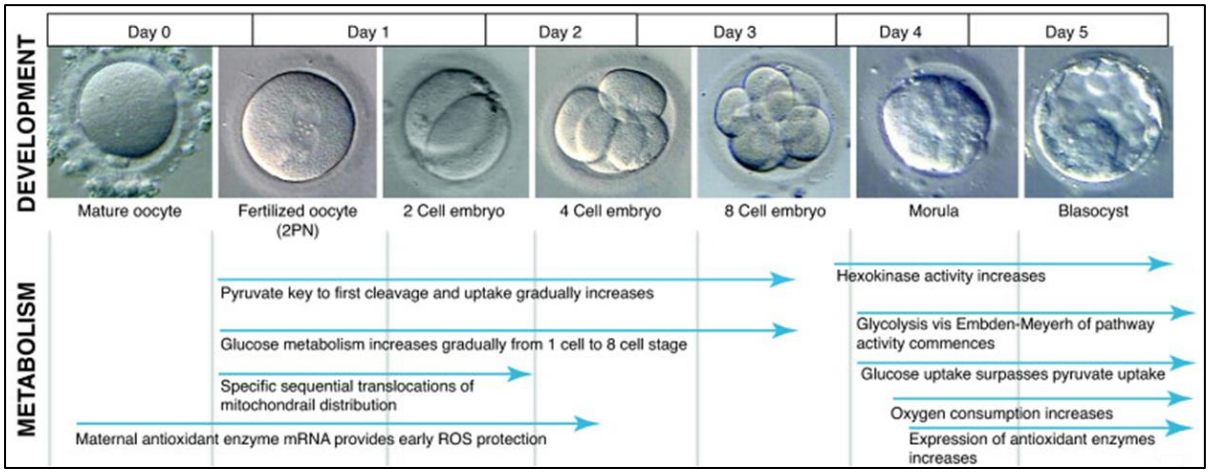

Figure 1.7. Preimplantation embryo development and metabolism [68].

1.3.2 Mitochondrial inheritance

The inheritance of the mitochondrial genome is distinct from that of nuclear genome. In mammals, after the entrance of sperm into the egg, both pro-nuclei contribute equally to the composition of zygote nucleus. However, mitochondria and their DNA are inherited exclusively from the population present in the egg at fertilization [69, 70], which is to ensure the mtDNA transmitted to the next generation is homoplasmic [71]. This uniparental inheritance is carried out by a ubiquitination mechanism. During early spermatogenesis, paternal mitochondria are labelled with ubiquitin when spermatogonia pass through the epididymis [72, 73]. After fertilization, mitochondria from the sperm and their DNA are destroyed within a few days, before ZGA for that particular species. In the mouse, sperm mitochondria and mtDNA are eliminated at 2-cell stage [74, 75], while in sheep and cattle, they can persist to 8-cell stage [73, 76], and in humans, the elimination tends to occur at 4-8-cell stage [77]. To ensure uniparental inheritance, ubiquitination modification is not the only mechanism to eliminate paternal mitochondria. LC3-dependent autophagy and proteosomal degradation are also involved in the removal of both paternal mitochondria and mtDNA [78-82]. In some cases, mitochondrial inheritance can also be both paternal and maternal, e.g., in inter-specific crosses, mussels and Drosophila [74, 76, 83-86]. An example of bi-parental inheritance of mtDNA in human was reported in 2018, showing that besides of maternal inheritance of mtDNA, paternal mtDNA could also be transmitted to the next generation [87]. The explanation behind this could

be that nuclear-encoded mitochondrial sequences (NUMTs) were passed from the paternal line [88, 89].

1.3.3 Mitochondrial DNA content

In mammalians, mature oocyte is the richest cell in mtDNA copy number comparing to other cell types, typically more than 100,000 copies. One possible explanation for the high mtDNA copy number is to make sure that not all the mitochondrial genes are influenced by a mutation at the same time and ensures the function of all the encoded proteins. This number is expanded from several thousand copies per PGC [90] and varies among different species and even individuals from the same species. Different research groups found different numbers using the same animal model (Table 1.1). All the numbers detected by different groups are based on Real-time PCR and one explanation of the massive variation in the same animal model among different groups could be the differences within the assays applied, such as DNA extraction methods, generation of DNA standards, primers, and PCR reagents. The variation is not only found in oocytes, but also during early embryonic development (Table 1.1). Mouse early embryos before implantation show a constant mtDNA content [90-92], indicating the mtDNA replication is not initiated until implantation, but it is interesting that mtDNA copy number at morula stage is decreased in porcine [93] and bovine [94] embryos, and is increased 1.5 to 3 folds comparing to oocytes at blastocyst stage, which suggests that mtDNA replication has already reinitiated

before implantation. What's more, mtDNA content can also be different between *in vivo*- and *in vitro*-derived embryos in rat; *in vitro*-derived embryos at morula and blastocyst stages show higher mtDNA copy number than *in vivo*-derived embryos and *in vitro* culture conditions lead to an increased mtDNA copy number after 8-cell stage, which is a different pattern from *in vivo* embryos [95]. Conversely, human *in vitro* matured oocytes have less mtDNA content than *in vivo* matured oocytes, and after fertilization by intracytoplasmic sperm injection (ICSI) followed by *in vitro* culture, a decline in mtDNA copy number in the ICM but not TE is found *in vitro* maturation group [96]. Fertilization techniques can also affect mtDNA content. For example, *in vitro* fertilized porcine embryos have more mtDNA copies at 4-cell stage, but fewer mtDNA copies at morula stage, compared with ICSI derived embryos [97].

The large amount of mtDNA and the fact that mtDNA replication is suppressed before blastocyst or implantation have given rise to the pervasive belief that mtDNA content is related not only to the fertilizability of oocyte, but also to the quality of early embryo development and subsequent implantation rate, which makes mtDNA content a potential biomarker [98, 99]. Comparing with unfertilized oocytes, there are more mtDNA copies in fertilized oocytes, which reflects a dependence of normal fertility on mtDNA content [99]. It is surprising that mouse oocytes with as few as 4000 mtDNA copies are still fertilizable, but unable to develop after implantation, and a threshold of 50000 copies of mtDNA

is required for postimplantation development [100], which further evidences that sufficient mtDNA content is important for oocyte fertility and embryo viability. But it does not mean that higher mtDNA copy number is better; human day-3 embryos that have massively increased mtDNA copy number consistently fail to implant [101]. Considering the variability of mtDNA copy number examined by different research groups, it is difficult to conclude the exact threshold of human oocyte mtDNA copy number for normal development, and even the threshold is confirmed, there is currently no practicable biopsy of oocyte mtDNA. Thus, as a potential biomarker, mtDNA content can be conducive to fundamental experiments but not to clinical analysis.

1.3.4 Mitochondrial DNA bottleneck and purifying selection

The mtDNA bottleneck theory proposes that the generation of large population of mtDNA in the oocyte involves subsampling of just a few mtDNA molecules from maternal germline. It was first described according to the observation of rapid segregation of mtDNA variants between generations in Holstein cows [102]. After fertilization of the oocyte, the total amount of mtDNA remains constant during early stages of embryo development. In blastocyst, the ICM but not the TE will develop into the embryo, which means only the mtDNA population in the ICM will contribute to the mtDNA pool of the next generation [103]. However, even mtDNA replication is initiated during blastocyst stage [91, 93, 94], the replication only takes place in the trophectodermal cells [44], which will finally

produce the placenta, whereas in ICM cells, which will develop into the embryo or embryonic stem cells (ESCs), there are few mitochondria and the quantity is likely to continue to decrease due to cell division. However there has been no comprehensive analysis directly comparing mtDNA in ICM and TE of individual embryos. It has been reported that there are only around 10-20 mitochondria and 30-45 mtDNA copies in human and mouse ESCs [104-106]. The limited quantity of mitochondria might be to control the number of mtDNA molecules available for subsequent clonal amplification and to reduce the risk of heteroplasmic transmission of mtDNA to the next generation.

Since the mtDNA bottleneck theory was raised, there are ongoing studies focusing on how and when the bottleneck happens. During early development, the primordial germ cells contain few mtDNA molecules. It has been calculated in mouse that in order to complete the genetic shift of mtDNA, there should be around 200 mtDNA copies in PGCs [107]. However, what is surprising is that absolute quantification using qRT-PCR showed there are approximate 2000 copies of mtDNA in primordial germ cell (PGC) [90], tenfold copies to achieve the observed genetic drift. Considering it is 100-fold lower than the mtDNA pool presented in the mature oocyte, it can still be called a bottleneck. Different research groups tried to discover the mechanism behind the mtDNA bottleneck theory but have failed to reach an agreement [90, 108-110]. The presence of a small amount of mtDNA at the early stages of PGCs has been concluded by two

groups [90, 109], but they have different arguments. One group explains that the low number of mtDNA molecules illustrates the mtDNA bottleneck [90], while another group argues that the bottleneck occurs through a preferential amplification of a subgroup of mtDNA [108, 110]. However, the third group proposes that the mtDNA bottleneck takes place after birth, also through a subselection of mtDNA molecules that populate the mtDNA pool of the mature oocytes [109]. How this preference happens and how the subgroup is determined are still unclear. Until now, questions related to mtDNA genetic bottleneck are not completely uncovered.

It is believed that the existence of mtDNA bottleneck is to ensure the maternal homoplasmic transmission of a single type of molecule [44], but mutations can still escape the bottleneck. Purifying selection is found to filter these escaped mutations during early embryonic development [103, 111, 112], and this selection has been proved to occur either positively or negatively [113, 114]. By tracking the evolution of mutations in mtDNA mutator mice which express a proofreading-deficient mitochondrial DNA polymerase, researchers found a rapid and strong elimination of potential deleterious mutations after backcrossing, which was a robust evidence of purifying selection [111]. And a recent study using the same mouse model shows that the purifying selection may happen post-implantation [115]. Another group also proved this selection using a mouse model introduced with a severe ND6 mutation, which was selectively excluded

during oogenesis within four generations [116]. What is unknown is the mechanism behind this purifying selection. One hypothesis is replicative advantage; the preferential replication of wildtype mtDNA with a stronger oxidative phosphorylation capacity, which is coupled to mitochondrial fitness [117]. Purifying selection could further be related to fusion and fission based mtDNA correction, which leads to selective disruption of dysfunctional mitochondria (mitophagy) [118].

1.3.5 Mitochondrial DNA replication

Mitochondrial DNA replication happens during oogenesis, resulting in a massive increase from a few hundred copies in PGC to hundreds of thousands in the mature oocyte. After fertilization, the replication remains quiescence and reinitiates at or after blastocyst stage. Over the last few decades, the mode of mtDNA replication has been argued. There are mainly 3 modes proposed which have been described in somatic cells; strand-displacement model [119], coupled leading and lagging strand model and RITOLS (RNA incorporation throughout the lagging strand) [120]. During the replication, a wide range of enzymes are characterized. The polymerase gamma (POLG), the hexameric Twinkle helicase and the mtDNA single-stranded DNA binding protein (mtSSB) constitute the minimal mtDNA replisome [121]. Other factors including mitochondrial transcription factor A (TFAM) [122], mitochondrial DNA-directed RNA polymerase (POLRMT) [123], and the Mitochondrial genome maintenance

exonuclease 1 (MGME1) [124] are found to assist mtDNA synthesis. A recent discovered eukaryotic primase-polymerase, PrimPol, is localized in both nucleus and mitochondrion, indicating its role in maintaining DNA integrity in both nDNA and mtDNA [125, 126]. Mutations in POLG and Twinkle can lead to large mtDNA deletions and further result in mitochondrial diseases [127-129]. Overexpression or knockout of TFAM in mouse result in an increase or decrease of mtDNA copy number, respectively, demonstrating the role of TFAM in direct regulation of mtDNA content [130]. PrimPol has also been shown to regulate mtDNA copy number and interact with many other mtDNA replication related proteins, including mtSSB, replication protein A (RPA, polymerase delta interacting protein 2 (PolDIP2) and Twinkle [131-133]. The pathways regulating mtDNA replication are being improved gradually, but how and why mtDNA replication does not occur until blastocyst of implantation is still unknown. As mentioned above, no mtDNA replication takes place during oocyte maturation and early embryo development. However, some factors that regulate mtDNA replication are also involved in establishing oocyte and embryo development competence. For example, Conditional knockout of TFAM in ovary results in defects in postimplantation development, but this defect may be due to decrease in mtDNA copy number in oocytes as the knockout starts from folliculogenesis [100]. Mutant in POLG leads to subfertility through inducing mtDNA mutations and decreased oocyte quality, indicating mtDNA replisome is important for oocyte competence [134, 135]. However, if other factors identified in other cells

also function in oocyte mtDNA replication or participate in the establishment of oocyte developmental competence is unknown.

1.3.6 Mitochondrial dynamics

1.3.6.1 Fission and fusion

Mitochondria are highly dynamic organelles in oocytes and embryos. They have the ability to constantly fuse (fusion) and divide (fission), in order to maintain normal function. Mitochondrial fusion is regulated by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (OPA1), which are all dynamin-related GTPase. They have different functions in regulating mitochondrial fusion. Mfn1 and Mfn2 are responsible for fusion of mitochondrial outer membranes [136-138], while OPA1 regulates the fusion of mitochondrial inner membrane. In mouse oocyte, overexpression of Mfn1 or Mfn2 results in mitochondrial aggregation, especially around nucleus during oocyte maturation, which can further disturb the spatiotemporal dynamic of the chromosomes and endoplasmic reticulum (ER) [139]. Oocyte-specific deletion of Mfn1, but not Mfn2, results in failure of oocyte growth and ovulation because of a depletion of ovarian follicular reserve [140, 141]. Mice deficient in either Mfn1 or Mfn2 die in midgestation, suggesting Mfn1 and Mfn2 mediated mitochondrial fusion is also essential for embryonic development [138]. In HeLa cells, loss of OPA1 disrupts the mitochondrial inner membrane structure and integrity, and further results in cytochrome c release and apoptosis [142], but its function in oocyte and embryos is unclear. Mitochondrial

fission is mediated by dynamin-related protein 1 (Drp1). Mitochondrion recruits Drp1 to the outer membrane to constrict itself and eventually to divide. In Drp1 KO oocytes, mitochondria are highly aggregated and multiorganelle aggregation can also be found, especially the ER [143], indicating a strong interaction between mitochondria and ER and this interaction is proved by the role of ER in defining the position of mitochondria fission site [144]. There is a hypothesis that mitochondria fusion and fission play a role of preserving mitochondria: depolarized mitochondria inactivate their ability of fusion or active fission capacity to prevent the dysfunctional mitochondria from fusing with healthy mitochondria. This hypothesis is partially proved by the study that photoactivated mitochondria showed continuous cycles of fission and fusion and daughter units with high membrane potential tended to undergo subsequent fusion, while the other with low membrane potential were degraded by autophagy [118]. During oocyte maturation and early embryo development, especially when embryonic cells start to differentiate, whether a similar quality control mechanism exists or not is unknown.

Disruption of mitochondrial fusion or fission has a profound effect on mtDNA. As an important step of mitochondria biogenesis, fusion and fission are required for maintaining the integrity of the mitochondrial genome in yeast [145]. In mammalian cells, deficiency in mitochondrial fission induces nuclear DNA damage [146] and mtDNA nucleoid clustering [147]. Silencing of Drp1 and hFis1,

which regulate mitochondrial fission, results in elevated level of mutant mtDNA in cells carrying both mutant and wildtype mtDNA [148]. Mitochondria undergo massive biogenesis during oogenesis, but it is unknown whether fission deficiency can affect mtDNA integrity or aggravate mtDNA mutation load during oogenesis and oocyte maturation.

1.3.6.2 Mitochondrial redistribution

During oocyte maturation and early embryo development, the distribution of mitochondria is stage specific, which is another feature of mitochondrial dynamics. It has been suggested that mitochondrial distribution plays an important role during oocyte maturation and embryo development [149-154]. However, the mitochondrial redistribution differs from one species to another. In mouse oocyte, Mitochondria remain distributed through the cytoplasm at GV stage and then accumulate around the first meiotic spindle, followed by dispersion across the cytoplasm at the time of polar body extrusion [155-157], while during bovine oocyte maturation, no significant change of mitochondrial redistribution was found [158]. In human oocyte, fewer mitochondria were found at the subcortical region of oocyte membranes at GV stage, but this region was filled with mitochondria after GVBD [159]. Also, no mitochondrial accumulation around MI spindle was observed and there was no marked change in mitochondrial distribution before and after first polar body extrusion [159]. During early embryo development, mitochondrial redistribution shows similar

pattern among different species, where mitochondria accumulate around the perinuclear region [151, 154, 160-162]. This kind of mitochondrial redistribution is mediated by microtubule, which can directly link to mitochondria [156, 163-165]. With a mathematical model, a recent study suggests that the polymerization of actin filaments may push mitochondria surrounding the spindle, which forms a counter force on the spindle and allows spindle to migrate to the cortex [166]. Thus, the interaction between mitochondria and cytoskeleton is important for the oocyte maturation.

1.3.6.3 Mitophagy

Mitophagy is the process that mitochondria are degraded by autophagy. When mitochondria are damaged or under stress, mitophagy occurs and promotes mitochondrial turnover and prevents accumulation of dysfunctional mitochondria which can result in degeneration of the cell. Besides of the role of selectively removing damaged mitochondria [167], mitophagy also serves as the regulator in adjusting mitochondrial quantities according to cellular metabolic needs [168]. In somatic cells, mitophagy is medicated by PTEN-induced putative kinase 1 (PINK1) and parkin proteins. PINK1 is processed by healthy mitochondria maintaining a normal membrane potential, which can be used for importing PINK1 into mitochondrial inner membrane, whereas dysfunctional mitochondria with insufficient membrane potential are not able to import PINK1 and accumulate PINK1 on the outer membrane, and these PINK1 proteins then recruit

parkin proteins to target dysfunctional mitochondria for degradation by autophagy [169, 170]. Loss function of PINK1 and parkin results in disrupted mitophagy, which leads to accumulation of damaged mitochondria and aggregation of proteins, and finally the Parkinson's disease [171, 172]. It was proposed that oocytes do not activate autophagy during oocyte maturation, while autophagy is crucial for embryo development [173-176]. By treating oocytes with Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or AntimycinA which artificially depolarizes mitochondria, no co-localization of mitochondria with autophagosomes and accumulation of PINK1 were observed and mtDNA copy number was unchanged, suggesting mitophagy was not activated in oocyte [177]. Thus, it is hypothesized that oocytes are not able to recognize and remove dysfunctional mitochondria through mitophagy. However, a recent study shows that, resveratrol (3,5,4′-trihydroxystilbene), a natural potential anti-aging polyphenolic compound, can induce mitophagy to delay postovulatory oocyte aging [178]. In resveratrol treated oocytes, mitochondria were isolated into an autophagosome, suggesting an enhanced mitophagy level. It was also demonstrated by an increased LC3 level, which is an autophagy marker [178]. This result is contrary to the previous findings. One explanation could be that CCCP induced mitochondrial depolarization is instant, which does not allow adequate time for the oocyte to respond, whereas aging caused mitochondrial defects result from long term effect, hence the oocyte has been preparing for unhealthy mitochondria for a long time and finally the mitophagy is promoted by

resveratrol. Mitophagy is confirmed to play an important role in embryos, especially the clearance of paternal mitochondria after fertilization, which occurs through autophagic degradation [179, 180]. As a quality control mechanism, it will be interesting to know if it can coordinate mitochondria with different health conditions during early development.

1.3.7 Mitochondrial heterogeneity

Mitochondrial heterogeneity can be defined as the variation of a mitochondrial property within a single cell, or at per-cell level, among same type of cells. This mitochondrial property can be mitochondrial membrane potential (MMP), mtDNA integrity, respiratory activity, redox state, uncoupling proteins and mitochondrial ROS level [156, 181-185]. It is well characterized that mitochondria are not only the main intracellular source of energy, but also important regulators in many cellular processes [186], including regulation of cellular redox state, Ca^{2+} homeostasis, cell communication, apoptosis and cell death [187-192]. Thus, inter-cellular heterogeneity of mitochondrial features can be a predictor of cell-physiological heterogeneity and cell fate. For example, mitochondrial respiratory chain functionality can influence stem cell differentiation [193] and cells with enhanced stemness have lower MMP [194]. Mitochondrial heterogeneity exists not only at inter-cell level, but also intracellularly. A growing body of evidence has shown that mitochondria at different regions within a single cell may possess different features, including

differences in morphology and organization, redox state, membrane potential and calcium transport [182-185, 195-198]. Mitochondrial subpopulations with different properties within single cell may participate in different physiological and pathological processes [183, 197] . Heterogeneity in MMP has been shown to be involved in transcript elongation rate and protein noise, and also prediction of cell cycle duration [199-201].

Mitochondrial heterogeneity also exists in single oocyte, which was first described that mitochondria with higher membrane potential are preferentially located at the cortical region, indicated by a MMP sensitive fluorescent reporter probe, 5,5′6,6′-tetra-chloro-1,1,3,3′-tetraethylbenzimidazolycarbocyanine iodide (JC-1) [150]. However, this indicator has been proved to have penetration problem in oocytes, which takes a long time to reach an equilibration across the oocyte, whereas the other MMP indicator, TMRM, does not have this problem [202]. TMRM shows that mitochondria with high membrane potential preferencially localized to the nuclear DNA [202]. But why highly-polarized mitochondria distribute around nDNA area is still unknown, and if mitochondrial heterogeneity in membrane potential contributes to oocyte maturation or early embryonic development remains to be determined. Heterogenous MMP is also found in blastocyst using JC1 as indicator, where mitochondria in the trophectoderm (TE, gives rise to the placenta) have higher membrane potential than those in the inner cell mass (ICM, gives rise to the embryo) [150]. But due

to the issue of JC1 described above, whether the high MMP found in TE is caused by the uneven distribution of the indicator needs more investigation. It is known that all the mitochondria in the blastocyst are from the oocyte due to elimination of paternal mitochondria and lack of mitochondrial biogenesis after fertilization [69, 91], but whether mitochondrial subpopulations with different membrane potential in the oocyte have different fates at blastocyst stage and if mitochondrial heterogeneity in membrane potential at blastocyst stage is required for normal post-implantation development are unclear.

1.3.8 Mitochondrial sorting

In stem cells, there is evidence showing mitochondria can be sorted dependent on their age [203]. The fates of old and young mitochondria were followed during the division of human mammary stem-like cells and an asymmetric apportioning of aged mitochondria between daughter cells was found. Daughter cells that maintained their stem cell traits received more newly generated mitochondria and fewer old ones. However, the underling mechanism which regulate the sorting is unclear. Currently there is no evidence indicating similar quality control mechanism in oocytes and embryos. Considering the unique meiotic division during oocyte maturation and cell differentiation before embryo implantation, we hypothesize that mitochondrial sorting exists in oocytes and embryos and play a role as an effective quality control mechanism. To be more specific, old or depolarized mitochondria will be sorted to polar bodies and TE cells, which do

not maintain any stem cell-like properties, and newly generated and healthy mitochondria are retained in the eggs and ICM cells, to make sure only those healthy mitochondria are inherited to the next generation. Furthermore, mtDNA mutations can be recognized by this mechanism and might also be sorted out of eggs and ICM to avoid transmission of mutant mtDNA to the offspring. It will also be interesting whether mitochondrial diseases and aging are related to dysfunction of this quality control mechanism if the hypothesis exists.

1.4 Mitochondria and ovarian aging

Female fertility declines with age, the result of the reduction of ovarian reserve and declined oocyte quality. The age-associated decline in oocyte quality has been considered to be linked to reduced mitochondrial functionality.

1.4.1 Mitochondrial dysfunction

The effects of ageing on oocyte mitochondria have been assessed through a number of aspects. MMP, the most commonly employed mitochondrial activity marker, is altered with aging in both human and mouse. More specific, MMP is negatively correlated with maternal aging in MII oocytes and preimplantation embryos from human [149]. Mouse oocytes with maternal aging showed similar negative correlation [204]. As the main function of mitochondria, ATP production is also affected, where old mouse oocytes showed reduced ATP level [204, 205]. Furthermore, disrupted ATP production can lead to meiotic spindle abnormalities at the MII stage [206], indicating aging caused spindle defects and

chromosomal misalignment in oocytes [204] may be mediated by mitochondrial dysfunction. Another important reason of the reduced fertility is attributed to aneuploidy when oocytes undergo the first meiotic division such that by age 40 around 60% of oocytes are thought to be aneuploid [207, 208], and recently a hypothesis was proposed that nuclear chromosomal abnormalities could be attributed to mitochondrial dysfunction.

1.4.2 Mitochondrial DNA abnormality

Mitochondrial DNA is also compromised in oocytes and embryos with maternal aging. The mtDNA content of oocytes from older women is significantly lower [209-211], and polar bodies from older women contain fewer mtDNA copies compared to younger women [212]. Not only in human, studies on aging models of other species also found similar results [213-215], indicating mtDNA insufficiency may be one of the reasons why aging affects oocyte quality. Considering oocytes can be dormant in ovarian for over 40 years and they are exposed to deleterious endogenous factors such as mitochondrial ROS, mtDNA integrity can also be disrupted and mtDNA mutations can gradually accumulate during this period. However, studies trying to uncover the link between mtDNA integrity and aging have drawn controversial conclusions. In some reports, oocytes with maternal aging showed more mtDNA common 4977-bp deletion [209, 216] and some unique point mutations [217], whereas others found aging had no significant influence on mtDNA integrity [218, 219]. The consequences

of reduced mtDNA and increased mutation load on oocytes of aged females is not known. To further investigate the causative role of mtDNA mutations in aging, a mutator mouse model with deficiency of POLG was developed [134, 220]. These mice harbor high levels of mtDNA point mutations and start to show a range of premature aging phenotypes after 6-7 month, indicating the involvement of mtDNA defects in decline of fertility with aging. The hypothesis is that oocyte or preimplantation embryo has the ability to sort mtDNA mutations and there is a quality control mechanism that mtDNA mutations can be excluded to those unconsidered daughter cells.

1.5 Conclusion

As the most organelle in cytoplasm, mitochondria and their own genome play a pivotal role during oocyte maturation and early embryo development. With the benefit of advanced imaging equipment and molecular techniques, researchers are able to discover more about mitochondrial function and underlying mechanisms in regulating early development, which are not fully uncovered. The relationship between oocytes and embryos competence and mitochondria function may be more complex than what we have already known. Future work can look at mitochondrial fate during oocyte maturation and early embryo development from single mitochondrion level, which can be really challenging. The role of mtDNA is also very important and can be a prime target for investigating mitochondrial function, especially with the benefit of Next

Generation Sequencing. Furthermore, aging caused decline of oocyte quality can be attributed to mitochondrial dysfunction from multiple aspects. More effort can be put on the underlying mechanism and potential clinical therapies in the future.

1.6 Aims

The overall aim of this project is to study mitochondrial function during mouse early development *in vitro*, including mitochondrial activity, mitochondrial genome and their correlation with oocyte and embryo competence and aging. Specifically, there are three sub aims.

1. To study MMP and mitochondrial heterogeneity in MMP in oocytes and their role during oocyte *in vitro* maturation.

2. To investigate possible mechanisms of how aging causes mtDNA mutations by studying some mouse model with defective mitochondrial function.

3. To study mitochondrial heterogeneity in MMP in blastocyst and the correlation between MMP and embryo competence.

Table1. mtDNA copy numbers in oocytes and preimplantation embryos among different mammalian species.

Species	Copy number								Reference
	Oocyte	Zygote	2-cell	4-cell	8-cell	16-cell	Morula	Blastocyst	
Homo Sapiens	84657								[221]
	143000								[222]
	163698								[99]
	193000								[98]
	~200000								[211]
	256000								[223]
	384251							241963	[224]
	598350								[209]
	697176				673722				[210]
							~20000-200000	~200000-950000	[225]
Mus Musculus		110500		116300	115700	116500	118500	138200	[92]
	84000								[226]
	131990								[227]
	147113								[228]
	157000	174000	172600	191600	168800				[110]
	175000								[109]
	215000								[229]
	238000								[230]
	249000		347700	196000	308600		244500	280800	[90]
	269386		270100		255129		229443	254414	[91]
								~300000	[231]
	~360000								[211]
	430000								[232]
	430000								[233]
	511765								[234]
								593367	[235]
Rattus Novegicus	110000								[236]
	147600		137018-149833		141882-164930		128995-166222	129400-166416	[95]
Sus scrofa	~40000								[237]
	138022								[238]

	147063								[97]
	239392-275132								[239]
	246984								[240]
	250457								[241]
	263191								[242]
	285000								[243]
	295671-699867								[244]
	323038-503263		564283	73561	21104	175454	131238	1254604	[93]
	347023								[245]
	400615	162324	180878					167287	[246]
	450000								[247]
	870000	580000		400000				470000	[248]
Bos taurus	134896-204173								[249]
	140733-529333								[250]
	260000								[251]
	271949							617467	[252]
	296000-380000								[253]
	329238								[254]
	361113								[255]
	373000		371000		135000	163000	180000	688000	[94]
	385332								[215]
	416559-510078								[256]
	807794								[214]
Ovis aries	242950-458600								[257]
	744633								[258]
	772896								[259]
Equus	860000								[260]
								260000-3291000	[261]

Chapter 2. Mitochondrial membrane potential and mouse oocyte competence

2.1 Introduction

During oogenesis and oocyte maturation *in vivo*, the oocyte is surrounded by cumulus cells. There is a bidirectional communication between oocyte and cumulus cells through gap junctions [262], which is crucial for the functionality of both cell types [263-265]. Since ATP generation within the oocyte relies on oxidative phosphorylation in mitochondria, metabolic support from cumulus cells through supplementation of metabolic substrates, such as pyruvate, becomes very important [55, 266-268]. Previous studies show that *in vivo* maturation or *in vitro* maturation in the presence of cumulus cells improves oocyte maturation and subsequent embryo development, as well as elevates oocyte ATP levels [18, 19, 186], which may suggest alterations in mitochondrial functions. However, there is no direct evidence showing whether *in vitro* maturation without cumulus cells has an effect on MMP or not.

MMP has been widely studied as a developmentally related factor in establishing oocyte and embryo competence [149, 150, 269-272]. These studies indirectly suggest that oocytes or embryos with high MMP have greater developmental potential. However, no direct link has been established between MMP and oocyte competence and it is not known whether oocytes with different level of MMP have

different competence and developmental potential. Specifically, can MMP at GV stage indicate oocyte developmental potential?

The MMP is established by proton pumps of the electron transport chain on the mitochondrial inner membrane. The MMP provides an electro-chemical gradient that drives ATP synthase activity. Thus, measurement of MMP is a well established means of investigating mitochondrial function. There are a number of MMP assays and most commonly used are "slow cationic dyes", including Rhodamine 123 (Rh123), $DiOC_6(3)$, Tetramethylrhodamine ethyl (TMRE) or methyl (TMRM) ester, Nonylacridine orange (NAO), Merocyanine 540, Saphranine O, JC-1 or JC-9 and many others [273].

MMP is known to influence mitochondrial and cellular behaviour in a number of ways, thus regulation of MMP is a means of maintaining a healthy cohort of mitochondria in a given cell. A high MMP is commonly associated with optimal cell function. For instance, mitochondria with lower MMP are less likely to fuse with the mitochondrial network and more likely to be removed by mitophagy [118]. Reducing the MMP with FCCP, a mitochondrial uncoupler, has been shown to decrease the rate of cell proliferation [274]. In contrast, some evidence suggests that restraining mitochondrial activity and maintaining a low MMP is also important for specific aspects of cell function. For example, T cells with low MMP exhibit decreased oxidative-stress and enhanced stemness [194], indicating that

MMP can function as an indicator of cell fate in some cell types. Further, mitochondrial subpopulations with different properties within a single cell may participate in different physiological and pathological processes [183, 197]. In neurons, heterogeneity in MMP has been shown to be involved in transcript elongation rate and protein noise, and also prediction of cell cycle duration [199-201].

Mitochondrial heterogeneity in membrane potential also exists in oocytes, whereas mitochondria with higher membrane potential are preferentially located at the cortical region, indicated by JC-1 staining [150]. But why highly-polarized mitochondria distribute in the pericortical domain is still unknown, and if mitochondrial heterogeneity in membrane potential contributes to oocyte maturation or early embryonic development remains to be determined. Heterogenous MMP is also found in the blastocyst, where JC-1 staining showed that mitochondria in the trophectoderm (TE, gives rise to the placenta) had higher membrane potential than those in the inner cell mass (ICM, gives rise to the embryo) [150]. Whether these mitochondrial subpopulations with different membrane potential arise due to sorting of oocyte mitochondria with differing membrane potential into different cell types or whether cell fate differentiation influences membrane potential is not known. Further, whether mitochondrial

heterogeneity in membrane potential at the blastocyst stage is required for normal post-implantation development are unclear.

In order to answer these questions pertaining to the role of mitochondrial function in oocyte and embryo development it is essential to have a reliable means of measuring MMP between cells and cellular sub-compartments. In this chapter, TMRM/mito-Dendra based ratiometric analysis using confocal imaging is developed for accurate measurement of MMP in oocytes. This ratiometric approach normalizes for the mass of mitochondria thereby allowing direct comparison of inter- and intra-cellular MMP. Using FCCP, I show the approach detects rapid changes in MMP in oocytes. Further, oocytes matured *in vitro* versus *in vivo*, which are known to have different developmental potential, have different levels of MMP, indicating MMP is linked to oocyte quality. As such, with this ratiometric analysis, a positive correlation between MMP and oocyte *in vitro* competence was then found. MMP is also spatially distributed in *in vitro* grown oocytes such that high MMP is found in specific sub-cellular compartments, which is not observed in *in vivo* grown oocytes and this spatial regulation of mitochondrial activity is important for oocyte *in vitro* maturation.

2.2 Materials and Methods

2.2.1 Animals

PhAM$^{loxP/loxP}$ mice (Jax mice stock No: 018385) [275] were crossed with transgenic mice that carried Gdf-9 promoter-mediated Cre recombinase which had a C57BL/6J background [276]. After multiple rounds of crossing, homozygous mutant female mice expressed a mitochondrial-specific version of Dendra2 green/red photo switchable monomeric fluorescent protein exclusively in oocytes (*PhAM*$^{loxP/loxP}$; *Gdf9*-Cre) (hereafter Dendra) were obtained. The mice were housed under controlled environmental conditions with free access to water and food. All animal experimentation was approved by Monash University Animal Experimentation Ethics Committee and was performed in accordance with Australian National Health and Medical Research Council Guidelines on Ethics in Animal Experimentation.

2.2.2 Oocyte collection and culture

Female mito-Dendra2 (hereafter Dendra) or C57BL/6 mice of 4-6 weeks old were administrated 10 IU PMSG by intraperitoneal injection. 48 hours later mice were culled by cervical dislocation and ovaries were collected into M2 medium (Sigma-Aldrich). Oocytes were released from ovaries by puncturing with a 27 gauge needle and GV stage oocytes were collected with mouth pipette (Sigma-Aldrich). Only oocytes with intact cumulus cells were collected and cumulus cells were

removed by repeated pipetting with a narrow bore pipette. Oocytes were arrested at GV stage in M2 medium containing 200 μM IBMX (Sigma-Aldrich).

For *in vitro* maturation, oocytes with or without cumulus cells were cultured in M16 medium (Sigma-Aldrich) under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air.

To collect *in vivo* matured oocytes, mice were administrated 10 IU human chorionic gonadotropin (hCG) 44-48 hours after PMSG injection. Oviducts were collected 14 h or 23 h after hCG injection and cumulus enclosed oocytes were released into M2 medium containing 300 μg/ml hyaluronidase (Sigma-Aldrich) for removal of cumulus cells.

2.2.3 TMRM labelling

Dendra or C57BL/6 oocytes at the desired stage were incubated in M2 medium containing 25 nM TMRM for 30 minutes at 37°C in the dark. Then oocytes were washed in fresh M2 medium 3 times before imaging.

2.2.4 Confocal imaging

Live oocytes labelled with TMRM were imaged at 37°C in a glass-bottomed dish using a laser-scanning confocal system (SP8, Leica). A 488 nm laser line and a 495-523 nm bandpass filter were used to image Dendra signal. A 552 nm laser line and a 563-627 nm bandpass filter were used to image TMRM signal. For

sequential scanning, the Hybrid detector (HyD) was used to acquire Dendra and TMRM signal sequentially. For simultaneous scanning, one PMT detector and one HyD were used to capture Dendra and TMRM signal simultaneously. Line average was set at 4 times for high quality images.

2.2.5 FCCP treatment

Dendra oocytes were placed in a 3 µL M2 medium drop after TMRM labelling. Then a volume of 3 µL M2 medium containing 2 µM FCCP was added into the drop to make a final concentration of 1 µM FCCP. Live cell imaging started immediately after the addition of FCCP.

2.2.6 Ratiometric analysis

Ratiometric analysis was performed using ImageJ software. Briefly, Dendra (green) and TMRM (red) channels of the confocal images were separated and converted to 32-bit images, and the background of each channel was set to Not a Number (NaN) using thresholding, which was determined visually. The same threshold was applied to other images from the same experiment. Then TMRM fluorescence value was divided by Dendra fluorescence value pixel by pixel using Image Calculation function in ImageJ. Calculated ratio image is shown by a preset lookup table in ImageJ called 16_colors, which shows higher value with warmer color.

2.2.7 Definition of ROIs for comparing MMP in oocytes at different stages.

For GV stage oocytes, a circular area 5 μm larger in diameter than the GV was defined as GV-surrounding area. The remainder of the oocyte was defined as non-GV-surrounding area. For MI stage oocytes, mitochondria in the area 10 μm larger in diameter than the spindle were defined as spindle-associated mitochondria and the reminder in the cytoplasm were non-spindle-associated mitochondria. For MII stage, the oocyte was divided into 3 parts equally along the axis crossing the central point of both the oocyte and the spindle. The cytoplasm where the spindle was located was defined as apsis area and the opposite one third was apoapsis area.

2.2.8 Statistical analysis

Data analysis was carried out using unpaired t-test, except for comparing TMRM/Dendra ratio between different regions in individual oocytes at different stages which used paired t-test. For experiments with three or more groups, Analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test was performed. Error bars on column graphs represent standard error of the mean (SEM). * represents a p value of < 0.05, ** represents a p value of < 0.01, *** represents a p value of < 0.001, **** represents a p value of < 0.0001.

2.3 Results

2.3.1 Optimization and validation of a reliable ratiometric method for measuring MMP in oocytes

2.3.1.1 Pixel-by-pixel ratiometric analysis corrects mitochondrial amount-dependent TMRM fluorescence intensity in oocyte

TMRM is a potentiometric cationic indicator that equilibrates across the mitochondrial inner membrane according to the MMP. As such, in resting cells TMRM accumulates in mitochondria, while depolarization causes loss of TMRM from the mitochondria. However, the TMRM signal intensity in confocal images reflects not only the MMP but also the mitochondrial mass in the imaging volume. As a result, inter- and intra-cellular differences in TMRM fluorescence are often simply due to the presence of more mitochondria in the region being imaged, rather than the MMP of individual mitochondria. This is particularly challenging in oocytes that have hundreds of thousands of dynamic mitochondria that are clustered in different regions of the cytoplasm. To overcome this issue and accurately measure MMP signal independent of mitochondrial mass, it is necessary to normalize TMRM fluorescence to a mitochondrial function-independent marker, for example, mitochondrially targeted Dendra.

The ability of this ratiometric approach to provide a reliable measurement independent of mitochondrial mass is shown in Figure 2.1A. A GV stage oocyte

from a Dendra mouse, which have endogenous green fluorescence localized to mitochondria, was loaded with TMRM. The mean TMRM fluorescence intensity of region b is higher than region a, but Dendra intensity is also higher (Figure 2.1B), indicating a higher density of mitochondria in this region. Here, pixel-by-pixel division of TMRM by Dendra intensities generates a ratio image that is pseudo-coloured according to the look up table (Figure 2.1C). This analysis shows that both regions have a similar TMRM/Dendra ratio, indicating similar MMP within each cluster of mitochondria (Figure 2.1C).

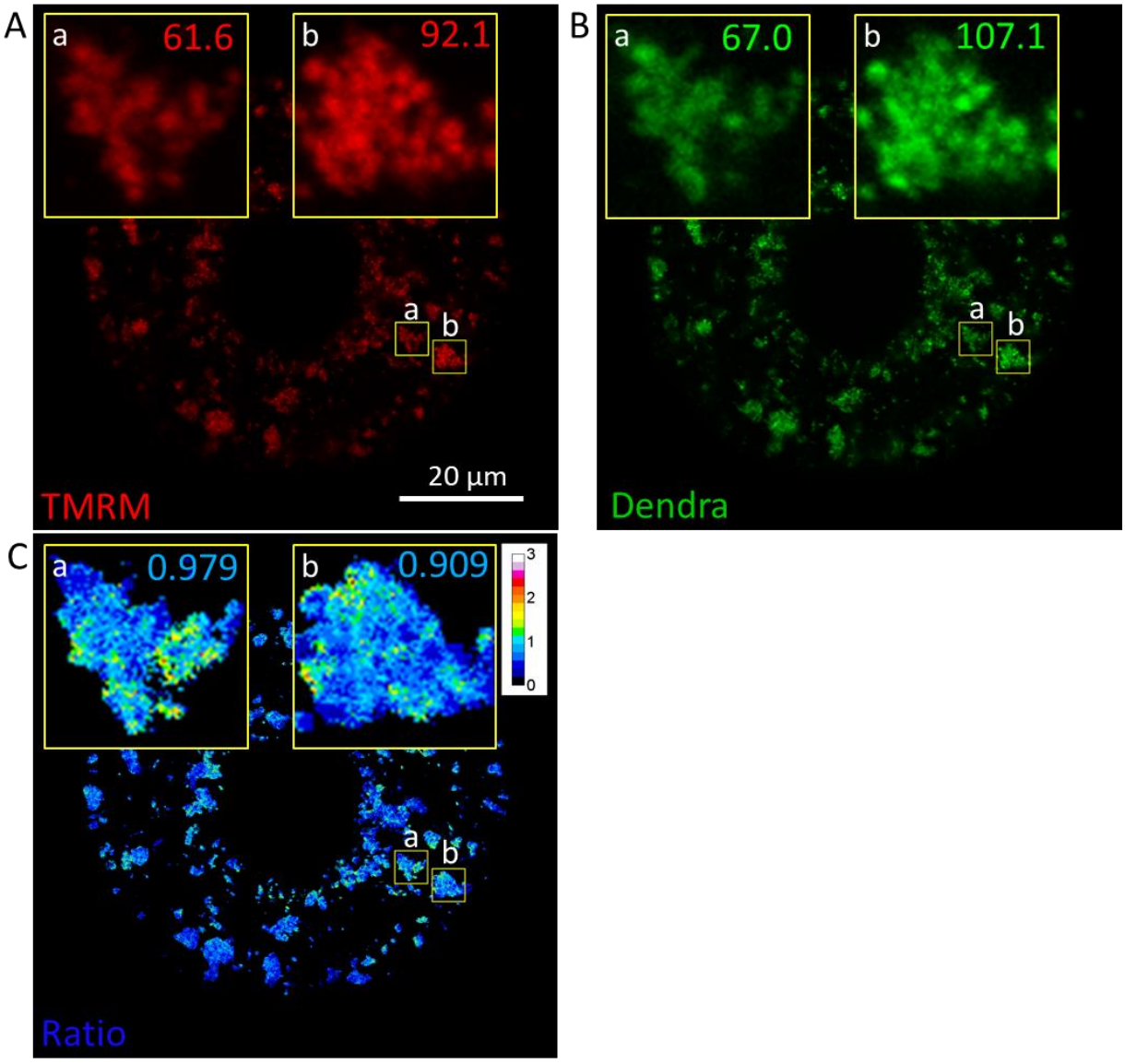

Figure 2.1. Ratiometric analysis can be utilized to normalize mitochondrial TMRM fluorescence intensity to density in oocytes. A) GV oocyte from Dendra mouse was labelled with TMRM (red). Numbers in panel a and b are corresponding mean TMRM fluorescence intensity. B) Dendra signal in the same oocyte with numbers in panel a and b corresponding to mean Dendra fluorescence intensity. C) Ratio image was produced by ratiometric analysis of TMRM and Dendra images and shown in pseudo colour. Numbers in panels a and b are corresponding mean TMRM/Dendra ratio.

2.3.1.2 Simultaneous scanning is required for accurate co-localization of TMRM and Dendra

Ratiometric analysis of MMP in GV stage oocytes shows a relatively consistent MMP across the oocyte (Figure 2.2A), as shown by TMRM/Dendra ratio. However, at the edges of mitochondrial clusters it was common to see an increased ratio signal, which suggested that MMP varied according to positions of mitochondria within the clusters. Alternatively, because TMRM signal and Dendra signal are captured sequentially, an edge effect could arise from slight movements of mitochondria or the oocyte during the time between acquisition of the two images. To eliminate the possibility that the increase in TMRM/Dendra ratio was due to mitochondrial or oocyte movement, we devised an image acquisition protocol in which both indicators were imaged simultaneously. In this imaging mode no edge effect was observed confirming that the increased ratio seen at the edges of the clusters is an artifact of slight changes in mitochondrial localisation between acquisition of the individual images rather than a difference in MMP (Figure 2.2B).

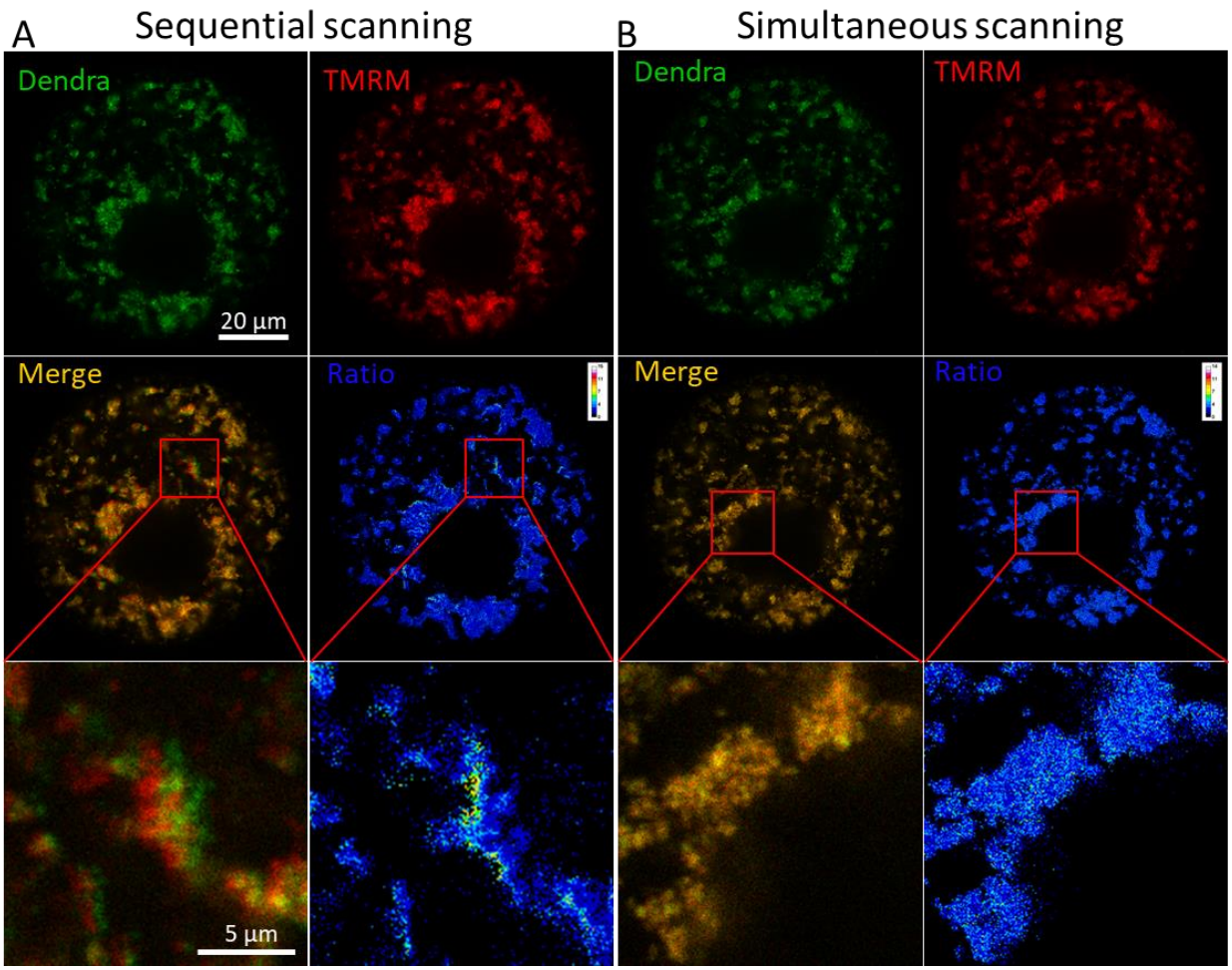

Figure 2.2. Simultaneous scanning ensures accurate co-localization of TMRM and Dendra. A) Sequential scanning results in artificial shifts in the localization of TMRM (red) and Dendra (green). GV oocyte from Dendra mouse was labelled with TMRM. Ratio image was produced by ratiometric analysis and shown in pseudo colour. B) The same oocyte as A was imaged using simultaneous scanning and images underwent ratiometric analysis. Tight colocalization was observed.

2.3.1.3 Ratiometric analysis faithfully detects FCCP-induced changes in MMP

To test if ratiometric analysis can reliably detect rapid alterations in MMP, Dendra oocytes labelled with TMRM were treated with FCCP, a mitochondrial uncoupler, which collapses the MMP. As shown in Figure 2.3A, the addition of FCCP caused a decrease in the TMRM signal while Dendra fluorescence intensity remained stable. A loss in MMP was reflected in the decreased TMRM/Dendra ratio (Figure 2.3B). Interestingly, mitochondria closest to the GV showed an initial increase in mean TMRM fluorescence intensity and TMRM/Dendra ratio within 4s of FCCP treatment (Figure 2.3C). It could be that the loss of MMP in the cortical mitochondria released TMRM that could then have been taken up by the mitochondria in the peri-nuclear region.

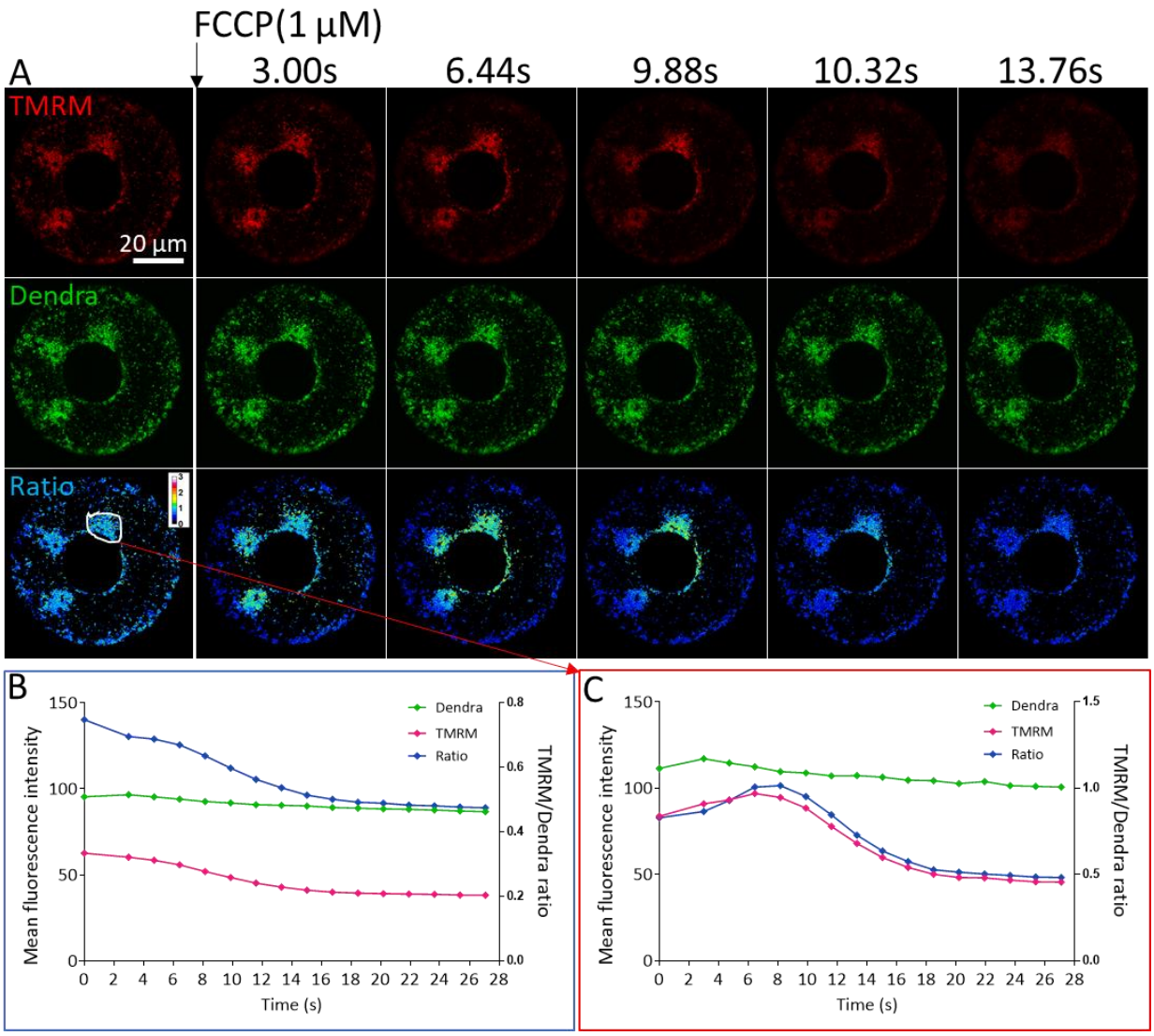

Figure 2.3. Ratiometric analysis can detect MMP decreases in oocyte. A) GV oocyte from Dendra mouse was labelled with TMRM and underwent live cell imaging immediately after FCCP treatment. Ratio image was produced by ratiometric analysis and shown in pseudo colour. B) Mean fluorescence intensity of TMRM and Dendra signal and TMRM/Dendra ratio of the whole oocyte. C) Mean fluorescence intensity of TMRM and Dendra signal and TMRM/Dendra ratio in the indicated Region of Interest.

2.3.1.4 Ratiometric analysis successfully detects postovulatory aging induced decrease in MMP

To verify that ratiometric analysis can detect physiological changes in MMP, postovulatory aged oocytes, which have previously been reported to show a decreased MMP [149, 277], were analysed. Fresh ovulated oocytes were collected from Dendra mice 14 hours after hCG injection (14h), and postovulatory-aged oocytes were collected 23 hours after hCG injection (23h). After TMRM loading and imaging, ratiometric analysis showed that aged oocytes had significantly lower MMP compared to recently ovulated oocytes (Figure 2.4). This result indicates that TMRM/Dendra ratiometric analysis is able to detect physiologically induced alterations in MMP in the oocyte.

Taken together, the above results demonstrate that TMRM/Dendra ratiometric analysis can accurately report dynamic changes in MMP as well as provide a means of comparing MMP in different populations of oocytes.

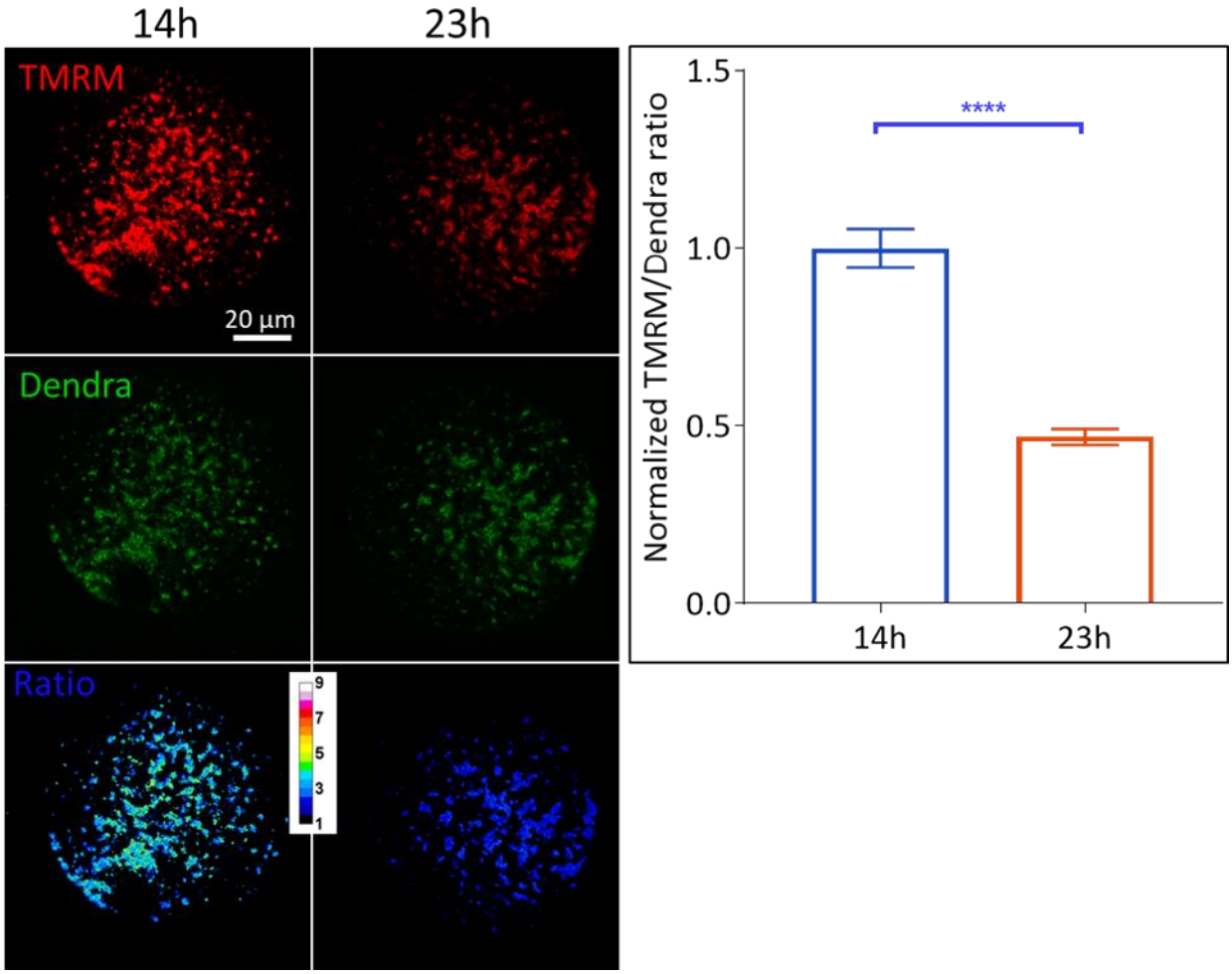

Figure 2.4. Physiological decreases in MMP can be detected by TMRM/Dendra ratiometric analysis. MII oocytes of Dendra mice collected 14h or 23h after hCG injection were labelled with TMRM. Ratio image was produced by ratiometric analysis and shown in pseudo colour. Bar graph shows mean normalized TMRM/Dendra ratio. Data shown as Mean ± SEM. N=16 at 14h and n=26 at 23h. Oocytes compiled from three replicate experiments, one mouse per treatment per experiment. Two-tailed T-test. ****, $p < 0.0001$.

2.3.2 MMP is upregulated in oocytes matured *in vitro* or in the absence of cumulus cells

2.3.2.1 *in vitro* matured oocytes have higher MMP than *in vivo* matured oocytes

Following validation of the ratiometric analysis methodology, it was first utilized to investigate if oocyte developmental competence is associated with mitochondrial activity. To do this, mitochondrial activity between *in vitro* and *in vivo* matured oocytes was compared, where the latter has been shown to have higher rates of fertilization and embryo development. For the *in vitro* maturation group, GV stage COCs were collected from Dendra mice, denuded and cultured in M2 media for 14 hours. *In vivo* matured oocytes were collected from superovulated Dendra mice 14 hours after hCG injection (*in vivo*). All oocytes from both maturation conditions underwent TMRM labelling at the same time. After TMRM/Dendra ratiometric analysis, the *in vitro* group showed a higher MMP than those matured *in vivo* (1 ± 0.04, n=12 vs. 0.63 ± 0.02, n=12, $p < 0.0001$) (Figure 2.5). This suggests that mitochondria of *in vitro* matured oocytes are more active than those of *in vivo* matured oocytes.

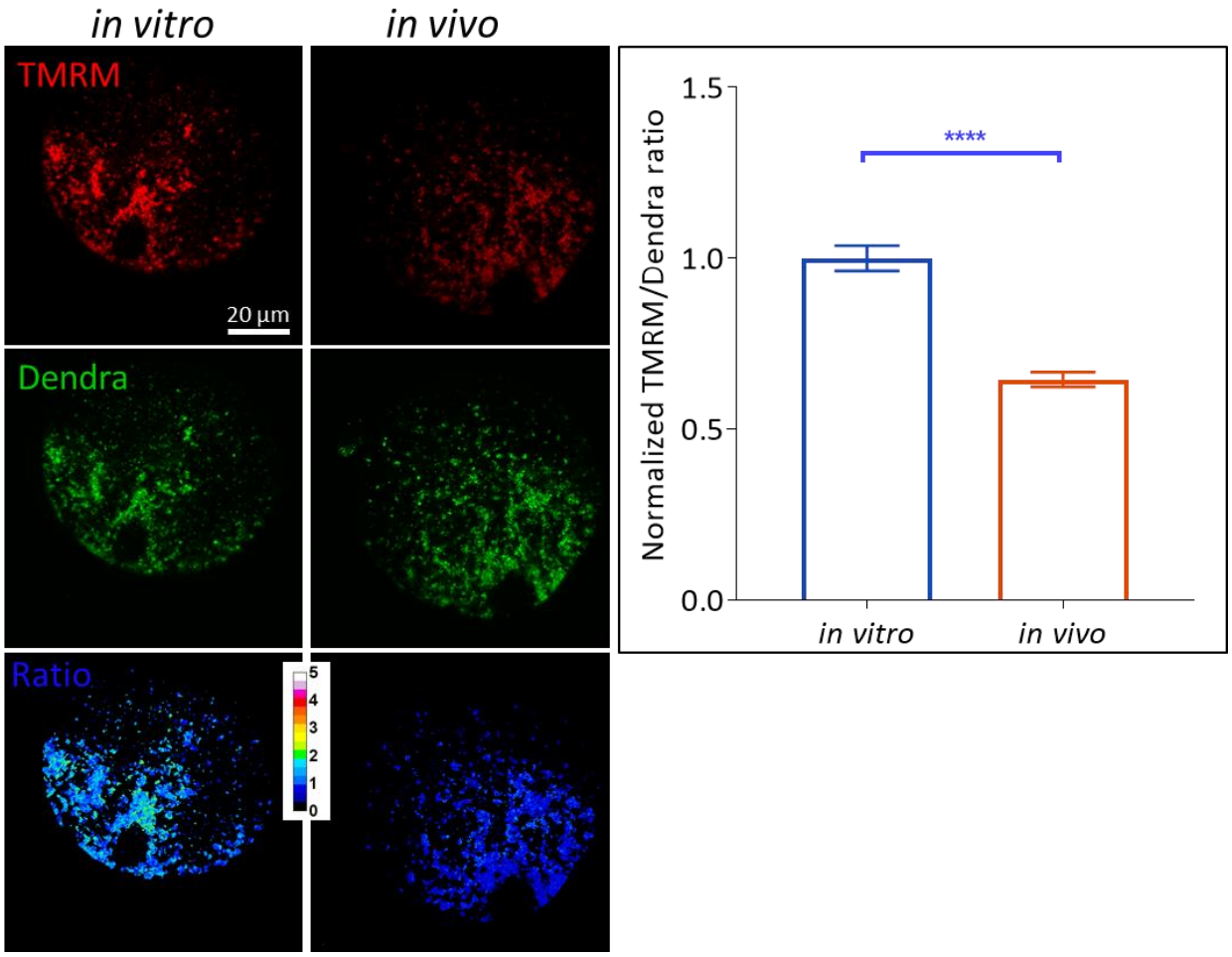

Figure 2.5. *in vitro* matured oocytes have higher MMP than *in vivo* matured oocytes. *in vitro* (n=12) and *in vivo* (n=12) matured MII oocytes of Dendra mice were labelled with TMRM. Ratio image was produced by ratiometric analysis and shown in pseudo colour. Bar graph shows mean normalized TMRM/Dendra ratio of two groups. Data shown as Mean ± SEM. One experiment. Two-tailed T-test. ****, $p < 0.0001$.

2.3.2.2 Oocytes matured without cumulus cells have upregulated MMP at MI and MII stages

Previous studies have also shown that the presence of cumulus cells during maturation improves *in vitro* maturation, fertilization and subsequent early embryo development [11]. Thus, I investigated whether the presence of cumulus cells during maturation leads to the differences in MMP. MMP of oocytes matured as denuded oocytes or COCs was compared first at the MI stage. COCs from a single Dendra mouse were collected and divided into 2 groups. Cumulus cells of one group were denuded. Both groups underwent *in vitro* culture under the same conditions. After 7.5 hours, half of the oocytes from both groups were collected for TMRM staining and comparison. Cumulus cells of the COC-matured group were removed and then oocytes of both groups were labelled with TMRM. After another 5 hours culture, when most oocytes reached MII stage, the MMP of the remaining oocytes from both groups was analysed. TMRM/Dendra ratiometric analysis showed that MI oocytes matured from denuded GV stage oocytes had a higher MMP than those grown from COCs (1 ± 0.04, n=22 vs. 0.85 ± 0.02, n=15, $p < 0.01$) (Figure 2.6A, C). Comparison of MMP at MII stage showed that MII oocytes matured from denuded GV oocytes also had significant higher MMP than those matured from COCs (1 ± 0.04, n=29 vs. 0.81 ± 0.06, n=16, $p < 0.05$) (Figure 2.6B, C). These data suggest, similar to the *in vivo* matured oocytes, that

maturation conditions that improve oocyte quality result in mature MII stage oocytes with lower MMP, and MMP of oocytes matured from denuded GV oocytes is upregulated by the MI stage compared to oocytes cultured with cumulus cells.

In all, results in this section show that oocytes cultured *in vitro* without cumulus cells have upregulated mitochondrial activity compared to those grown *in vivo* or with cumulus cells.

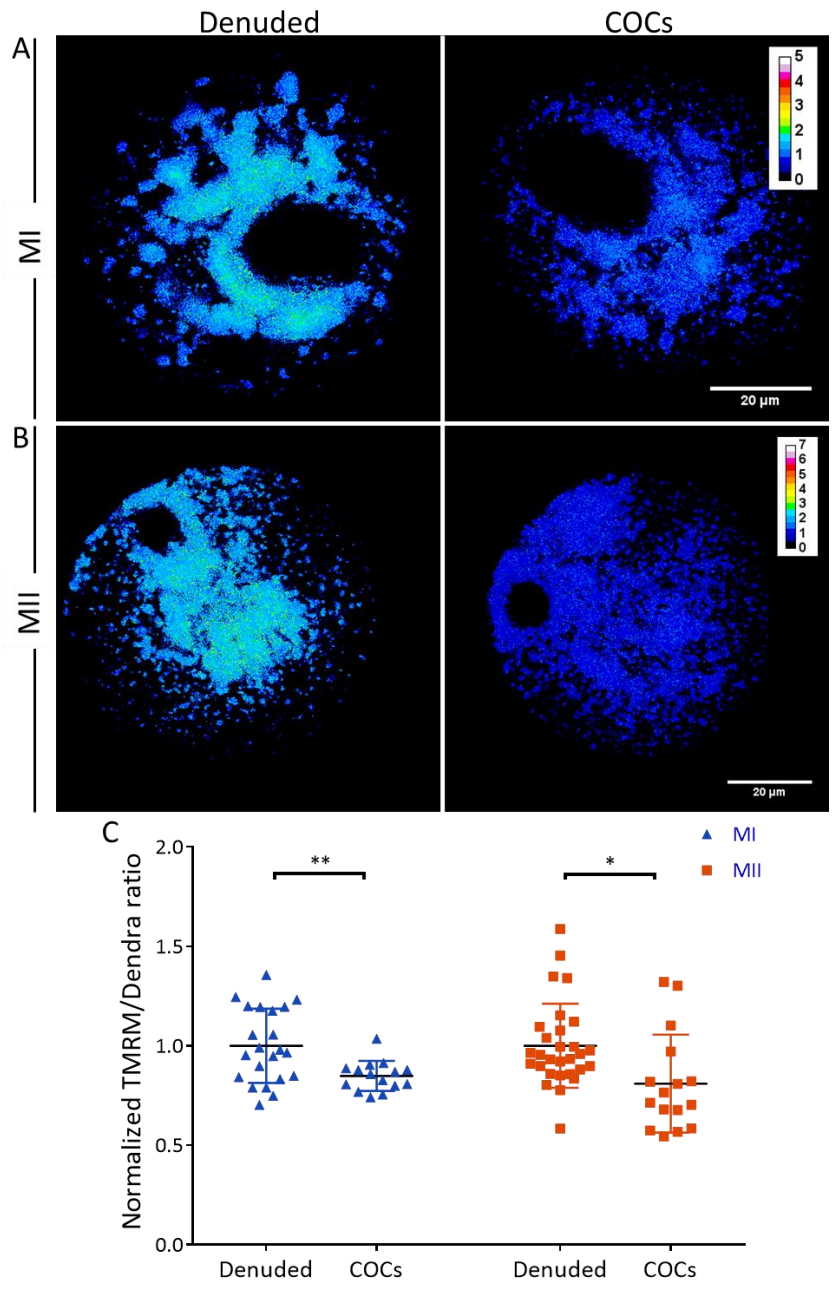

Figure 2.6. Oocytes grown without cumulus cells (denuded) have upregulated MMP at MI and MII stage. A) TMRM/Dendra ratio image of MI oocytes grown from denuded GV oocyte or COCs. B) TMRM/Dendra ratio image of MII oocytes matured from denuded GV oocyte or COCs. C) Normalized TMRM/Dendra ratio of MI or MII oocytes grown from denuded GV oocytes or COCs. Oocytes compiled from three replicate experiments. One mouse per experiment. Two-tailed T-test. **, $p < 0.01$; *, $p < 0.05$

2.3.3 The ability to upregulate MMP in association with sorting at MI is associated with improved maturation

2.3.3.1 The ability of oocytes to complete maturation *in vitro* correlates with a higher MMP at the GV and MI stages

I next sought to determine whether high mitochondrial activity contributes to oocyte maturation *in vitro*. During *in vitro* maturation, oocytes either complete maturation and progress to MII stage (typically 70-80%) or arrest at MI stage (20-30%). The reasons underlying this MI arrest have been linked to abnormalities in cell cycle control [278], but the possibility that mitochondrial function may contribute has not been explored. Comparison of MMP between denuded oocytes and cumulus-enclosed oocytes suggests that mitochondria from oocytes that lack the support from cumulus cells are more active. To investigate whether high mitochondrial activity contributes to oocyte maturation *in vitro*, MMP was measured in oocytes and correlated with their subsequent maturation. GV oocytes from individual Dendra mice were loaded with TMRM and a ratiometric image was acquired prior to being transferred to the incubator in individual drops of culture medium. After 14 hours of *in vitro* culture maturation stage was recorded and related back to the original ratiometric image of MMP. The majority of oocytes progressed to MII stage (79/117, 67.52%) and some were arrested at MI stage (38/117, 32.48%). As shown in Figure 2.7A, B, oocytes reaching the mature MII stage have, on average, a higher MMP at the GV stage (1.11 ± 0.03, n=79)

than those oocytes that arrest at MI stage (0.80 ± 0.03, n=38) (Figure 2.7C). These results suggest that oocyte capacity for complete *in vitro* maturation to MII is associated with higher MMP at the GV stage.

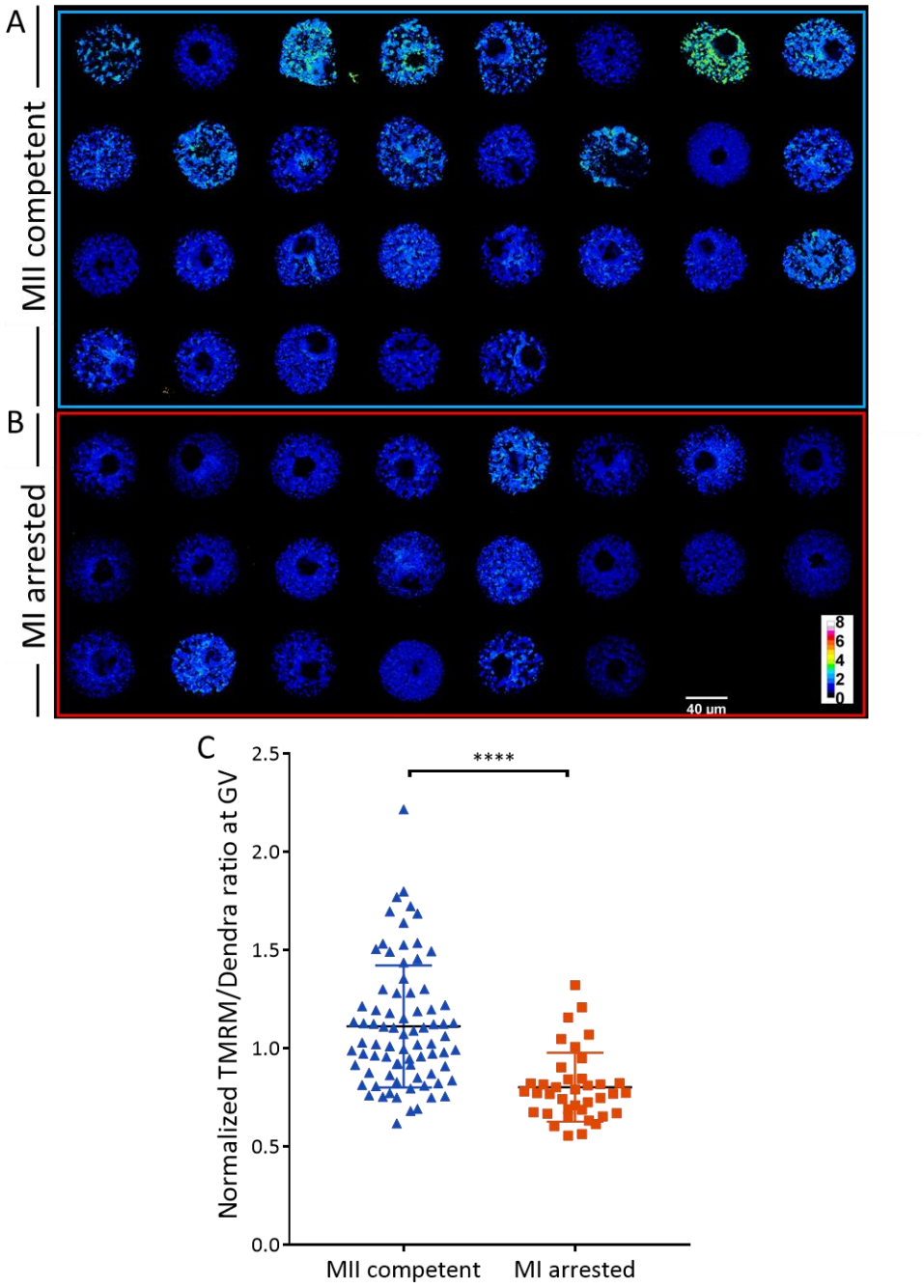

Figure 2.7. MMP at GV stage is related to oocyte competence *in vitro*. A) Ratio images of GV oocytes which subsequently reached MII stage after 14 h culture *in vitro*. B) Ratio images of GV oocytes which were arrested at MI stage after 14 h culture *in vitro*. C) Oocytes matured (n=79) or arrested at MI stage (n=38) after 14 h culture and their corresponding TMRM/Dendra ratio. Three replicates. One mouse per experiment. Two-tailed T-test. ****, $p < 0.0001$.

Whether MMP at MI stage correlates with oocyte developmental competence *in vitro* was also investigated. During *in vitro* maturation, mouse oocytes normally reach MI stage at ~8h and proceed to anaphase I at ~9 h. To measure MMP at MI stage, GV oocytes were cultured *in vitro* for 7.5 h and then loaded with TMRM for 30 minutes, after which oocytes were placed into individual drops of culture medium and a ratiometric image of each oocyte was acquired. Oocytes then underwent an additional 6 h culture *in vitro* at which time maturation stage was recorded and related back to the ratiometric image at MI stage. As expected, the majority of oocytes reached MII stage (89/139, 64.03%) and some were arrested at MI stage (50/139, 35.97%). As shown in Figure 2.8A, B, oocytes progressing to MII stage have, on average, higher MMP at the MI stage (1 ± 0.02, n=89) than those arrested at MI stage (0.68 ± 0.03, n=50) (Figure 2.8C). These results indicate that oocytes with the ability to maintain a high mitochondrial activity have greater potential to proceed to maturation.

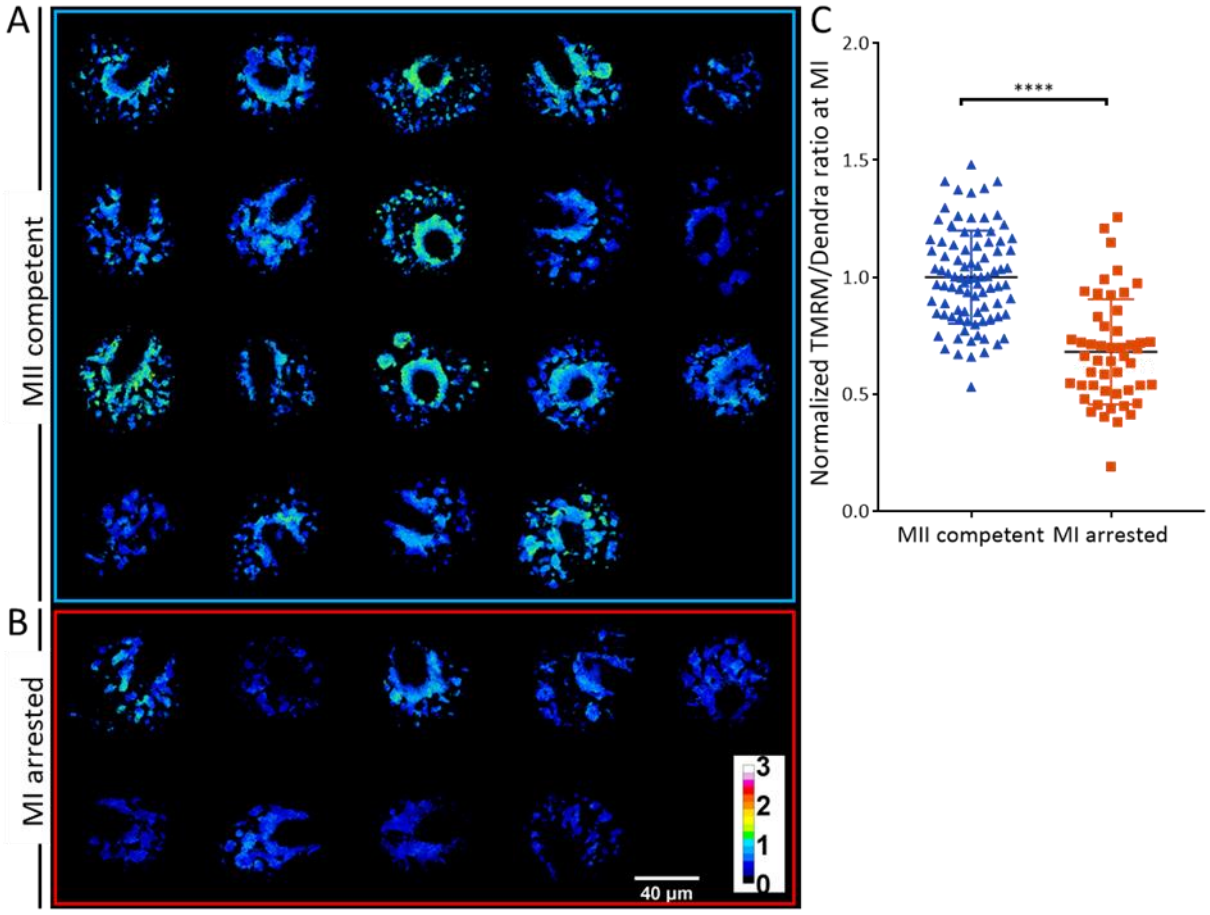

Figure 2.8. Oocyte competence *in vitro* is associated with upregulated MMP at MI stage. A) Ratio images of MI oocytes which reached MII stage after 6 h *in vitro* culture. B) Ratio images of MI oocytes which were arrested at MI stage after 6 h culture *in vitro*. C) Oocytes matured (n=89) or arrested at MI stage (n=50) after 6 h culture and their corresponding TMRM/Dendra ratio. Oocytes compiled from three replicate experiments. One mouse per experiment. Two-tailed T-test. ****, $p < 0.0001$.

2.3.3.2 Mitochondrial accumulation around MI spindle correlates with oocyte competence *in vitro*

In each of the previous experiments, it was observed that mitochondria accumulated around the MI spindle during *in vitro* maturation, consistent with previous findings [155, 156].To see if this mitochondrial reorganization also happens *in vivo*, oocytes from Dendra mice were collected 8 h after hCG administration, when most oocytes are at MI stage. For comparison, GV oocytes were collected from another Dendra mouse and cultured *in vitro* for 8 h. Surprisingly, mitochondrial accumulation around spindle was not observed in *in vivo* grown MI oocytes (Figure 2.9A) and mitochondria were generally observed dispersed throughout the cytoplasm. Quantification of the percentage of spindle-associated mitochondria showed that 57% of mitochondria were around the spindle in MI oocytes matured *in vitro*, whereas only 34% of the mitochondria were found near the spindle in MI oocytes matured *in vivo* (Figure 2.9B). These data suggest that the *in vitro* environment alters not only MMP but also mitochondrial redistribution during *in vitro* maturation.

To investigate whether the aggregation of mitochondria around the MI spindle is an important feature for completion of maturation to MII *in vitro*, spindle-associated mitochondria were measured at the MI stage and correlated with subsequent MII maturation. Dendra oocytes at GV stage underwent *in vitro*

cultured in individual drops of medium, and after 8 h, each oocyte was imaged and the percentage of spindle-associated mitochondria was calculated. oocytes were then cultured in the same drop for another 6 h at which time maturation stage was recorded and related back to the ratiometric image at MI stage. Most oocytes reached MII stage (89/139, 64.03%) and some were arrested at MI stage (50/139, 35.97%). As shown in Figure 2.10A, B, oocytes that complete maturation showed greater mitochondrial accumulation around the spindle at MI stage, compared to those arrested at MI stage which showed less accumulation and more dispersed mitochondria through the cytoplasm. Quantitation of the proportion of mitochondria around the spindle demonstrated that, where on average MII competent oocytes have larger proportion of spindle-associated mitochondria ($52.26 \pm 1.29\%$, n=89) than those arrested at MI stage ($40.19 \pm 1.86\%$, n=50) ($p < 0.0001$) (Figure 2.10C). These results suggest that oocyte *in vitro* competence is associated with a mitochondrial aggregation around the MI spindle.

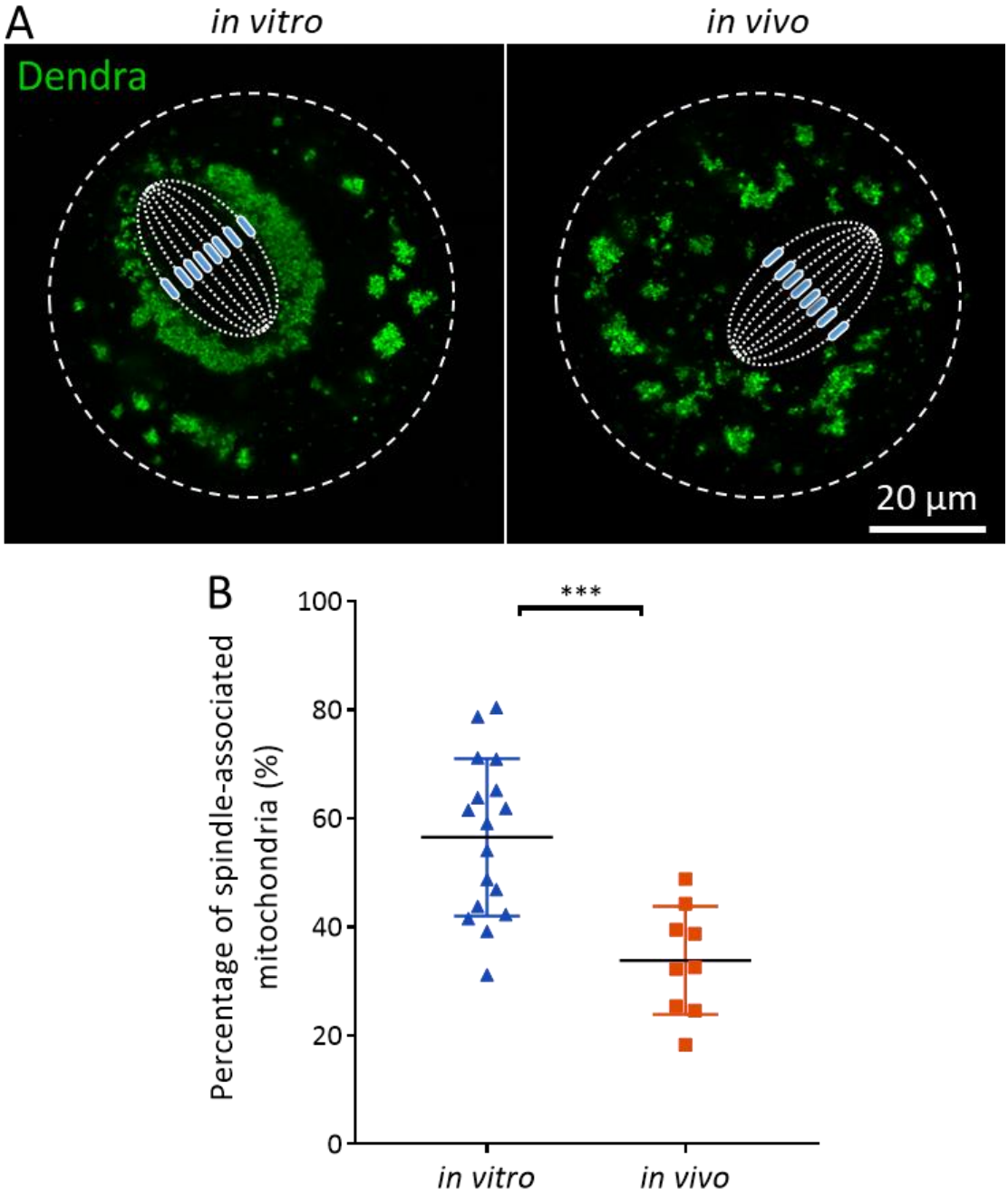

Figure 2.9. *In vivo* grown MI oocytes do not show mitochondrial accumulation around spindle. A) Mitochondrial distribution of MI oocyte derived from *in vitro* culture or *in vivo* growth. B) Percentage of mitochondria around spindle area of Dendra MI oocytes grown *in vitro* or *in vivo*. Oocytes are from single mouse per treatment group. One experiment. Two-tailed T-test. ***, $p < 0.001$.

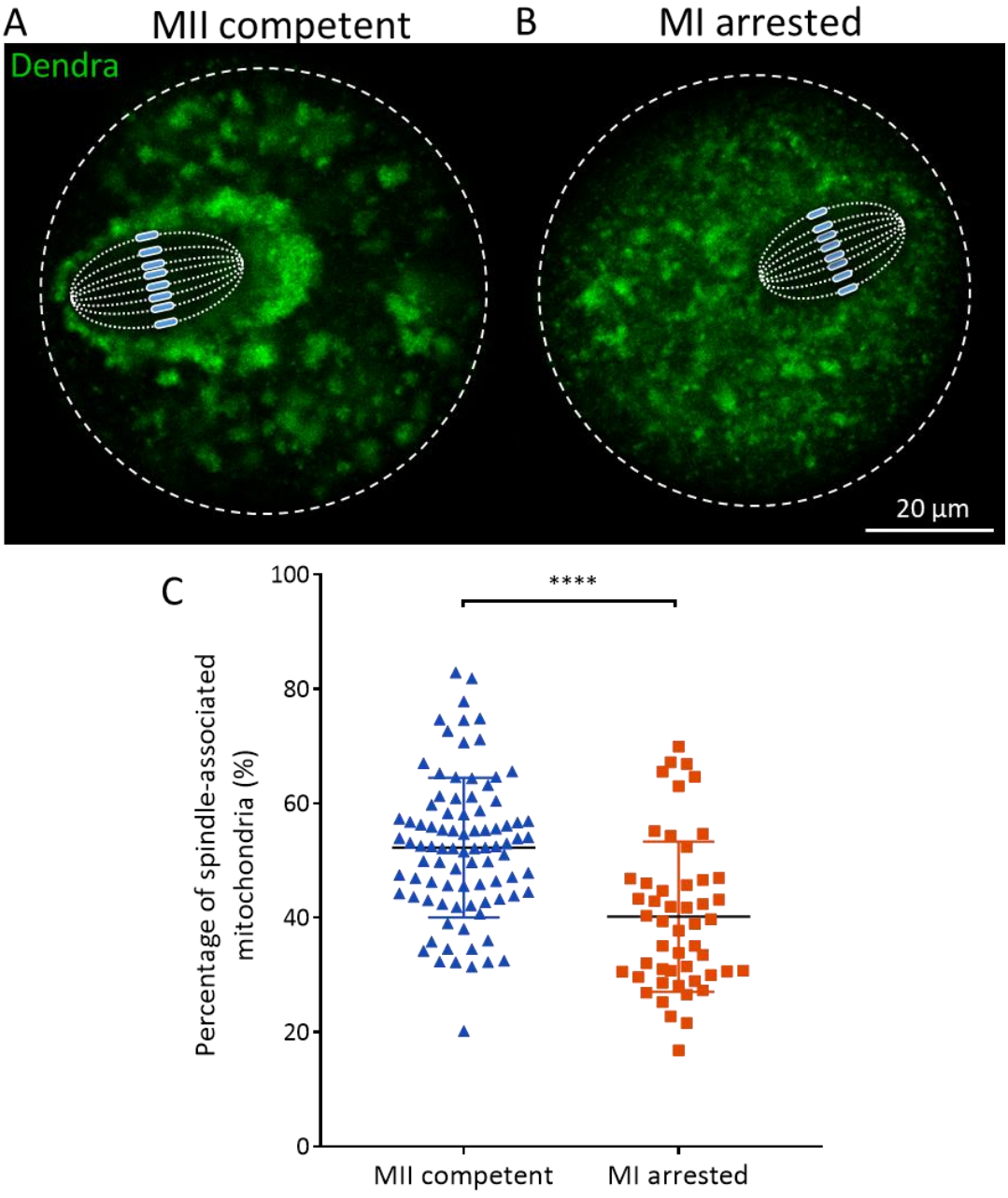

Figure 2.10. Oocyte competence *in vitro* is associated with larger proportion of spindle-associated mitochondria. A) Mitochondrial distribution of MI oocytes which reached MII stage after 6 h culture *in vitro*. B) Mitochondrial distribution of MI oocytes which were arrested at MI stage after 6 h culture *in vitro*. C) Oocytes matured to MII (n=79) or arrested at MI stage (n=38) after 14 h culture and their corresponding percentage of spindle-associated mitochondria. Three replicates. Two-tailed T-test. ****, $p < 0.0001$.

2.3.3.3 Spindle associated mitochondria have upregulated membrane potential

To investigate differences in activity between spindle-associated mitochondria and cytoplasmic mitochondria, MMP was compared between these two groups using ratiometric analysis. After *in vitro* culture of denuded GV stage oocytes for 8h, oocytes reached MI stage, and as expected, most mitochondria were accumulated around the spindle. Ratiometric analysis showed that spindle-associated mitochondria had higher membrane potential than cytoplasmic mitochondria (1 vs. 0.78 ± 0.02, n=32, $p < 0.0001$) (Figure 2.11A). Similar results were also found between GV-associated mitochondria and cytoplasmic mitochondria at GV stage (1 vs. 0.92 ± 0.02, n=28, $p < 0.001$) (Figure 2.11B), and mitochondria around the MII spindle area and those at apoapsis area (1 vs. 0.94 ± 0.02, n=16, $p < 0.01$) (Figure 2.11C). Although the biological relevance of the small differences seen at the GV and MII stages is uncertain, this data indicates the intriguing possibility that at mitochondrial activity may be spatially regulated in oocytes.

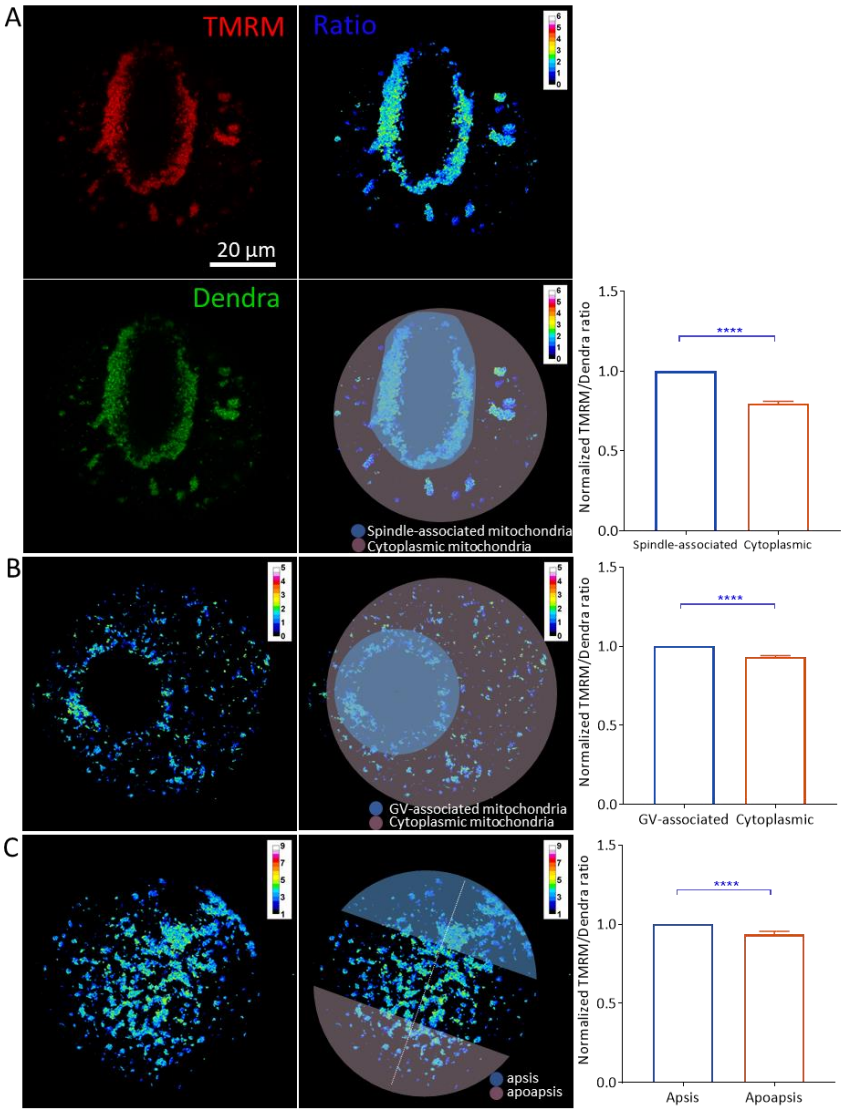

Figure 2.11. Mitochondria around nuclear DNA area have upregulated membrane potential. A) Mitochondria around the MI spindle have higher membrane potential. Bar graph shows mean normalized TMRM/Dendra ratio of Spindle-associated region (light blue) and cytoplasmic region (pink), as shown in the image. n = 28. B) GV-associated mitochondria have higher membrane potential. Bar graph shows mean normalized TMRM/Dendra ratio of GV-associated region (light blue) and cytoplasmic region (pink), as shown in the image. n = 32. C) Mitochondria around MII spindle area have higher membrane potential than those at apoapsis area. Bar graph shows mean normalized TMRM/Dendra ratio of apsis region (light blue) and apoapsis region(pink), as shown in the image above. n = 16. All data shown as Mean ± SEM. Oocytes compiled from three replicate experiments. Two-tailed T-test. ****, $p < 0.0001$.

2.3.3.4 Oocyte competence *in vitro* is related with MMP based mitochondrial sorting at MI stage

To investigate if MMP based mitochondrial sorting at MI stage during *in vitro* maturation is important for oocyte development, ratiometric assessment of specific mitochondrial localization in MI oocytes was related to subsequent maturation. GV oocytes were cultured *in vitro* for 7.5 h and then loaded with TMRM for 30 minutes, after which oocytes were placed into individual drops of culture medium and a ratiometric image of each oocyte was acquired. Oocytes underwent an additional 5 h culture *in vitro* at which time maturation stage was recorded and related back to ratiometric image at MI stage. As shown in Figure 2.12A, oocytes that reached MII stage and oocytes that failed to mature had accumulations of mitochondria around the spindle at MI stage. However, only those oocytes that completed maturation showed MMP based localisation of mitochondria, where mitochondria around the spindle had significantly higher MMP than cytoplasmic mitochondria (1 vs 0.88 ± 0.01, $p < 0.01$, n=3) (Figure 2.12B). Oocytes that were arrested at MI stage did not show the same pattern, specifically, MMP was generally lower and there was no significant difference in MMP between spindle-associated mitochondria and cytoplasmic mitochondria (0.68 ± 0.04 vs 0.66 ± 0.04, n=3) (Figure 2.12B). These results suggest that oocyte mitochondria may undergo

an MMP-based sorting during maturation and that an increase in MMP around MI spindle is essential for the establishment of oocyte competence *in vitro*.

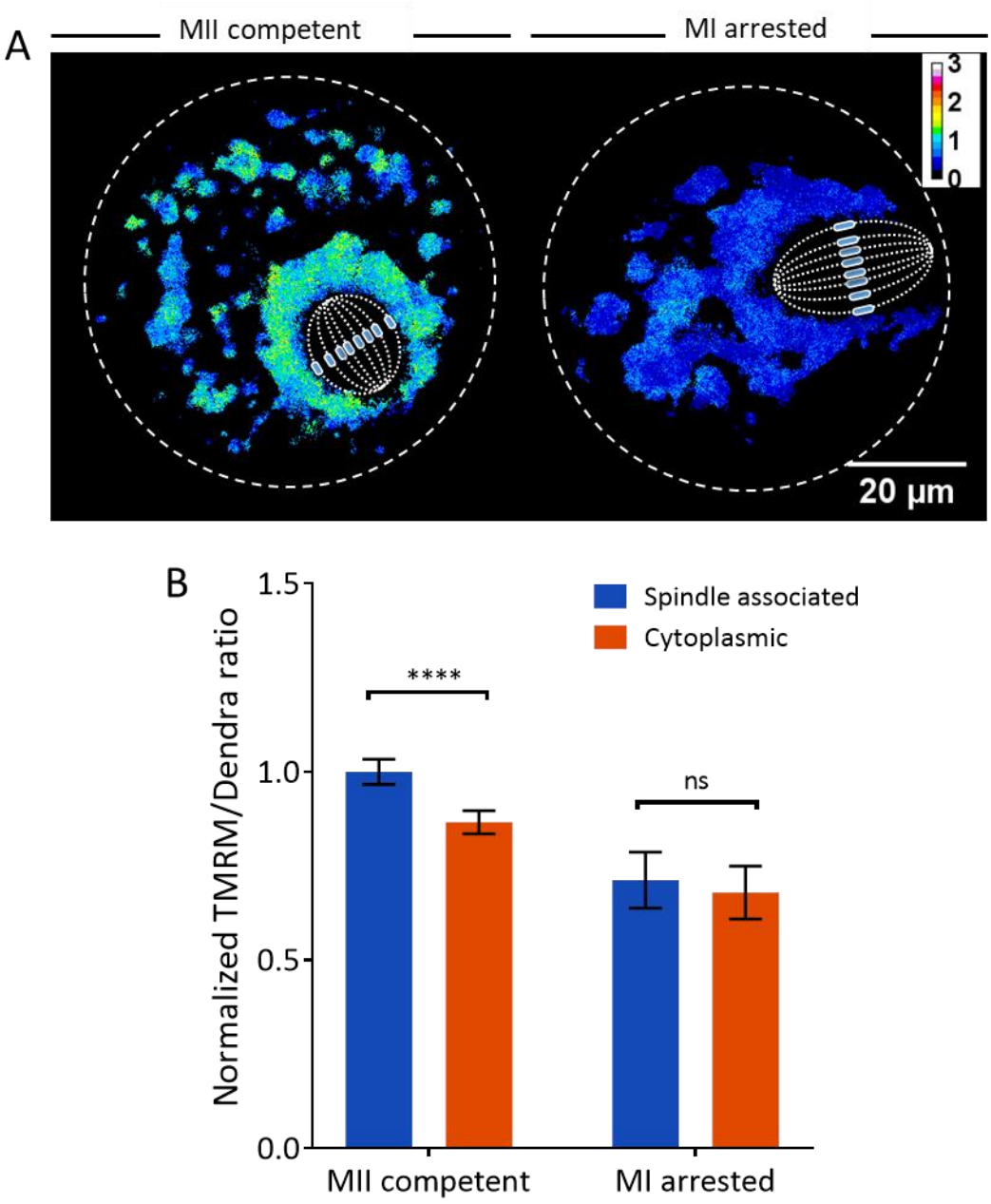

Figure 2.12. Oocyte competence *in vitro* is related to MMP based mitochondrial localisation at MI stage. A) Ratio images of MI oocytes (8 h after *in vitro* culture) which reached MII stage or were arrested at MI stage after additional 6 h culture *in vitro*. B) TMRM/Dendra ratio of spindle-associated or cytoplasmic mitochondria of MI oocytes (8 h after *in vitro* culture) which reached MII stage (n=89) or were arrested at MI stage (n=50) after additional 6 h culture *in vitro*. Three replicates. Two-tailed T-test. **, $p < 0.01$.

2.4 Discussion

Results in this chapter describe a method that can precisely measure mitochondrial heterogeneity in membrane potential at both the intra- and inter-oocyte level. This method can detect dynamic alterations of MMP induced by treatment with a mitochondrial uncoupler and MMP differences caused by different physiological conditions. With this method, I found that *in vitro* matured oocytes have higher MMP than *in vivo* matured oocytes, which is at least in part appears to be caused by the absence of cumulus cells. This indicates that oocyte mitochondria are highly sensitive and adaptive to *in vitro* environments, and may occur due to differences in metabolites, oxygen, temperature or possibly as part of a stress response. There was a consistent relationship between MMP at GV and MI and oocyte developmental competence *in vitro,* with high mitochondrial activity associated with improved rates of maturation. Mitochondrial distribution and spatial regulation of mitochondrial activity are also important for the establishment of oocyte *in vitro* competence, which correlates with larger proportion of spindle-associated mitochondria and enhanced mitochondrial activity around MI spindle.

There is great interest in improved technologies to culture oocytes *in vitro* for clinical therapeutics and animal applications. Although *in vitro* culture is able to produce matured oocytes for fertilization and subsequent embryo development, it cannot completely simulate *in vivo* environment, which makes *in vitro* matured

oocytes less robust than those matured *in vivo* [18]. However, the alterations that occur during *in vitro* maturation that cause this difference are unclear. To investigate this, I started by comparing mitochondrial activity between *in vitro* and *in vivo* matured oocytes. *In vivo* matured oocytes have significantly lower MMP than *in vitro* matured oocytes (Figure 2.13). Because the *in vitro* maturation occurs in the absence of cumulus cells, this difference in MMP may be due to loss of support from cumulus cells. Thus, I further compared MMP between oocytes matured with or without cumulus cells and, as hypothesized, found those matured with cumulus cells also have significantly lower MMP (Figure 2.13), suggesting that communication between the oocyte and cumulus cell does affect oocyte mitochondrial activity. This decrease in MMP suggests that conditions that improve oocyte quality (*in vivo* maturation, presence of cumulus cells) may in fact lead to a counterintuitive decrease in ATP level. However, previous studies have reported a higher ATP level in *in vitro* matured cumulus-enclosed oocytes compared to denuded oocytes [19]. One explanation may lie in the fact that ATP levels are reduced after blocking gap junctions between the oocyte and cumulus cells [19], suggesting that cumulus cells play an important role in supplying pyruvate or ATP directly to the oocyte. This additional metabolic support may reduce the need for oocyte mitochondria to generate ATP and thereby maintain a lower MMP, and at the same time explain the high MMP found in denuded oocytes.

Besides, this low MMP state indicates that mitochondria in oocytes matured with cumulus cells are in a relatively quiet status and this quiescence may play an important part in preparing oocytes for subsequent development. This idea is consistent with the “quiet embryo hypothesis”. This hypothesis points out that a "quiet" rather than "active" metabolism is best for optimal development of early mammalian embryos [279].

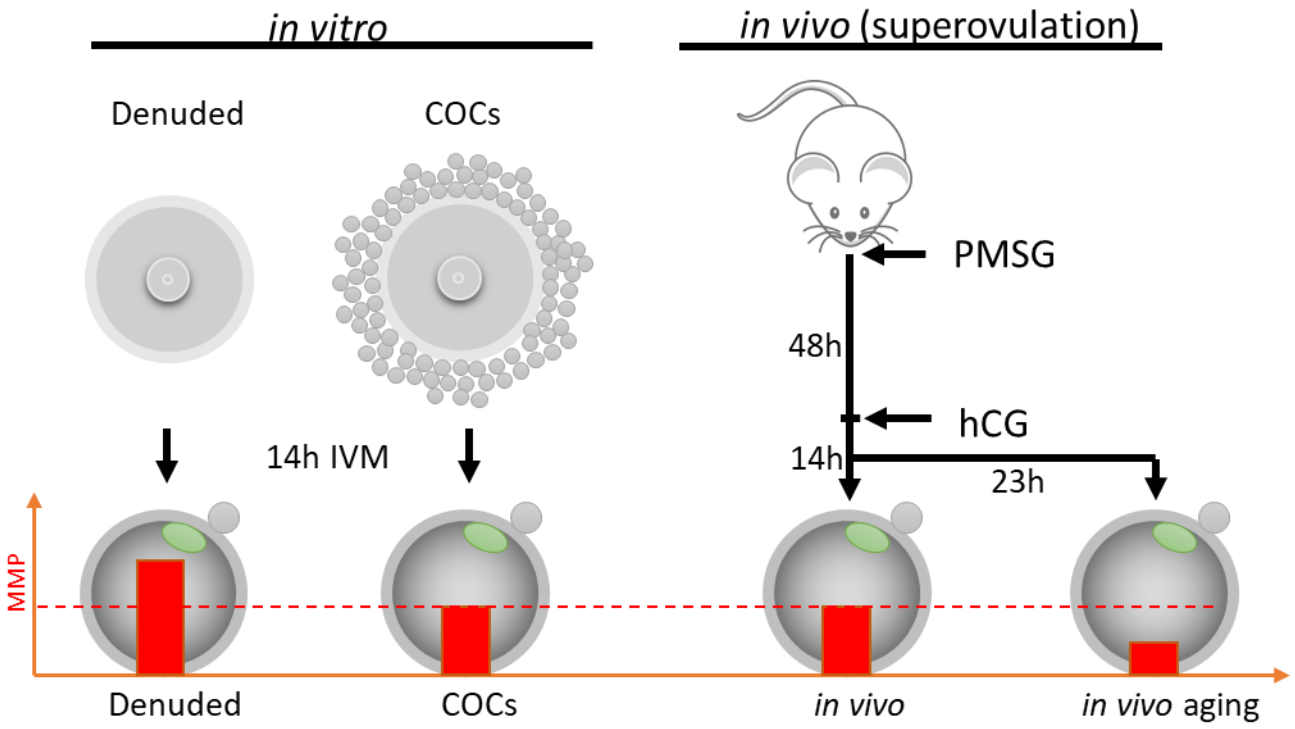

Figure 2.13. Diagram showing MMP of MII oocytes mature from different conditions.

Mitochondrial function is tightly related to oocyte quality [280]. However, there is no evidence showing the direct relation between MMP and oocyte *in vitro*

competence. Indirect evidence has shown that decreased MMP could be one of the reasons why aged oocytes have diminished quality [281]. Here, through tracing oocyte development *in vitro* after measurement of mitochondrial activity at the GV or MI stage, I found that oocytes with high MMP at GV or MI stage tended to have greater maturation potential. This result directly shows that MMP is linked to oocyte competence during *in vitro* maturation. A similar relationship has been reported between MMP at 2-cell stage and embryo developmental competence, where individual mice that have ≥ 80% blastocyst rate have significantly higher MMP at 2-cell stage than those with < 80% blastocyst rate [272]. This suggests mitochondria in oocytes and embryos need to maintain a relatively high membrane potential to fulfill the ATP demand of intracellular processes during subsequent *in vitro* development. Interestingly, this result is contradictory with the above "quiet hypothesis", where the more competent oocytes matured in vivo shows lower MMP. Comparing to in vivo matured oocytes, oocytes grown in vitro without CCs need to adapt themselves to the in vitro condition and generate ATP on their own to meet the energy requirement for intracellular events, therefore, during in vitro situation, their mitochondrial activity becomes important and the higher their mitochondrial activity is, the more competent they are.

While comparing MMP at MI stage, it was noticed that the majority of mitochondria accumulated around the MI spindle, which is consistent with

previous findings [155, 156]. Surprisingly, no mitochondrial accumulation around the spindle was found in *in vivo* matured MI oocytes, suggesting this specific mitochondrial distribution seen in *in vitro* matured oocytes may be important for oocyte *in vitro* competence. Comparison of the proportion of mitochondria around the MI spindle between MII competent and MI arrested oocytes indicates that aggregation of mitochondria around the spindle promotes progression to MII. This relationship may reflect the high ATP demand of events such as spindle formation and migration and that during *in vitro* conditions, mitochondria are needed closer to the source of consumption. Alternatively, because mitochondrial trafficking is an active process driven by ATP consuming motor proteins [282], the increased aggregation may reflect a 'healthier' oocyte which is also more likely to undergo maturation *in vitro*. Further work is needed to work out the nature of the MI arrest that is seen when mitochondria fail to aggregate. Possibilities include spindle defects that cause MI arrest due to the Spindle Assembly Checkpoint, failure of spindle migration, or an uncoupling of nuclear and cytoplasmic events necessary for successful completion of MI.

MMP can be indicative of oocyte *in vitro* competence but considering the large number of mitochondria in a single oocyte, it is important to understand whether mitochondrial subpopulations exist in relation to their functionality. Mitochondrial heterogeneity in membrane potential within single oocytes was first reported in

2002 [283] using JC1 as the MMP indicator. This study shows that the J-aggregate indicative of high MMP is found mainly in the pericortical region of GV and MII stage oocytes. Subsequent studies with JC1 consistently reported this increased peri-cortical MMP in oocytes and preimplantation embryos [150] such that it has become widely accepted. It has been suggested that the increase may reflect a higher oxygen concentration at the cortex while at the MII stage it may reflect a specific metabolic requirement for regulation of Ca^{2+} oscillations at fertilization.

The data presented above, however, using a more robust potentiometric dye, TMRM, shows no evidence for increased MMP in the peri-cortical regions. In contrast, we find a small increase of 5-10% in MMP being apparent around the GV or MII spindle while in mitochondria surrounding the MI spindle the data show a marked increase in MMP of approximately 20% over peripherally located mitochondria. The difference between these two staining patterns likely reflects the behavior of JC1 in cells. It is recognized as being slowly permeant in other cell types [284] and the distribution of J-aggregates is time dependent, requiring more than 2 hours to approach equilibration [202]. The ratiometric approach I have developed using TMRM and Dendra is not subject to such equilibration issues or the non-linearity associated with J-aggregate formation, and as such provides a more reliable approach to reporting spatial heterogeneity in MMP within cells.

Our finding in MI stage oocytes that the MMP in the vicinity of the spindle is higher than in peripherally located mitochondria indicates that increased ATP production may be generated in this region, perhaps in response to the metabolic demands of spindle formation [285]. This increase in MMP around the MI spindle has also been shown to be correlated with the ability of oocytes to complete *in vitro* maturation. A previous study shows that insufficient mitochondria-derived ATP caused by mitochondrial uncouplers can induce disruption of mouse MII spindles [206], but whether this physiologically low mitochondrial activity around MI spindle can lead to deficit of ATP and further impairs MI spindle formation is unknown. Besides, failure of maturation may be attributed to unsuccessful cytokinesis. It has been reported that mitochondria enrich at the cleavage furrow during somatic cell division via a microtubule-mediated mechanism to fuel cytokinesis [286-288]. Similar mitochondrial accumulation has been found around the contractile ring in oocytes during first polar body extrusion [156], but whether this accumulation is important for oocyte cytokinesis is unknown and whether depolarization of mitochondria, especially around MI spindle, can cause failure of relocation of mitochondria to contractile ring area and block cytokinesis needs more investigation.

Mitochondria from *in vitro* matured oocytes are highly active, which may produce more ROS with potentially deleterious consequences for the oocytes.

Mitochondrial quiescence revealed by low membrane potential is also present in other cell types, for instance, T cells with low MMP have decreased oxidative stress, reduced levels of DNA-damage, increased long-term *in vivo* persistence and superior anti-tumour activity compared with high MMP counterparts [194]. The increased mitochondrial activity in the vicinity of the meiotic spindle carries with it some risk of increased local ROS generation which could compromise spindle function and DNA integrity. An alternative mechanism explaining the increased MMP around the spindle may be that there exists a stochastic variability in MMP in mitochondria and that those with higher MMP are preferentially trafficked by dynein-based mechanisms. Finally, the close localization of mitochondria and ER around the meiotic spindle [156] may lead to a local Ca^{2+}-stimulated increase in mitochondrial activity resulting in an increase in MMP [289]. Future work will be undertaken to investigate these possibilities.

2.5 Summary

In vitro matured oocytes feature a dramatic mitochondrial reorganization with mitochondrial accumulation around the MI spindle, and greater MMP compared to *in vivo* matured oocytes. This high loading of mitochondrial machinery in *in vitro* matured oocytes may create more ROS, affect mitochondrial function and thereby influence oocyte quality. However, this higher mitochondrial activity is also associated with successful completion of maturation suggesting upregulation of MMP is required for oocytes to complete *in vitro* maturation under these conditions. Most importantly, MMP-based nuclear biased localisation of mitochondria in *in vitro* matured oocytes correlates with oocyte *in vitro* competence suggesting that oocyte mitochondria may undergo a mechanism of selective intracellular sorting of mitochondria under certain conditions.

Chapter 3. The control of mtDNA fidelity in oocytes: effect of maternal age, defects in mitochondrial dynamics and mtDNA replication errors

3.1 Introduction

Mitochondria have their own genome, in the form of a circular double-stranded DNA. A fully-grown oocyte contains a large number of mtDNA copies, ranging from 80,000 to 700,000 copies according to different studies [98, 99, 209, 210, 221-224]. MtDNA encodes necessary subunits contributing to Complexes I, III, IV and V of the ETC. It also encodes 22 tRNAs and 2 rRNAs for its own transcriptional and translational machinery. Owing to its proximity to the site of ROS production and the absence of histones, mtDNA is susceptible to mutations. A number of diseases have been reported to be related to mtDNA mutations, such as MELAS (mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptoms) [290], LHON (Leber's hereditary optic neuropathy) [291], and MERRF (myoclonic epilepsy with ragged red fibres) [292]. Considering that mitochondria present in the early embryo and subsequent adult are derived solely from the oocyte, mtDNA mutations can lead to mitochondrial genetic diseases. Thus, it is important to investigate what factors can induce mtDNA mutations in oocytes, and in particular what kind of mitochondrial dysfunction can introduce mtDNA variants.

Maternal aging is associated with multiple types of mitochondrial dysfunction in oocytes and early embryos, including decreased MMP and ATP production [149, 204]. Ovarian aging is also associated with decreased mtDNA content and increased mtDNA variants in oocytes, including common deletions and SNPs [209, 213, 216, 217]; yet, how and when mtDNA variants in aged oocytes are generated is unknown. A previous study shows that oocytes from aged mice exhibit aggregated mitochondria, which may be due to diminished mitochondrial fission, as shown by repressed expression and phosphorylation of Drp1, a fundamental component of mitochondrial fission [143, 205]. As an important step of mitochondria biogenesis, fusion and fission are required for maintaining the integrity of the mitochondrial genome in yeast [145]. In mammalian cells, deficiency in mitochondrial fission induces nuclear DNA damage [146] and mtDNA nucleoid clustering [147]. Silencing of Drp1 and hFis1, which regulate mitochondrial fission, results in elevated level of mutant mtDNA in cells carrying both mutant and wildtype mtDNA [148]. Mitochondria undergo massive biogenesis during oogenesis, but it is unknown whether fission deficiency can affect mtDNA integrity or aggravate mtDNA mutation load during oogenesis and oocyte maturation.

Mitochondrial biogenesis is accompanied by mtDNA replication. A study investigating the interplay between mtDNA copy number and mtDNA mutation

load showed that mice harbouring a specific mtDNA C5024T mutation have more mtDNA copies [293]. Besides, modulation of mtDNA copy number via manipulating Tfam level in adult mice harbouring C5024T mutation reveals that increasing mtDNA copy number has no effect on mtDNA heteroplasmy level, whereas down regulating mtDNA copy number results in a strong selection against mutated mtDNA in proliferating tissues [293]. Knockout of Tfam during oogenesis also reduces mtDNA copy number in matured oocytes [100]. Interestingly, low mtDNA content does not affect oocyte maturation and preimplantation development but impairs post-implantation development [100]. The reason why Tfam KO during oogenesis leads to failed post-implantation development is unknown and it is not known if reduced mtDNA copy number has an effect on mtDNA integrity in oocyte.

It is well known that mtDNA mutations are induced when there are defects in mtDNA polymerase gamma (Polg), an enzyme that regulates mtDNA replication, repairs mtDNA base excision and proofreads mis-incorporated nucleotides. Mice with mutant Polg (Mutator mice) exhibit elevated mtDNA mutations, impaired fertility and premature ageing [134, 135, 294-299]. Thus, this mouse provides a useful model for understanding mtDNA mutations in oocytes.

To determine the mechanistic links between mtDNA mutation load and aging, a panel of mouse models were used. This includes old and young mice, oocyte-

specific Drp1 KO mice, oocyte-specific Tfam KO mice and Mutator mice. Oocytes from these mice were examined for mtDNA mutation load. The results showed that oocytes from oocyte-specific Drp1 KO mice and oocyte-specific Tfam KO mice are not prone to accumulate mtDNA mutations and old mice and mutator mice showed more mutations but distinct mtDNA mutation spectrums.

3.2 Materials and Methods

3.2.1 Animals

Drp1$^{loxP/loxP}$ mice [300], *Tfam*$^{loxP/loxP}$ mice (Jax mice stock No. 026123) [301] and *PhAM*$^{loxP/loxP}$ mice (Jax mice stock No: 018385) [275] were crossed with transgenic mice that carried Gdf-9 promoter-mediated Cre recombinase which had a C57BL/6J background [276]. After multiple rounds of crossing, homozygous mutant female mice lacking *Drp1* (*Drp1*$^{loxP/loxP}$; *Gdf9*-Cre) (hereafter Drp1$^{-/-}$) or *Tfam* (*Tfam*$^{loxP/loxP}$; *Gdf9*-Cre) (hereafter Tfam$^{-/-}$) or expressing a mitochondrial-specific version of Dendra2 green/red photo switchable monomeric fluorescent protein exclusively in oocytes (*PhAM*$^{loxP/loxP}$; *Gdf9*-Cre) (hereafter Dendra) were obtained. Mice that do not carry the Cre transgene are referred to as *Drp1*$^{loxP/loxP}$ (hereafter *Drp1*$^{fl/fl}$) or *Tfam*$^{loxP/loxP}$ (hereafter *Tfam*$^{fl/fl}$) were used as controls. To produce homozygous Polg$^{mut/mut}$ mice, heterozygous Polg$^{wt/mut}$ females were first generated by crossing Polg$^{wt/mut}$ males with Polg$^{wt/wt}$ females (Jax mice stock No. 017341). Then Polg$^{wt/mut}$ males were crossed with Polg$^{wt/mut}$ females to produce homozygous Polg$^{mut/mut}$ mice. Polg$^{wt/wt}$ females from the crossing of Polg$^{wt/mut}$ males with Polg$^{wt/wt}$ females were used as controls. The mice were housed under controlled environmental conditions with free access to water and food. All animal experimentation was approved by Monash University Animal Experimentation Ethics Committee and was performed in accordance with Australian National

Health and Medical Research Council Guidelines on Ethics in Animal Experimentation.

3.2.2 Oocyte collection and culture

C57BL/6, Dendra, Drp1 or Tfam mice of desired age were culled by cervical dislocation and ovaries were collected into M2 medium (Sigma-Aldrich). Oocytes were released from ovaries by puncturing with a 27 gauge needle and GV stage oocytes were collected with mouth pipette (Sigma-Aldrich). Only oocytes with intact cumulus cells were collected and cumulus cells were removed by repeated pipetting with a narrow bore pipette.

For GV oocytes from old Dendra mice, mice of 12-18 months old were administrated 10 IU PMSG by intraperitoneal injection. 48 hours later mice were culled and GV oocytes were collected.

For *in vitro* maturation of old Dendra oocytes, oocytes without cumulus cells were cultured in M16 medium (Sigma-Aldrich) under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air for 14 h.

3.2.3 DNA extraction and mtDNA amplification

In experiments examining the effects of age, total DNA was extracted from single oocytes (collected from 6-8 weeks or 12-16 months old mice) using a QIAamp DNA Micro Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Each sample was eluted with 10 µl DNase-free water. mtDNA was amplified by two-step Long PCR reaction using 2 sets of overlapping primers, each spanning half of the mitochondrial genome. For the first step of Long PCR, each reaction of 50 µl consisted of 5 µl total DNA, 1x High Fidelity PCR buffer, 2 mM MgSO4, 0.2 mM dNTPs, 1U of Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) and 0.2 µM each of the forward and reverse primer (A forward CCGTGCTACCTAAACACCTTATC and A reverse CGTCCGTACCATCATCCAATTA; B forward CCCTTCATCCTTCTCTCCCTAT and B reverse GTGGGATCCCTTGAGTTACTTC). Reaction conditions were 94°C for 2:00, 94°C for 0:15, 57°C for 0:30, 68°C for 10:00 (10 cycles), 68°C for 10:00, held at 4°C. 10 µl of the first PCR product was used as template for a longer second run consisting of an additional 35 cycles.

For experiments examining oocytes of $Drp1^{-/-}$, $Tfam^{-/-}$ and mutator mice, a pool of 20 oocytes from each mouse (4 weeks old) were transferred into 10 µl lysis buffer (50 mM Tris-HCl (pH=8.0), 1mM EDTA, 0.5% Tween-20, and 200 µg/ml proteinase K). Then each sample was incubated at 55°C for 2 h, followed by inactivation of proteinase K at 95°C for 10 min. MtDNA was amplified by Long PCR reaction using 2 sets of overlapping primers, each spanning half of the mitochondrial genome. Each reaction of 50 µl consisted of 5 µl total DNA, 1x

High Fidelity PCR buffer, 2 mM MgSO4, 0.2 mM dNTPs, 1U of Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) and 0.2 μM each of the forward and reverse primer (A forward CCGTGCTACCTAAACACCTTATC and A reverse CGTCCGTACCATCATCCAATTA; B forward CCCTTCATCCTTCTCTCCCTAT and B reverse GTGGGATCCCTTGAGTTACTTC). Reaction conditions were 94°C for 2:00, 94°C for 0:15, 57°C for 0:30, 68°C for 10:00 (20 cycles), 68°C for 10:00, held at 4°C.

3.2.4 Next-Generation Sequencing and NGS data analysis

Long PCR products were confirmed by gel electrophoresis (aging experiment) or Bioanalyzer (Drp1, Tfam and Mutator experiment) and sent to Micromon, the sequencing platform in Monash University, for sequencing using NGS. All library preparation and sequencing were carried out by the experts in the platform. For NGS data analysis, reads were aligned to mouse mitochondrial genome (NC_005089.1) using BWA v0.7.17 invoking 'mem'. Aligned reads were sorted and indexed using Samtools v1.8. Variant calling was performed using VarScan v2.3.138 (minimum depth = 1,500, supporting reads = 10, base-quality = > 30 and variant threshold = 1.0%). Genetic variant annotation and functional effect prediction were done by SnpEff [302].

3.2.5 TMRM labelling

Dendra or Drp1 oocytes at GV stage were incubated in M2 medium containing 25 nM TMRM for 30 minutes at 37°C in the dark. Then oocytes were washed in fresh M2 medium 3 times before imaging.

3.2.6 Immunofluorescence and imaging

Live oocytes labelled with TMRM were imaged at 37°C in a glass-bottomed dish using a laser-scanning confocal system (SP8, Leica). A 552 nm laser line and a 563-627 nm bandpass filter were used to image TMRM signal.

MII oocytes from Dendra mice were fixed in a 4% paraformaldehyde containing 2% Triton X-100 in PBS for 20 min at room temperature (RT), followed by blocking performed in PBS with 10% BSA and 2% Tween for 60 min at RT. The following antibodies were used for immunolabeling: 1:200 Drp1 antibody (Cell Signalling Technology (#8570)), 1:200 phosphor-Drp1 (p-Drp1) antibody (Cell Signalling Technology (#3455)) and 1:200 α-tubulin (Thermo Fisher Scientific (#322588)). ThermoFisher Scientific secondary donkey anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 555 and 647 (A-31570; A-31573, ThermoFisher Scientific) were used at 1:500. DNA was labelled using 10-min incubation in Hoechst 33342 (10 µg/ml; Sigma-Aldrich). Fluorescence images of fixed oocytes were acquired at room temperature in a glass-bottomed dish using a

laser-scanning confocal microscope imaging system (SP8; Leica). Image acquisition was performed using Las X software (Leica).

3.2.7 mtDNA copy number assay

Individual oocytes were transferred into 10 µl lysis buffer (50 mM Tris-HCl (pH=8.0), 1mM EDTA, 0.5% Tween-20, and 200 µg/ml proteinase K). Then each sample was incubated at 55°C for 2 h, followed by inactivation of proteinase K at 95°C for 10 min. Genomic DNA was diluted 5 times for quantitative PCR. A plasmid (gift from Rebecca L. Robker) containing 12S ribosomal (r)RNA region of mtDNA was used as quantification standards. The standard stock was serially diluted for use in the standard curve. Real-time fluorescence-monitored quantitative PCR using the primer pair 5'-CGT TAG GTC AAG GTG TAG CC-3' and 5'-CCA AGC ACA CTT TCC AGT ATG-3' was performed in Stratagene Mx3000P (Agilent Technologies). Each reaction of 20 µl consisted of 10 µl SYBR Green, 100 nM each of the forward and reverse primer and 2 µl diluted DNA sample. Standard curves were created for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve.

3.2.8 Statistical analysis

Data analysis was carried out using unpaired t-test. Error bars on column graphs represent standard error of the mean (SEM). * represents a p value of < 0.05, ** represents a p value of < 0.01, *** represents a p value of < 0.001, **** represents a p value of < 0.0001.

3.3 Results

3.3.1 Old oocytes exhibit more mtDNA variants

To confirm the effect of aging on oocyte mtDNA variant load, NGS was performed on single oocytes from young (4-6 weeks) and old (12-16 months) mice. Firstly, the average variant rate per base pair was calculated for substitutions, deletions and insertions. Old oocytes had marginally lower substitution rate than young oocytes (0.90 ± 0.01%, n=23 vs. 1.01 ± 0.03%, n=15, p = 0.0002) (Figure 3.1A) which is unexpected as it has never been reported in other cell types. But a higher deletion rate was found in old oocytes (0.006 ± 0.0001%, n=23 vs. 0.005 ± 0.0001%, n=15, p < 0.001) (Figure 3.1B). There was no difference in insertion rate between young and old groups (0.0014 ± 0.0001%, n=15 vs. 0.0015 ± 0.0002%, n=23) (Figure 3.1C).

Because variant load per base pair does not show significant point variants, next we performed variant calling on each sample. After applying appropriate statistical and biological filters as described in methods, single nucleotide polymorphisms (SNPs) at > 1% heteroplasmy were observed in both groups. No difference was found in the total number of SNPs per oocyte between young and old groups (1.1 ± 0.4, n=15 vs. 2.7 ± 1.1, n=23) (Figure 3.2A). However, old oocytes had significantly higher mean variant load of SNPs than young oocytes (13.46 ± 3.59%, n=23 vs. 1.52 ± 0.47%, n=15, p < 0.05) (Figure 3.2B). Further, 57% of old oocytes

were carrying a unique SNP, p12581 C > A substitution, which was located in the ND5 gene (Figure 3.2C). Besides SNPs, insertions and deletions (Indels) that exceeded a 1% threshold were also detected in both groups. There was no difference in the number of Indels per oocyte between young and old groups (3.8 ± 0.6, n=15 vs. 4.2± 0.3, n=23) (Figure 3.3A), but the mean variant load of Indels in old oocytes was significantly higher than young oocytes (8.25 ± 0.74%, n=23 vs. 5.32 ± 0.67%, n=15, $p < 0.01$) (Figure 3.3B). The frequency of each Indel was compared and old oocytes showed significantly higher p5171 deletion than young oocytes (31.76 ± 1.02%, n=19 vs. 24.10 ± 0.23%, n=10, $p < 0.0001$) (Figure 3.3C).

The above results indicate that mtDNA variants exist in individual oocytes of both young and old mice, and old oocytes have higher variant load than young oocytes. Also, intriguingly, p12581 C > A substitution was found exclusively in old oocytes.

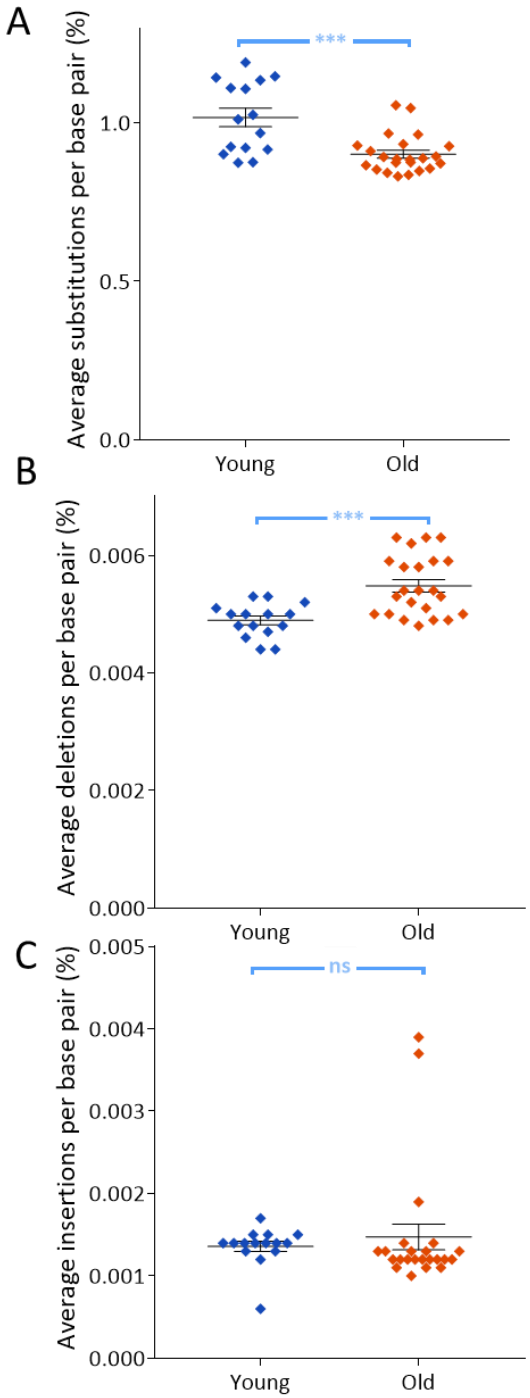

Figure 3.1. mtDNA variants per base pair in individual oocytes from young and old mice. A) Average mtDNA substitutions per base pair. B) Average mtDNA deletions per base pair. C) Average mtDNA insertions per base pair. Data were derived from 15 single young oocytes and 23 single old oocytes. Two-tailed T-test. ***, $p < 0.001$.

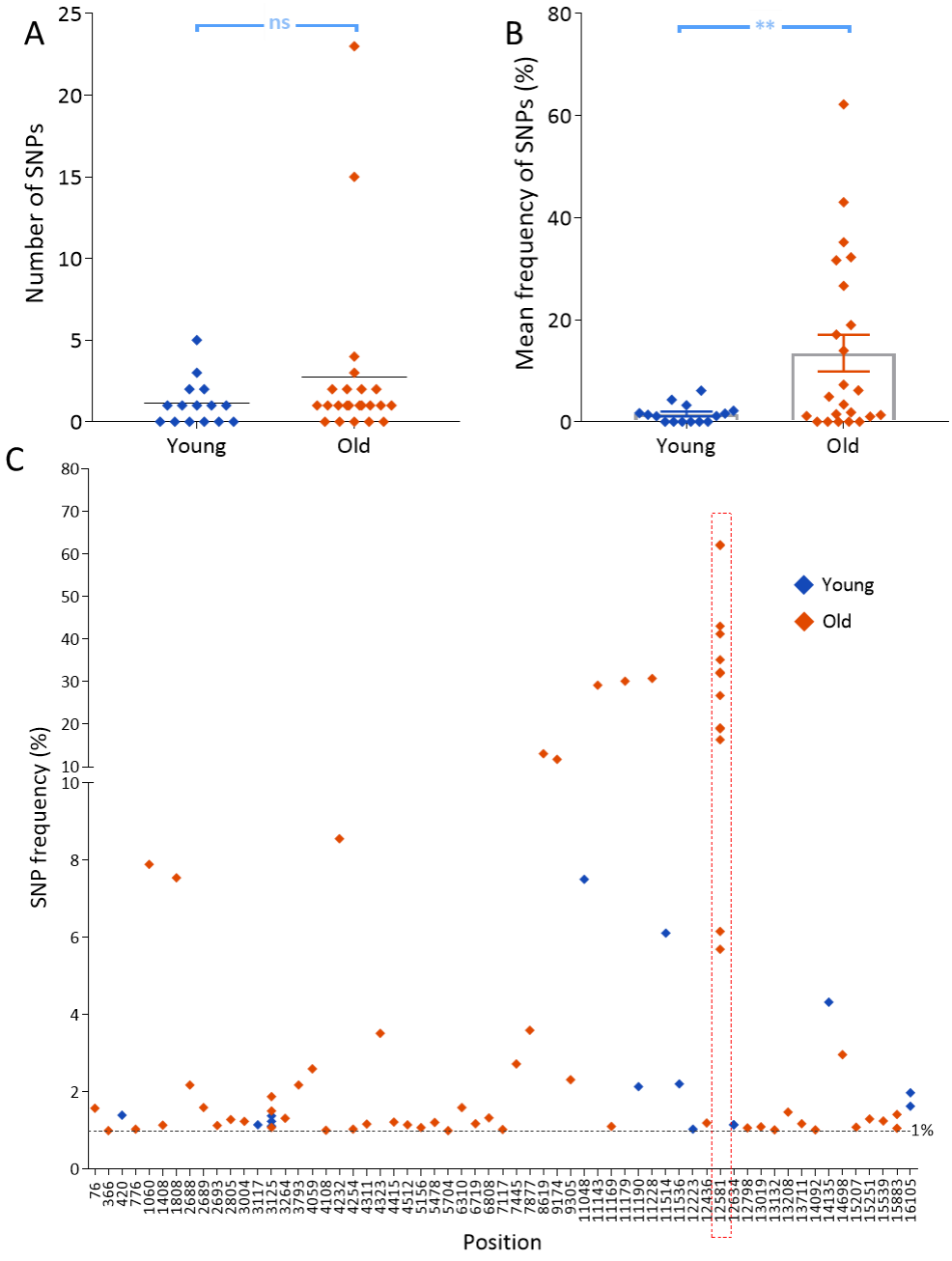

Figure 3.2. mtDNA SNPs of individual young and old oocytes exceeding the assay detection threshold of >1%. A) Number of SNPs with variant load over 1% detected in young and old oocytes. B) Mean variant load of SNPs. Two-tailed T-test. **, $p < 0.01$. C) Frequency of all mtDNA SNPs with variant load over 1% found in both groups. SNP at position 12581(orange box) was found in most old oocytes but not young oocytes. Data were derived from 15 single young oocytes and 23 single old oocytes.

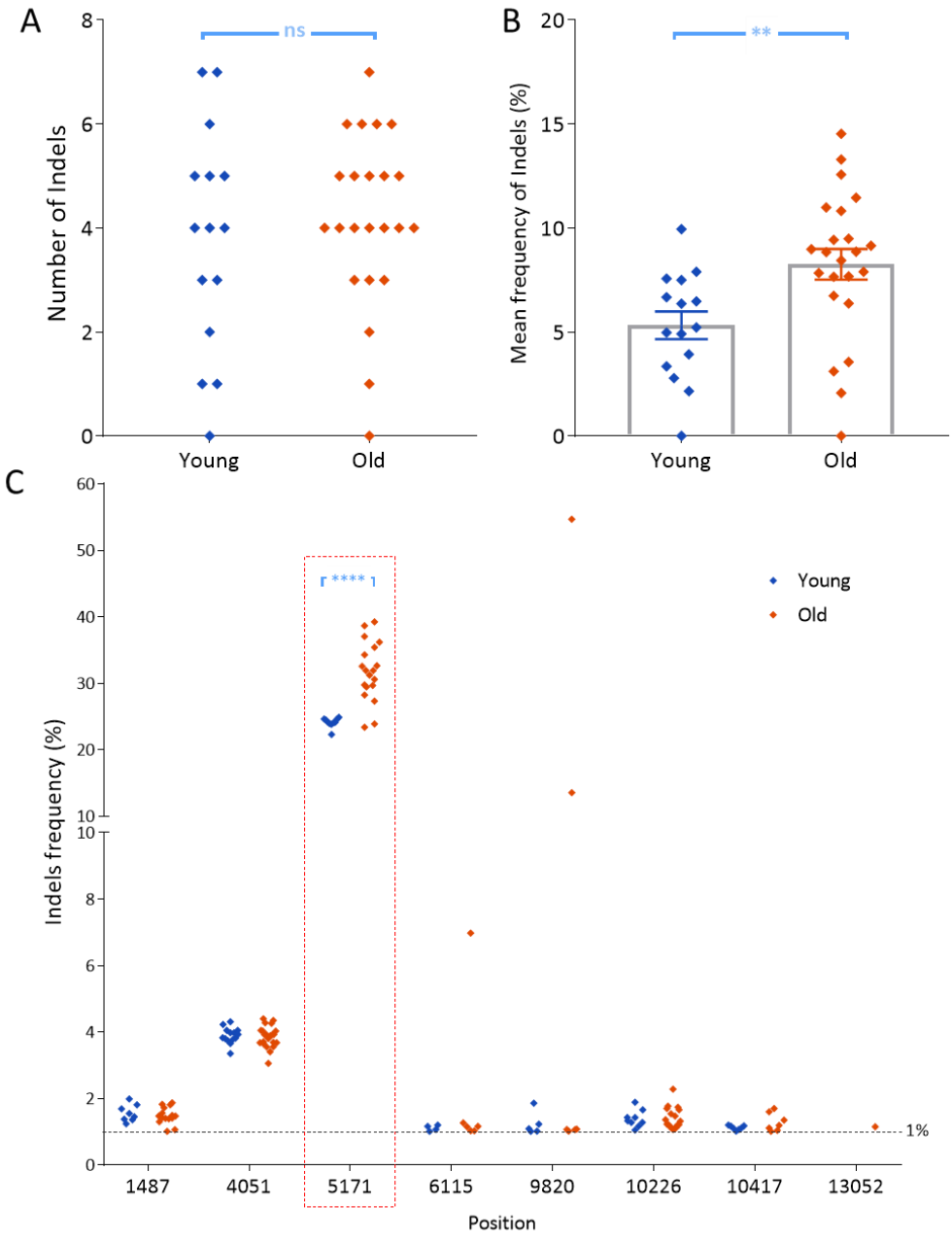

Figure 3.3. mtDNA Indels of young and old individual oocytes exceeding the assay detection threshold of >1%. A) Number of Indels with variant load over 1% detected in young and old oocytes. B) Mean variant load of Indels with variant load over 1%. C) Frequency of all mtDNA Indels with variant load over 1% found in both groups. Old oocytes had significantly higher variant load of p5171 deletion (orange box). Two-tailed T-test. **, $p < 0.01$. ****, $p < 0.0001$. Data were derived from 15 single young oocytes and 23 single old oocytes.

3.3.2 Old and Drp1$^{-/-}$ oocytes show larger mitochondrial clusters at GV stage and old oocytes show lower phospho-Drp1 level in mitochondria than young oocytes

A previous study has reported that aging is associated with aggregations of mitochondria in oocytes and speculated this could be due to decreased fission, regulated by p-Drp1 [143]. To investigate this, GV oocytes from young and old mito-Dendra mice were collected and imaged. As shown in Figure 3.4A, young oocytes show dispersed mitochondrial distribution, while mitochondria form clusters in old oocytes. A similar phenotype is also observed in Drp1$^{-/-}$ oocytes, in which mitochondria aggregate and form larger clusters than those in Drp1$^{fl/fl}$ oocytes (Figure 3.4B).

Mitochondrial aggregation in old oocytes is suggestive of a deficiency in fission, and which was similar to Drp1 KO oocytes. To determine whether mitochondrial fission is disrupted in old oocytes we investigated Drp1 and p-Drp1 levels in old oocytes. Mito-Dendra was used as a reference for normalizing Drp1 and p-Drp1 fluorescence. As shown in Figure 3.5A, mitochondria were aggregated in the ooplasm with some evidence of accumulation around the MII spindle. Drp1 co-localized with mitochondria and the total Drp1 level was similar between young and old oocytes, as shown by Drp1/Dendra ratio (Figure 3.5B). However, phosphorylated Drp1 was decreased in old oocytes, as shown by reduced p-Drp1

fluorescence and p-Drp1/Dendra ratio (Figure 3.5B). This result is consistent with previous observations [143], which indicates deficiency in Drp1 activity might be one of the reasons why aging affects competency of oocytes.

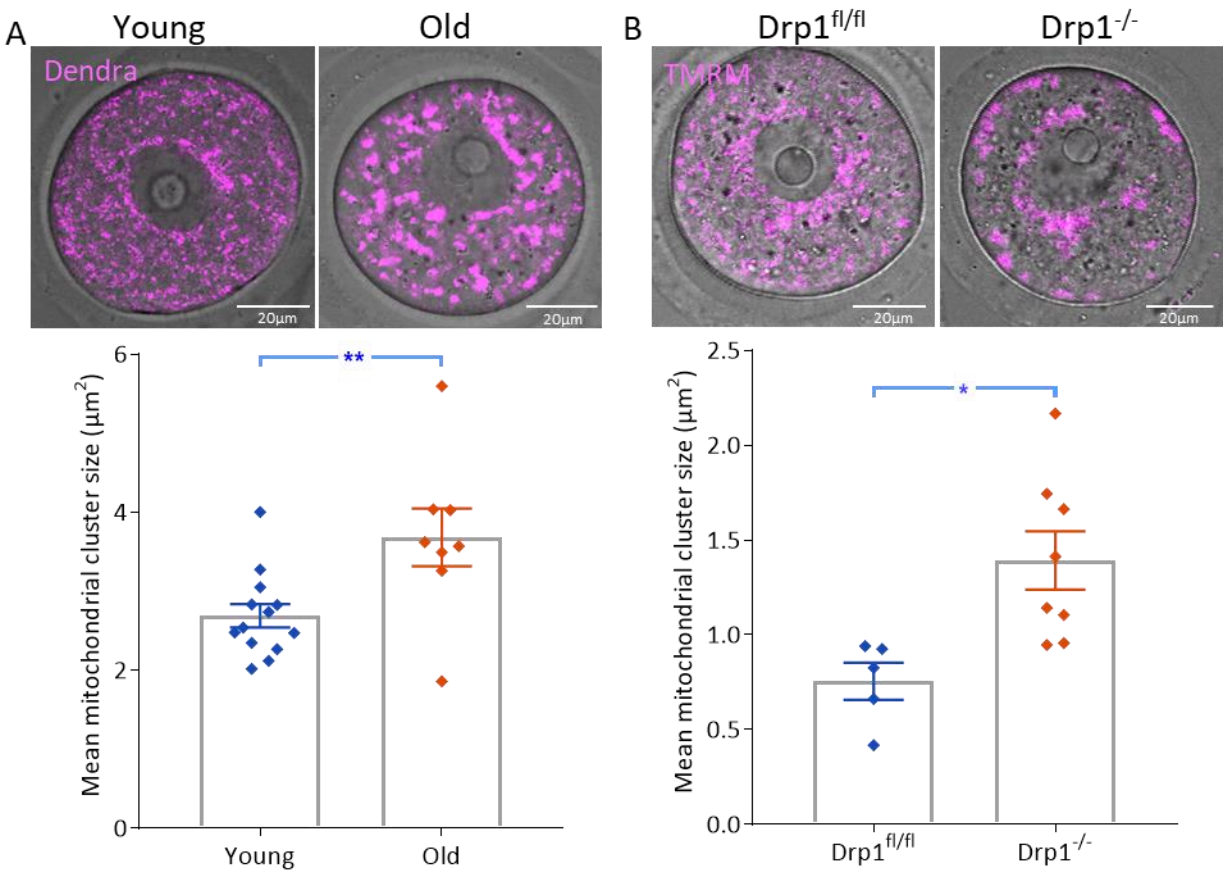

Figure 3.4. Mitochondrial cluster size in GV oocytes from old or Drp1-/- mice. A) Oocytes from old mice (n = 8) show larger mean mitochondrial cluster size than young mice (n = 13). ** $p < 0.01$. B) $Drp1^{-/-}$ oocytes (n = 8) show larger mean mitochondrial cluster size than oocytes from $Drp1^{fl/fl}$ littermates (n = 5). Two-tailed T-test. * $p < 0.05$.

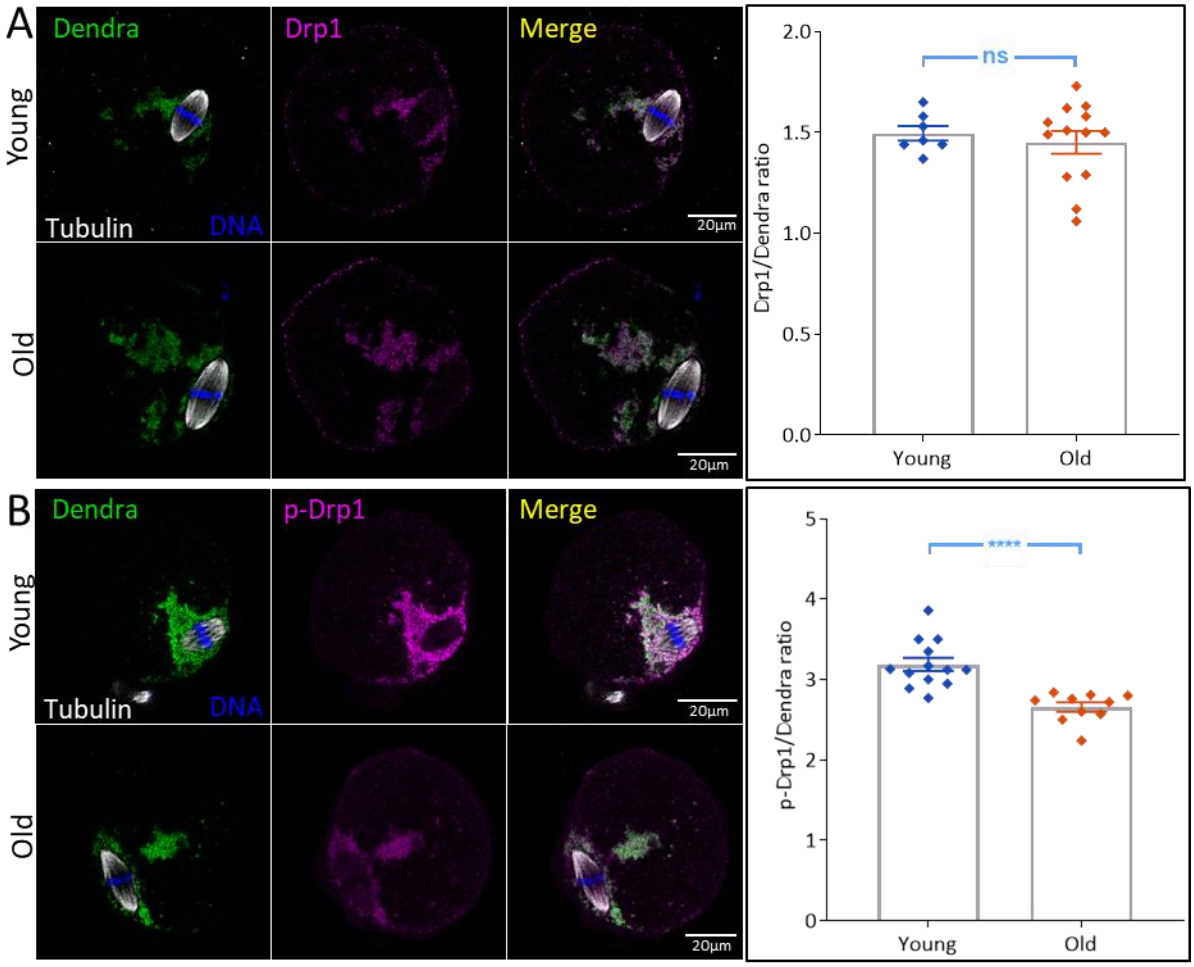

Figure 3.5. Old oocytes show lower phospho-Drp1 level in mitochondria. A) Representative confocal images of MII oocytes from young and old mice stained with Drp1 (magenta) and mitochondria labelled with Dendra (green). Quantification showed no difference in Drp1/Dendra ratio between young (n = 7) and old (n = 13) oocytes. B) Representative confocal images of MII oocytes from young and old oocytes stained with p-Drp1 antibody. p-Drp1/Dendra ratio was significantly lower in old oocytes (n = 13) than young oocytes (n = 10). Two-tailed T-test. **** $p < 0.0001$.

3.3.3 No significant difference of mtDNA variants found between Drp1$^{fl/fl}$ and Drp1$^{-/-}$ oocytes

It has been reported that silencing of Drp1 and hFis1 leads to elevated levels of mutant mtDNA in cells carrying both mutant and wildtype mtDNA [148]. To investigate if mitochondrial fission deficiency can influence mtDNA variants in oocytes, we performed NGS on mtDNA from Drp1$^{fl/fl}$ and Drp1$^{-/-}$ GV oocytes. In this design, pools of 20 GV oocytes rather than single oocytes were sequenced. This was done to minimize sequencing costs but also to generate representative samples of oocytes. In total, two Drp1$^{fl/fl}$ and two Drp1$^{-/-}$ samples were sequenced. No differences were found in the variant load per base pair between Drp1$^{fl/fl}$ and Drp1$^{-/-}$ oocytes, including substitutions (0.367 ± 0.08%, n=2 vs. 0.342 ± 0.044%, n=2) (Figure 3.6A), deletions (0.0032 ± 0.0001%, n=2 vs. 0.0031 ± 0.0001%, n=2) (Figure 3.6B) and insertions (0.0012 ± 0.0001%, n=2 vs. 0.0010 ± 0.0001%, n=2) (Figure 3.6C). After filtering by setting the threshold at 1%, SNPs were identified in both groups. No differences were found in the number of SNPs or the mean frequency of the SNPs between Drp1$^{fl/fl}$ and Drp1$^{-/-}$ oocytes ((1.5 ± 0.5, n=2, vs. 1.0 ± 1.0, n=2) (Figure 3.6D) (1.11 ± 0.05%, n=2 vs. 0.97 ± 0.97%, n=2) (Figure 3.6E) respectively). Both groups had p1924 A > C substitution, but one of the Drp1$^{-/-}$ samples had a unique p3069 G > A substitution with frequency at 2.72%, and one of the Drp1$^{fl/fl}$ samples also had a unique SNP; p16105 T > C substitution

with frequency at 1.22% (Figure 3.6F). There was also no difference in Indels between $Drp1^{fl/fl}$ and $Drp1^{-/-}$ oocytes, neither number of Indels (3, n=2, vs. 2, n=2) (Figure 3.6G) nor mean frequency of Indels (7.67 ± 0.63%, n=2 vs. 8.80 ± 0.16%, n=2) (Figure 3.6H). However, $Drp1^{fl/fl}$ oocytes exhibited a particular deletion at position 9820 with mean frequency at 5.02% (Figure 3.6I). To confirm those unique SNPs and Indels in each sample, a bigger sample size is needed. However, taken together, the above results suggest that oocytes with mitochondrial fission deficiency are not prone to accumulate mtDNA mutations.

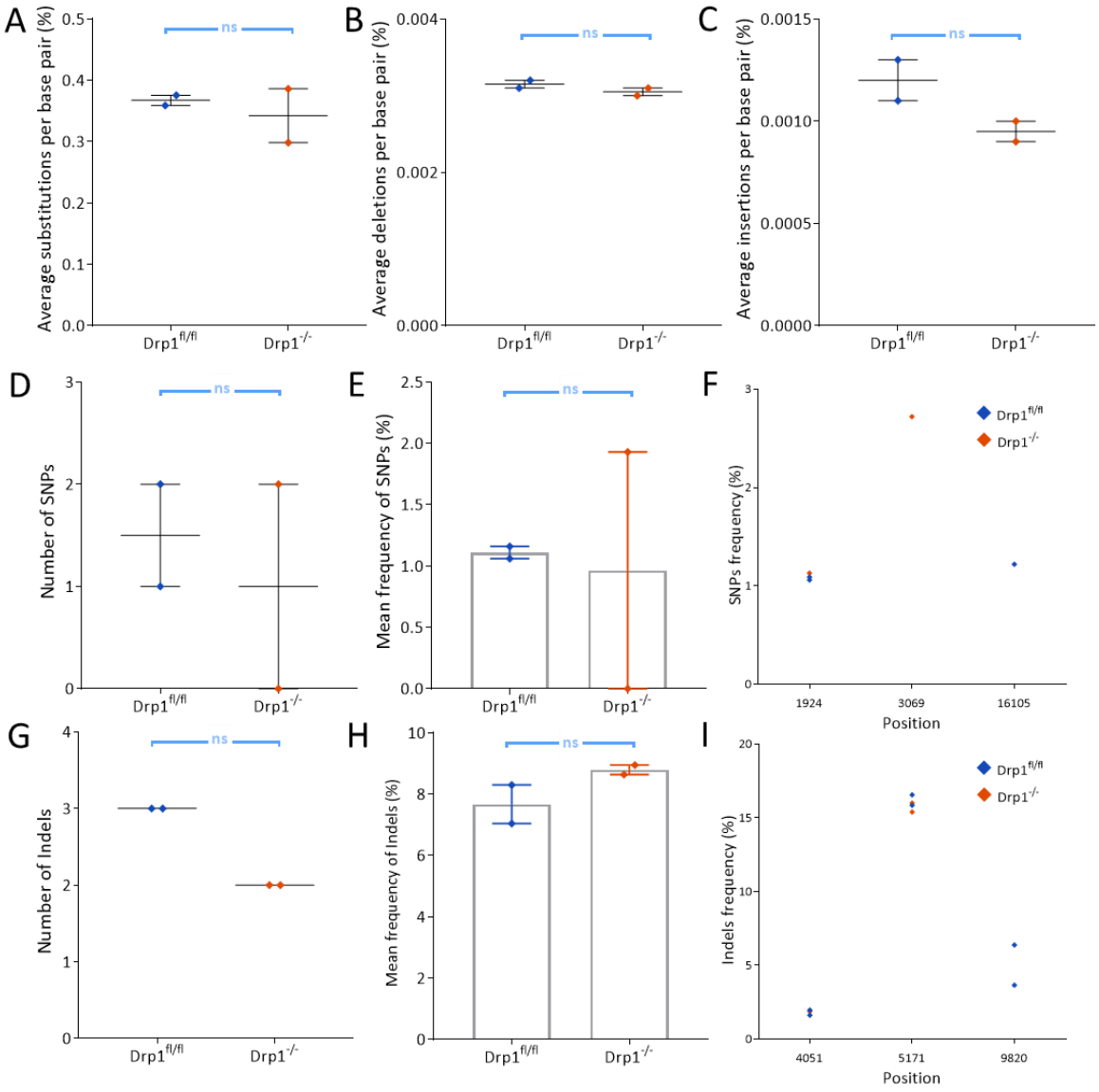

Figure 3.6. mtDNA variants of Drp1fl/fl and Drp1-/- oocytes. A) Average mtDNA substitutions per base pair. B) Average mtDNA deletions per base pair. C) Average mtDNA insertions per base pair. D) Number of SNPs with variant load over 1%. E) Mean variant load of SNPs with variant load over 1%. F) Frequency of all mtDNA SNPs with variant load over 1% found in both groups. G) Number of Indels with variant load over 1%. H) Mean variant load of Indels with variant load over 1%. I) Frequency of all mtDNA Indels with variant load over 1%. All data was analysed by t-test and no significant differences found.

3.3.4 No significant difference of mtDNA variants found between $Tfam^{fl/fl}$ and $Tfam^{-/-}$ oocytes

Maternal aging is associated with decreased mtDNA copy number in oocytes [209, 214] and decreased mtDNA content is known to affect the competency of oocytes [98, 210]. I also confirmed that GV oocytes from aged mice (12-16 months) have significantly lower mtDNA copy number than young (6-8 weeks) counterparts (641657 ± 63658, n=15 vs 231357 ± 98913, n=12, $p < 0.01$) (Figure 3.7A). To investigate the relationship between low mtDNA and mtDNA integrity, the Tfam gene was deleted during oogenesis, a developmental phase during which mitochondria normally undergo massive biogenesis, including mtDNA replication. In oocytes from $Tfam^{-/-}$ mice, mtDNA copy number was significantly lower than $Tfam^{fl/fl}$ oocytes (465411 ± 29859, n=12 vs 41862 ± 2993, n=12, $p < 0.0001$) (Figure 3.7B), which is consistent with a previous study [100]. Low mtDNA content has been reported to be associated with higher mtDNA oxidative damage in tumour cells [303] but there is no evidence showing whether there is a correlation between low mtDNA copy number and mtDNA integrity in oocytes. To investigate this, mtDNA of oocytes from $Tfam^{fl/fl}$ and $Tfam^{-/-}$ mice was sequenced. In total 3 pools of $Tfam^{fl/fl}$ oocytes and 3 pools of $Tfam^{-/-}$ oocytes were sequenced. There was no difference in the variant load per base pair between $Tfam^{fl/fl}$ and $Tfam^{-/-}$ oocytes, including substitutions (0.313 ± 0.025%, n=3 vs.

0.311 ± 0.020%, n=3) (Figure 3.8A) and deletions (0.0047 ± 0.0001%, n=3 vs. 0.0051 ± 0.0002%, n=3) (Figure 3.8B). But $Tfam^{-/-}$ mice showed slightly higher insertion rate in oocytes than $Tfam^{fl/fl}$ mice (0.0013 ± 0.0001%, n=3 vs. 0.0012 ± 0.0001%, n=3) (Figure 3.8C). After filtering by setting the threshold at 1%, only one SNP was found in one of the $Tfam^{-/-}$ samples (Figure 3.8D). Two identical Indels were found in all the samples from both groups (Figure 3.8E) and there was no difference in the mean frequency of the Indels between $Tfam^{fl/fl}$ and $Tfam^{-/-}$ (10.78 ± 0.65%, n=3 vs. 9.99 ± 0.11%, n=3) (Figure 3.8F). The above results suggest that oocytes with low mtDNA copy number are not prone to accumulate mtDNA mutations.

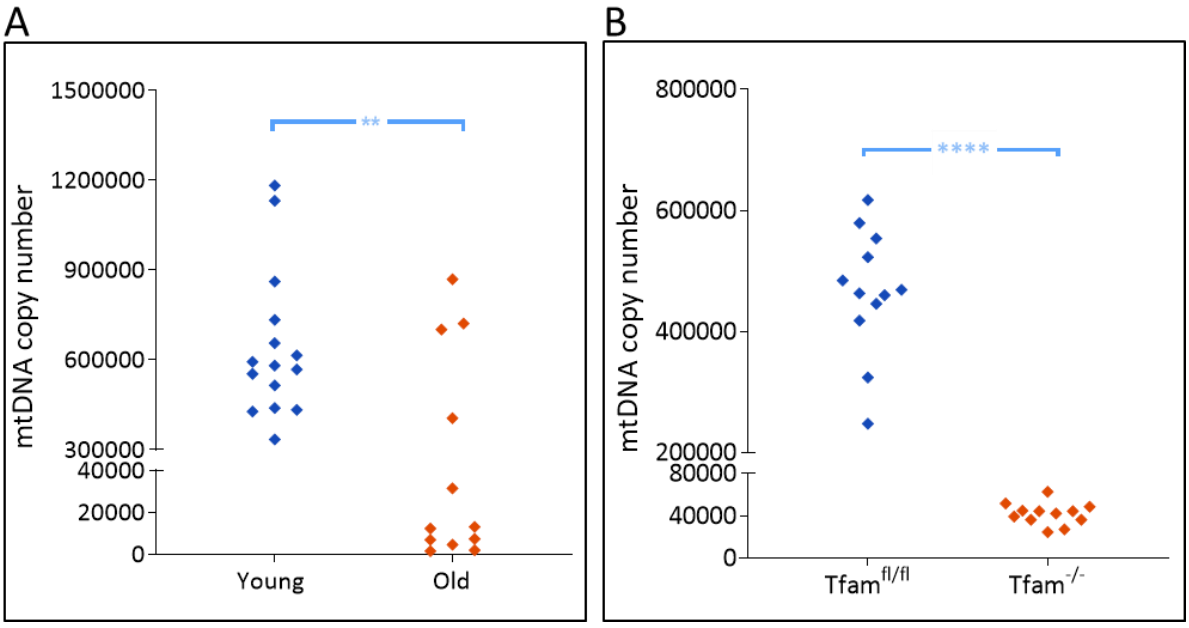

Figure 3.7. MtDNA copy number of oocytes from old or Tfam-/- mice. A) mtDNA copy number of young (n = 15) and old (n = 12) oocytes. ** $p < 0.01$. B) mtDNA copy number of $Tfam^{fl/fl}$ (n = 12) and $Tfam^{-/-}$ (n = 12) oocytes. Two-tailed T-test. **** $p < 0.0001$.

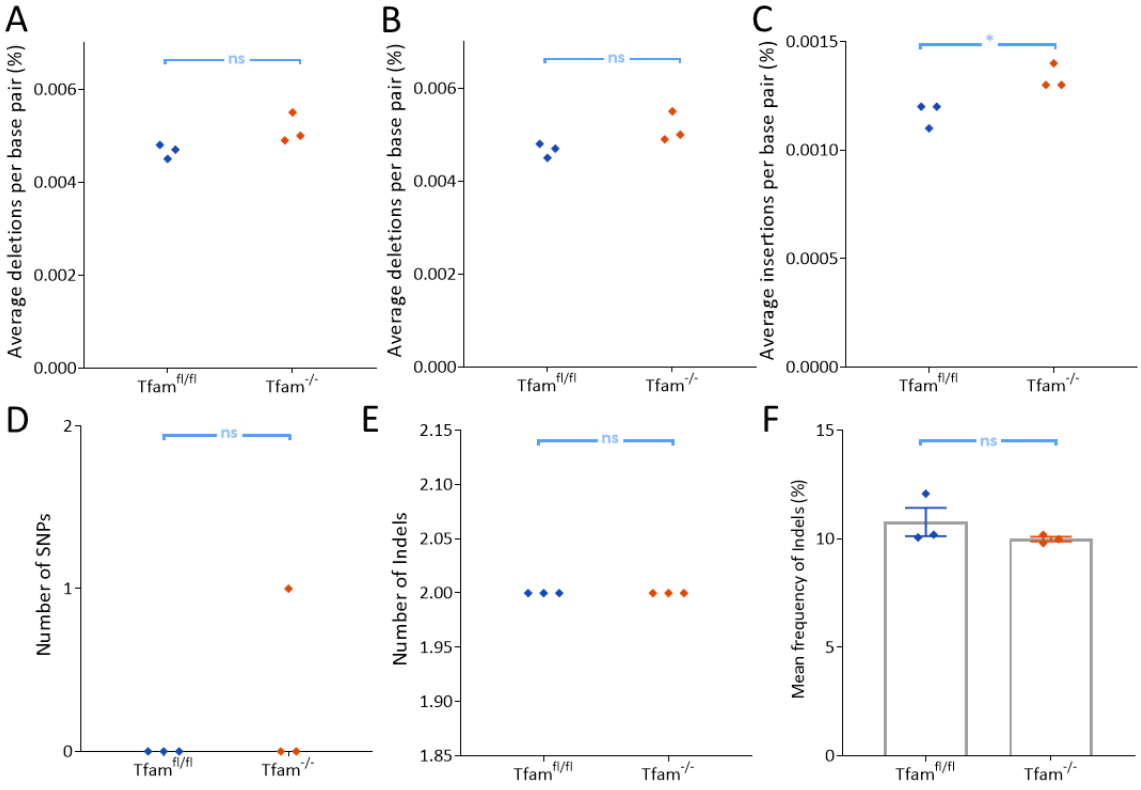

Figure 3.8 mtDNA variants of Tfamfl/fl and Tfam-/- oocytes. A) Average mtDNA substitutions per base pair. B) Average mtDNA deletions per base pair. C) Average mtDNA insertions per base pair. D) Number of SNPs with variant load over 1%. E) Number of Indels with variant load over 1%. F) Mean variant load of Indels with variant load over 1%. I) Frequency of all mtDNA Indels with variant load over 1%. Two-tailed T-test *, $p < 0.05$.

3.3.5 The effect of mtDNA proofreading deficiency on mtDNA integrity

A previous study shows that aging can cause increased mtDNA mutation load in oocytes [217]. A widely used a mouse model of ageing, the 'mutator mouse', has a deficiency in mtDNA proofreading, which causes mtDNA variants to accumulate in multiple tissues, and which leads to a premature ageing phenotype [134, 296]. To investigate if there are any similarities in oocyte mtDNA mutations between aged and mutator mice and how mtDNA proofreading deficiency impacts oocyte mtDNA integrity, mtDNA of oocytes from mutator mice was sequenced. In total 3 pools of oocytes from $Polg^{wt/wt}$ mice and 6 pools of oocytes from $Polg^{mut/mut}$ mice were compared. Oocytes from $Polg^{mut/mut}$ mice showed higher variant load per base pair than those from $Polg^{wt/wt}$ mice, including substitutions ($0.320 \pm 0.0047\%$, n=3 vs. $0.372 \pm 0.010\%$, n=6, $p < 0.01$) (Figure 3.9A), deletions ($0.0046 \pm 0.0001\%$, n=3 vs. $0.0062 \pm 0.0002\%$, n=6) (Figure 3.89, $p < 0.01$) and insertions ($0.0013 \pm 0.0001\%$, n=3 vs. $0.0020 \pm 0.0001\%$, n=6, $p < 0.0001$) (Figure 3.9C). $Polg^{mut/mut}$ mice also showed more SNPs and Indels in oocytes than $Polg^{wt/wt}$ mice (Figure 3.9D&E). Both groups had Indels at position 4051 and 5171 and $Polg^{mut/mut}$ oocytes exhibited significantly higher insertion rate at position 4051 than $Polg^{wt/wt}$ oocytes (Figure 3.9F). $Polg^{mut/mut}$ oocytes also showed Indels at other positions but not consistently among all the samples (Figure 3.9F). Interestingly, most of the SNPs found in $Polg^{mut/mut}$ oocytes are unique to each sample and not

shared with other samples (Figure 3.10). These results indicate that oocytes with mutant Polg have more random mtDNA mutations and amplify existing mutations. Comparing to the mutations found in old oocytes (Figure 3.2C, Figure 3.3C), Indels at position 4051 and 5171 are the common variants found in both experiments, which are also detected in Drp1 and Tfam experiments, suggesting they are common mutations in C57BL/6J background. Thus, aging induced mtDNA mutations may not be due to mtDNA proofreading deficiency.

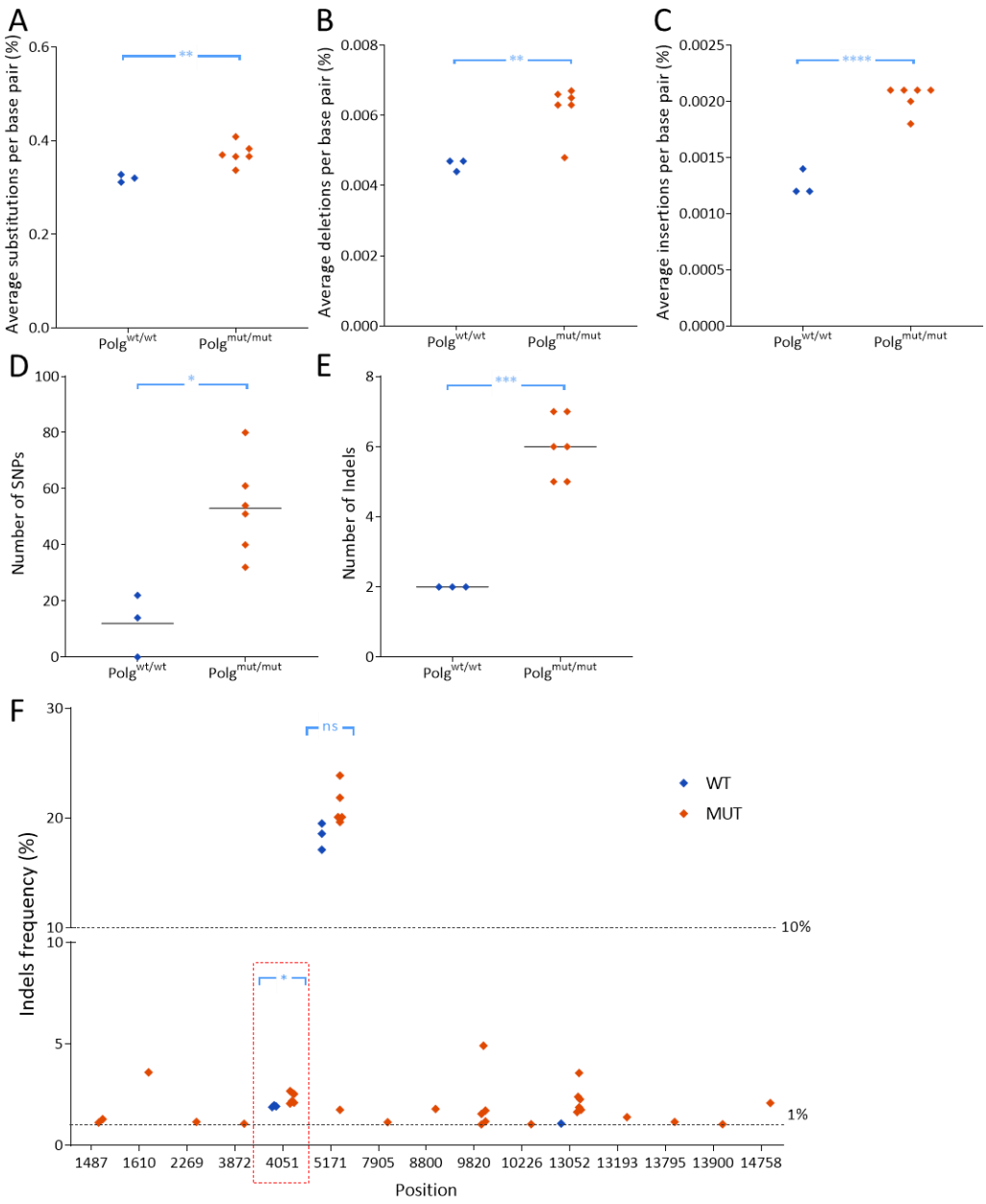

Figure 3.9. mtDNA variants of Polgwt/wt and Polgmut/mut oocytes. A) Average mtDNA substitutions per base pair. Two-tailed T-test. **, $p < 0.01$. B) Average mtDNA deletions per base pair. Two-tailed T-test. **, $p < 0.01$. C) Average mtDNA insertions per base pair. Two-tailed T-test. ****, $p < 0.0001$.D) Number of SNPs with variant load over 1%. Two-tailed T-test. *, $p < 0.05$. E) Number of Indels with variant load over 1%. Two-tailed T-test. ***, $p < 0.001$. F) Mean variant load of Indels with variant load over 1%. Two-tailed T-test. *, $p < 0.05$.

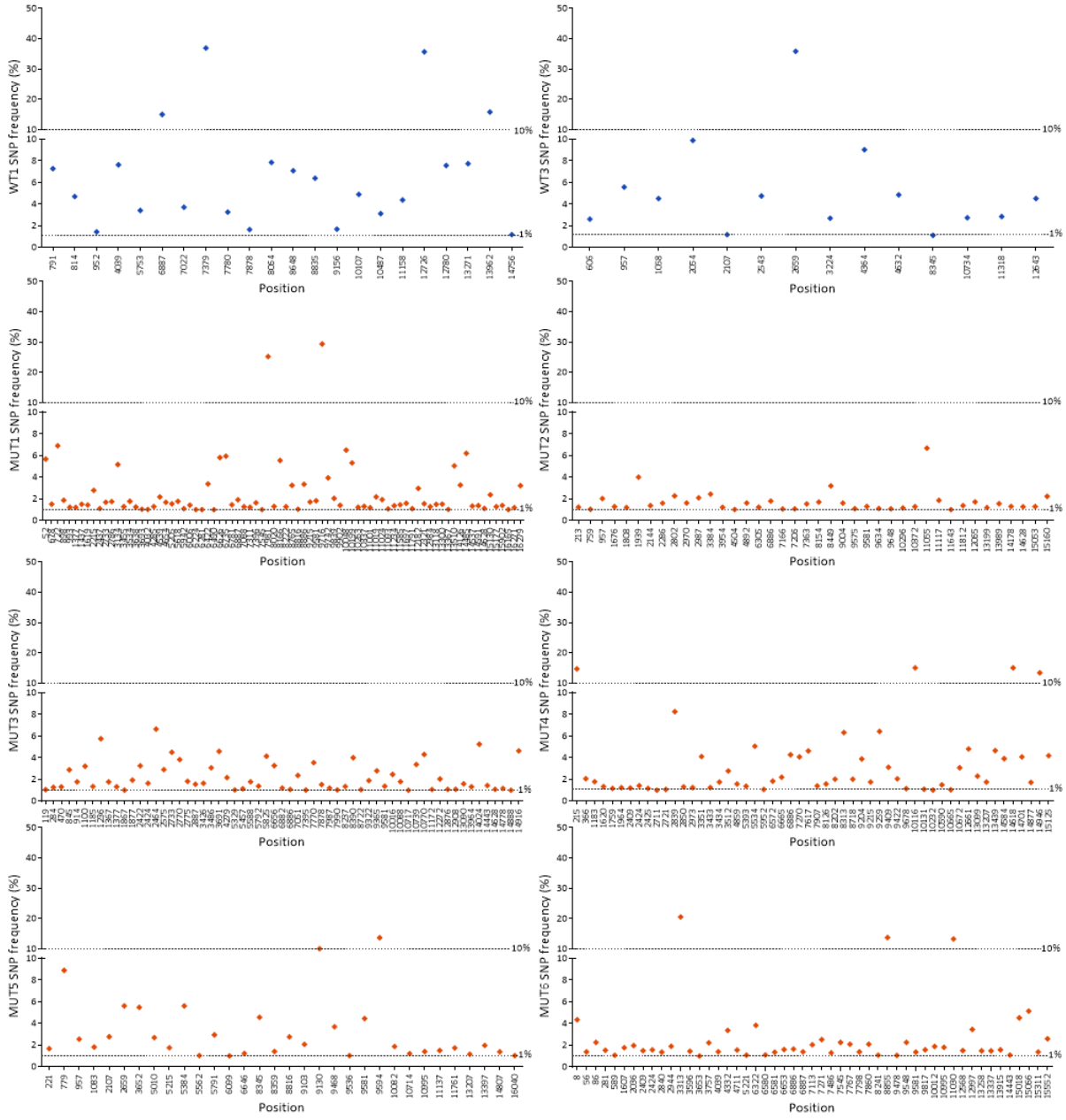

Figure 3.10. Frequency of all mtDNA SNPs with variant load over 1% found in two representative Polg$^{wt/wt}$ and all Polg$^{mut/mut}$ samples.

To see if these SNPs and Indels have an impact on protein function, SnpEff, a genomic variant annotation and functional effect prediction toolbox, was utilized to further analyse the sequencing data. According to the effect on protein coding, the variants are divided into four categories: High (the variant is predicted to have high (disruptive) impact on the protein, ie protein truncation, loss of function or triggering nonsense mediated decay); Moderate (a non-disruptive variant that might change protein effectiveness); Low (deemed to be mostly harmless or unlikely to change protein behaviour); and Modifier (usually non-coding variants or variants affecting non-coding genes, where predictions are difficult or there is no evidence of impact). As shown in Figure 3.11A, $Polg^{mut/mut}$ oocytes showed more mtDNA mutations in all four categories compared to $Polg^{wt/wt}$ oocytes. However, no difference was found in the proportion of variants with different severity between $Polg^{wt/wt}$ and $Polg^{mut/mut}$ oocytes (Figure 3.11B). Also, there was no difference in the mean frequency of mutations in the different severity categories (Figure 3.11C). Cumulatively, these results show that $Polg^{mut/mut}$ oocytes have more mtDNA variants than $Polg^{wt/wt}$ oocytes and that oocytes with mutant Polg have random mtDNA mutations with no preference on severity.

Since mutations with Moderate or High predicted impact may affect protein function, next I focused on the mutations in these two categories. As shown in Figure 3.12A, $Polg^{mut/mut}$ oocytes exhibited more variants located in mt-ND1, mt-

ND2, mt-CO1, mt-ND3, mt-ND4 and mt-Cytb compared to $Polg^{wt/wt}$ oocytes. Correspondingly, $Polg^{mut/mut}$ oocytes have more mtDNA variants located in genes that encode complex I, complex III and complex IV than $Polg^{wt/wt}$ oocytes and COMIII mutations were exclusively found in $Polg^{mut/mut}$ oocytes (Figure 3.12B). Among all the variants with Moderate or High effect impact, more than half resided in the genes of COMI in both $Polg^{wt/wt}$ and $Polg^{mut/mut}$ oocytes (Figure 3.12C). Affected genes that located in COMIV and COMV were at similar proportions between the two groups, but COMIII mutations account for 10% of the total mutations in $Polg^{mut/mut}$ oocytes, which was not seen in $Polg^{wt/wt}$ oocytes (Figure 3.12C). After comparing the mean frequency of variants affecting ETC complexes, a difference between the two groups was only found in COMIII due to zero variants in $Polg^{wt/wt}$ oocytes affecting COMIII; and no difference was seen in COMI, COMIV or COMV (Figure 3.12D).

Taken together, the above results suggest that oocytes with mtDNA proofreading deficiency are prone to accumulate mtDNA variants which are likely to affect protein coding and function.

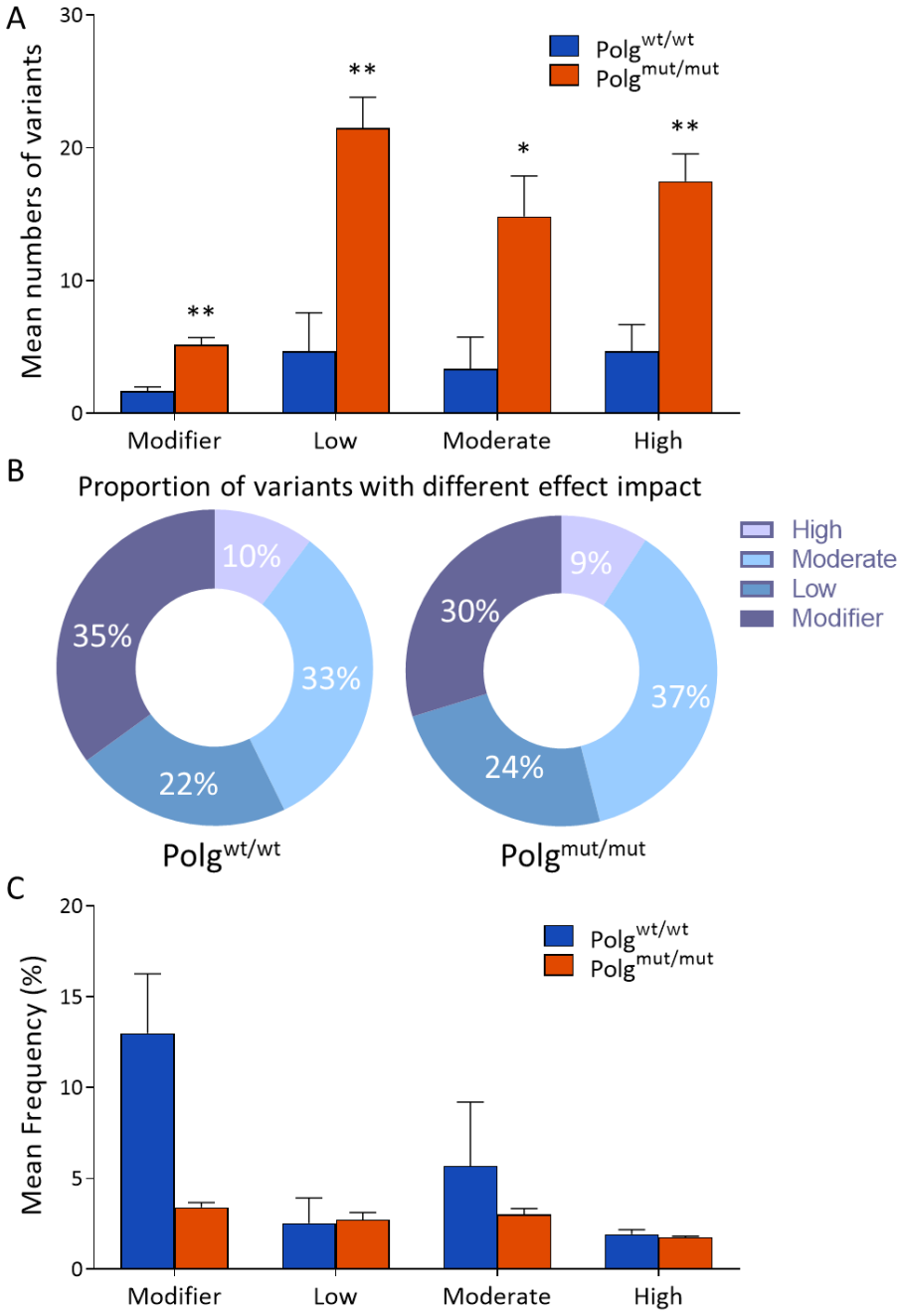

Figure 3.11. Impact of mtDNA mutations in Polgmut/mut oocytes. A) Mean numbers of variants with different effect impact in Polg$^{mut/mut}$ oocytes (n = 6) compared to Polg$^{wt/wt}$ oocytes (n = 3). Two-tailed T-test. *, $p < 0.05$. **, $p < 0.01$. B) Proportion of variants with different effect impact in both Polg$^{wt/wt}$ and Polg$^{mut/mut}$ groups. C) Mean frequency of mutations with different effect impact in Polg$^{mut/mut}$ oocytes compared to Polg$^{wt/wt}$ oocytes.

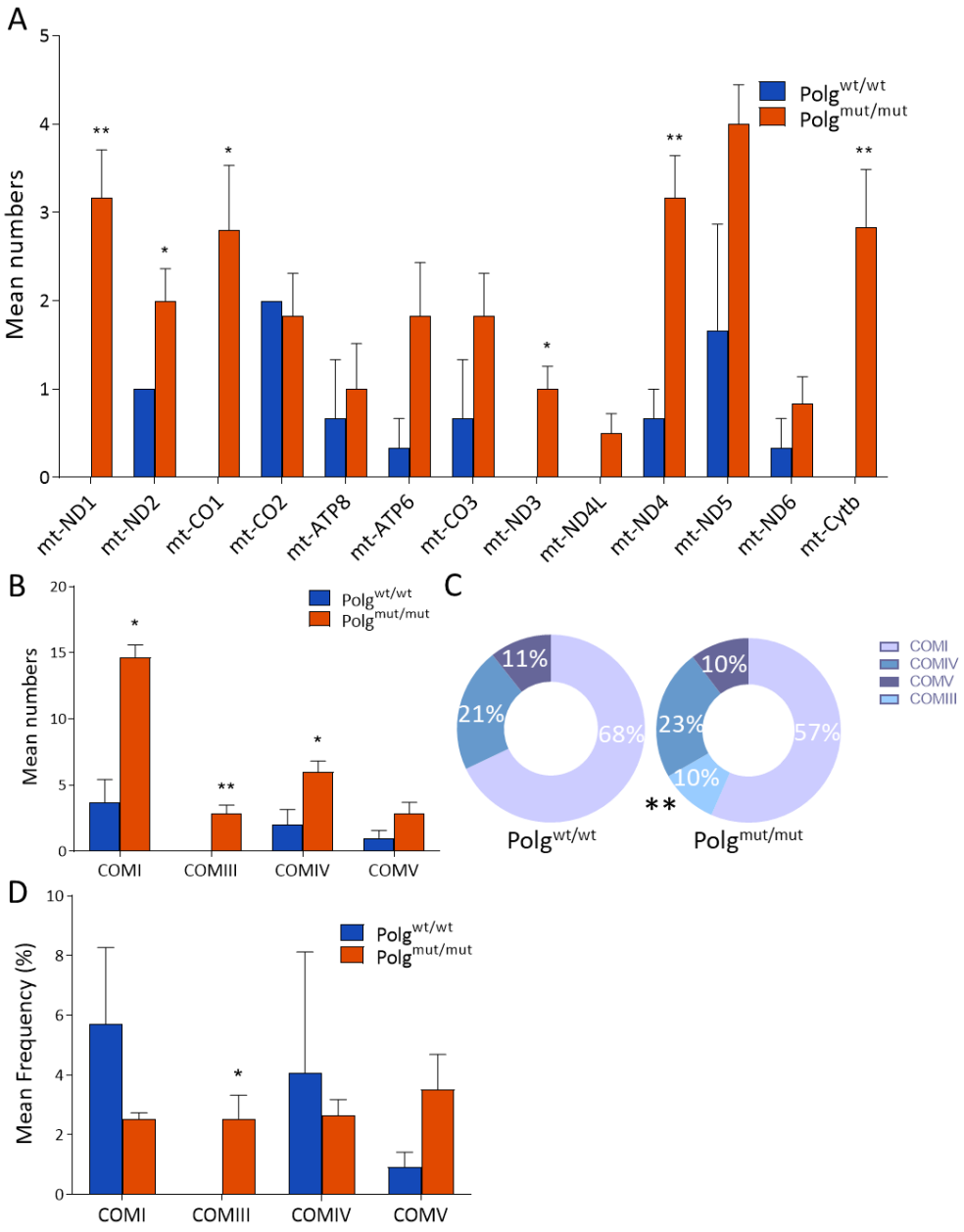

Figure 3.12. Distribution of mtDNA mutations with moderate or high effect impact. A) Mean numbers of variants with moderate or high effect impact in protein-coding genes in Polg$^{mut/mut}$ oocytes (n = 6) compared to Polg$^{wt/wt}$ oocytes (n = 3). B) Mean numbers of variants with moderate or high effect impact in ETC complexes in Polg$^{mut/mut}$ oocytes compared to Polg$^{wt/wt}$ oocytes. C) Distribution of variants with moderate or high effect impact in ETC complexes in Polg$^{mut/mut}$ and Polg$^{wt/wt}$ oocytes. D) Mean frequency of mutations with moderate or high effect impact on ETC complexes. Two-tailed T-test. *, $p < 0.05$. **, $p < 0.01$.

3.4 Discussion

The aim of this chapter was to investigate the effect of different mitochondrial dysfunctions on mtDNA integrity, especially the changes invoked by maternal aging. In summary, I first confirmed that maternal aging affects mtDNA integrity in oocytes, but surprisingly, the effects observed in old oocytes were not recapitulated in either Drp1 or TFAM knockout oocytes. As predicted, I did detect an increase in mitochondrial mutation rate in 'mutator' oocytes, demonstrating the validity of our analysis of mtDNA sequencing. These findings suggest oocytes from old mice are prone to increased mtDNA mutation, but that a failure of mitochondrial fission, or decreased mtDNA copy number are not likely the cause.

MtDNA is unstable relative to nuclear DNA due to its proximity with ETC, lack of protective histones and DNA repair machineries, which can result in accumulative mtDNA variants in multiple tissues with age [304]. Ovarian aging affects oocyte mitochondria morphologically and functionally. With increased age, MMP and ATP production are affected in oocytes [149, 204, 205, 277], which may further lead to failure of chromosome segregation and poor embryonic development [305-308]. In this study, by comparing the mtDNA spectrum of oocytes from both young and old mice, I found old oocytes have higher average deletions per base pair, suggesting more mtDNA deletions occurring in aged oocytes. Interestingly, the average substitutions per base pair of old oocytes is

lower than young oocytes, which is hard to explain and to my knowledge has never been previously reported. SNPs with frequency over 1% were found in both groups. Two samples from the old oocyte group have more than 10 SNPs, suggesting some individual oocytes might be highly sensitive to aging, which may result in the birth of pups with a high mtDNA variant load. The remaining old oocytes had similar numbers of SNPs as young oocytes, indicating a low level mtDNA heteroplasmy, which has been shown to be common even in healthy human individuals [309].

Whether these SNPs are pathogenic needs more analysis. Among all the oocytes tested, most point mutations were only found in one or two samples (Figure 3.2C), except mutations at position 3125 and 12581. The former was found in some of the young and old oocytes with SNP frequency ranging from 2-4%, whereas the latter was found exclusively in more than half of the old oocytes. The mutation at 12581 is within the ND2 gene, but to my knowledge has not been previously reported and is predicted to be a synonymous mutation with no effect on protein coding.

Indels are also found in both groups. Although there is no difference in the number of Indels, old oocytes do have higher frequency, which suggests aging might affect the proof-reading function to mtDNA and deteriorate the existing mtDNA insertions or deletions. A previous study has shown that mtDNA deletions are common in tissues of aged but not young mice, but with restricted methodology,

only large fragment deletion was reported [310]. Other research in a mouse model which has the same genetic background as old mice used in our studies did not detect any deletions using NGS on oocyte mtDNA [135], hence further experiments are needed to confirm our novel mtDNA Indels and investigate their potential effects on oocyte competency.

The first mitochondrial dysfunction that was studied for its impact on mtDNA mutation load was mitochondrial fission deficiency as this shared defect was first noticed while comparing mitochondrial distribution between young and old GV oocytes. I found mitochondria in old oocytes formed large clusters, which might be partially contributed to lower rates of fission in old oocytes. In support of this idea is the observed reduced level of Drp1 phosphorylation, as shown in the results and previous study [143]. Although mitochondrial fission and fusion has not been directly studied in oocytes, deletion of Drp1 during oogenesis can alter mitochondrial distribution in fully-grown oocytes and leads to failure of embryonic development [143], suggesting mitochondrial fission is important for developmental competence of oocytes. It has been reported that shRNA-induced Drp1 downregulation leads to increased mutant mtDNA in rhabdomyosarcoma cells [148], indicating mitochondrial fission might play a role in eliminating deleterious mtDNA. In Drosophila, overexpression of Drp1 also favors the selection against mutant mtDNA in the germline [311]. On the basis of these

findings, we anticipated a potential increase in mtDNA variants in Drp1 KO oocytes, however, the results in this chapter clearly demonstrate that the absence of Drp1 during oogenesis has no detectable effect on mtDNA integrity.

An explanation for the apparently different findings is that previous studies tested for the elimination or reduction in a known level of mtDNA heteroplasmy, while heteroplasmy levels in young oocytes may be too low to see any beneficial effect of Drp1. Extrapolating to old oocytes, mitochondria may gain moderate levels of mtDNA mutation during their long-lasting resting state in the primordial follicle, in which case testing if Drp1 levels can affect mtDNA variant load in old oocytes may be a more telling experiment. In addition, it would be interesting to generate mouse models that generate higher levels of mtDNA mutations and test if any deficiency in mitochondrial fission can impact the levels of mutant mtDNA during early development.

Oocytes increase their mitochondrial mass via mitochondrial biogenesis during the oocyte growth phase, a process presumably involving co-ordinated mitochondrial fission and mtDNA replication. Inhibiting mtDNA replication during oocyte growth via cell-specific targeted deleted of Tfam results in an approximate 10-fold decrease in mtDNA copy number in GV oocytes ([100] and Fig 3.7). However, TFAM KO oocytes with low mtDNA content undergo apparently normal maturation, fertilization and preimplantation embryo development, but fail during

post-implantation development [100]. A previous study shows that mice with conditional Tfam KO in heart and skeletal muscle have reduced mitochondrial encoded proteins and a concomitant decrease in the activities of complex I and IV [312]. Moreover, Tfam heterozygous null mice have increased oxidative mtDNA damage and ROS production in MEFs and intestinal tissues [313] Considering aged oocytes have lower mtDNA copy number compared to young oocytes and harbor mtDNA mutations, we proposed that there might be a correlation between low mtDNA content and mtDNA mutation load. However, in Tfam KO oocytes with only 10% of mtDNA left in the oocyte, no detectable increase in mtDNA variants was apparent. One explanation could be that the mtDNA copy number present in the primary oocyte is sufficient for oocyte and early embryonic development, thus even without mtDNA replication during oogenesis, mitochondria are not overly stressed and excessive ROS are not generated. In this case it could be hypothesised that there is either a remarkable level of redundancy in the ATP generating capacity, and ETC function in oocytes, or that there is an increased translation from the existing mtDNA in Tfam KO oocytes sufficient to compensate for the reduced levels of mtDNA. Another possibility could be that cumulus cells play a role in compensating for any energy shortage if oocyte mitochondria are deficient in ATP production due to insufficient synthesis of ETC

proteins. Thus, it might be worthwhile to determine if mitochondria in the cumulus cells of oocyte-specific Tfam KO mice are more active and generate more ROS.

The findings outlined above indicate that mitochondrial fission deficiency and low mtDNA content appear to not be the cause by which aging introduces mtDNA mutations in oocytes. One factor that is known to induce mtDNA variants in oocytes is mtDNA proofreading deficiency [135, 314]. This has been achieved by creating a mutation in the proof-reading domain of the DNA polymerase Polg. In this chapter, the mtDNA sequence spectrum of oocytes with proofreading deficiency was studied. Interestingly, compared to young mice in the first experiment and WT mice in the second and third experiment, $Polg^{wt/wt}$ oocytes exhibit more mtDNA mutations. The genotyping results were confirmed that the mice did carry the WT Polg alleles. This likely reflects that their WT mothers were born from heterozygous mothers and inherited a small number of mtDNA mutations that were passed in the germline to the pups. In any case, the $Polg^{wt/wt}$ oocytes exhibited dramatically different mutation spectrum compared to $Polg^{mut/mut}$ oocytes. Previous study shows that liver of $Polg^{mut/mut}$ mice have around 6 times the mtDNA mutations of that in $Polg^{wt/wt}$ mice [315], and possibly higher in heart [294], while our studies in oocytes showed an approximate 3-fold increase. Although the results may vary depending on the standard used to identify mtDNA mutations, oocytes harbouring deficient Polg alleles seem to have fewer mutations

compared to somatic tissues. One explanation for this difference may be the relative quiescence of primordial follicles before oocyte growth and development is initiated, compared to more highly proliferative and metabolically active tissues.

MtDNA mutation load not only varies among tissues in $Polg^{mut/mut}$ mice but also with aging. Old $Polg^{mut/mut}$ mice (9 months old) have 10 times more mtDNA mutations in their skin than when they were 2 months old [296]. WT mice also show more mtDNA mutations when they are aged, but the increase is much reduced (on average 0.9 mutation increased from 0) compared to that seen in $Polg^{mut/mut}$ mice (on average 4.3 mutations increased from 0.3) [296]. In this chapter, aging is shown to aggravate the pre-existing mtDNA deletion at position 5171, which is a synonymous variant. However, deficiency in mtDNA proofreading causes deterioration of pre-existing mtDNA deletion at position 4051, which is a non-synonymous variant and has 'HIGH' effect on protein coding. The other pre-existing mtDNA deletion at position 5171 is not elevated in $Polg^{mut/mut}$ oocytes. However, based on these two variants alone, it is hard to conclude that lacking the capacity of mtDNA proofreading can selectively amplify pathogenic mtDNA variants rather than synonymous ones.

That $Polg^{mut/mut}$ mice exhibit a premature aging syndrome [134, 316], demonstrates that mtDNA mutations underlie at least some aspects of physiological ageing. Even $Polg^{wt/wt}$ mice with maternally transmitted mtDNA mutations have mild

aging phenotypes [283], suggesting that mtDNA mutations and aging could mutually affect each other. But from the results in this chapter, both aging and Polg$^{mut/mut}$ mouse models showed random mtDNA mutations and rarely had common mtDNA variants in oocytes, except the 2 Indels which can also be found in young or WT mice. Although the level of Polg in old mice is unknown, the mtDNA mutations found in aged mice is not likely to be caused by compromised mtDNA proofreading.

Non-synonymous protein coding mutations take up around half of the total mutations in oocyte, but the mean frequency is relatively low, and whether they can result in pathogenic phenotypes needs more investigation. The heteroplasmy level of mtDNA mutations in Polg$^{mut/mut}$ mice does elevate with aging [296], which might hit a certain threshold at a certain age and cause premature aging phenotypes. Among those mtDNA variants found in Polg$^{mut/mut}$ oocytes, the non-synonymous protein coding ones are distributed at different genes and have a preference to genes that encode COMI. In a previous report, oocytes from Polg$^{wt/mut}$ mice also show relatively more mutations located in COMI genes [314]. However, in somatic tissues, an equal distribution of germline mtDNA mutations between the COMI and COMIV genes was found and no mutations are distributed at COMIII and COMV [296]. It is not clear why germline cells and somatic cells have this differing distribution of mtDNA mutations, but given their frequency is low, these

mutations may not cause disruption in ETC function. Future studies using homozygous mutator mice with a greater mtDNA mutation load in oocytes, would be useful to determine if there is a threshold mtDNA variant threshold at which oocyte fertility is compromised.

3.5 Summary

Based on the findings that organisms with advanced age have more mtDNA variants, it has been proposed that ageing causes an increase in mtDNA mutations, which in turn further exacerbate ageing phenotypes. Excessive ROS production has been proposed to be the link between ageing and mtDNA mutations, although other mitochondria-related activities may also be involved. Findings in this Chapter indicate that other mitochondrial dysfunctions including mitochondrial fission deficiency and mtDNA replication deficiency, both of which are also features of oocyte ageing, do not cause an increase in mtDNA mutations. Finally, mice with deficiency in mtDNA proofreading show premature aging phenotypes but their mtDNA mutation spectrum is different from that of oocytes from aged mice.

Chapter 4. Mitochondrial activity based mitochondrial sorting during early embryo development

4.1 Introduction

After fertilization, mammalian zygote undergoes three cell divisions to form eight blastomeres. Following the compaction of the 8-cell embryo, a compact mass called morula forms, which is the first embryonic stage where mammalian cells can be characterized as internal or external. The outer layer blastomeres, which will develop into trophectoderm which gives rise to the placenta, exhibit different cell polarity, protein abundance and gene expression with those on the inner part of the embryo. These inner cells will give rise to the inner cell mass and ultimately the developing fetus [22, 23, 317]. Since mitochondrial biogenesis remains quiescent during preimplantation development [90, 91], mitochondria present in the zygote are apportioned into these first embryonic lineages via an apparent passive process as the cells undergo consecutive cell divisions. Given the segregation of the lineages at this stage of development, it would be advantageous to the organism if there is a mechanism to ensure the 'healthiest' mitochondria are inherited by the cells that end up in the ICM and subsequent fetus. Whether the embryo has the ability to sort mitochondria and allocate them into different cell populations according to their health is unknown.

It has been shown that mitochondria distributed at different positions in a single oocyte have different membrane potential, which is indicative of mitochondrial heterogeneity in activity [150, 202]. However, the two studies show controversial results about the distribution of mitochondria with different membrane potential. The first one utilized JC-1 to investigate if there are domains of mitochondria with low or high membrane potential in oocytes and found that red fluorescence signal, which is indicative of high-polarized mitochondria, was found in the pericortical cytoplasm, whereas low-polarized mitochondria were found in the middle area of the oocytes [150]. The recent paper using TMRM as MMP indicator found an opposite result - mitochondria with higher membrane potential tend to accumulate around nDNA, such as GV, MI spindle or MII spindle [202]. Besides, it points out that JC-1 distribution in the oocytes is dependent on the concentration and loading time, where higher concentration and longer equilibration time leads to a more unified distribution of both green and red fluorescence [202]. Thus, the higher MMP found in the cortical area of the oocyte in the first study may be contributed to insufficient equilibration of the indicator [150]. Zygote inherits all the mitochondria from the oocyte, hence mitochondrial heterogeneity in activity should also be presented in early embryos. Similarly, JC-1 shows that mitochondria with high membrane potential are preferentially located at the pericortical region at one cell or 2-cell stage and in TE at blastocyst stage [150].

But whether the difference in JC-1 staining is due to the uneven loading of the indicator or actual difference in mitochondrial activity needs more investigation.

To test if there is any sorting of mitochondria based on their activity, first I tried multiple methods to label TMRM in blastocyst, including loading TMRM to the blastocyst directly, staining ICM isolated by immunosurgery with TMRM, and labelling TMRM from 8-cell stage. However, these methods cannot reach an agreement on the difference in MMP between ICM and TE. Live cell confocal imaging from 8-cell to blastocyst stage showed a negative correlation between mitochondrial activity and embryo competence *in vitro*, where competent embryos show lower MMP during *in vitro* culture.

4.2 Materials and Methods

4.2.1 Animals

PhAM$^{loxP/loxP}$ mice (Jax mice stock No: 018385) [275] were crossed with transgenic mice that carried Gdf-9 promoter-mediated Cre recombinase which had a C57BL/6J background [276]. After multiple rounds of crossing, homozygous mutant female mice expressing a mitochondrial-specific version of Dendra2 green/red photo switchable monomeric fluorescent protein exclusively in oocytes (*PhAM*$^{loxP/loxP}$; *Gdf9*-Cre) (hereafter Dendra) were obtained. The mice were housed under controlled environmental conditions with free access to water and food. All animal experimentation was approved by Monash University Animal Experimentation Ethics Committee and was performed in accordance with Australian National Health and Medical Research Council Guidelines on Ethics in Animal Experimentation (Animal Ethics ID 15094).

4.2.2 Embryo collection

To obtain 8-cell embryos from C57BL/6 mice, female mice were time mated with male mice and female mice were culled by cervical dislocation at day E2.5. Oviducts were collected into M2 medium (Sigma-Aldrich) and embryos were flushed from the oviducts with a 27 gauge needle. Eight-cell embryos were collected with mouth pipette (Sigma-Aldrich).

To obtain blastocysts from C57BL/6 and Dendra mice, female mice were time mated with male mice and female mice were culled by cervical dislocation at day E3.5. Uteri were collected into M2 medium (Sigma-Aldrich) and embryos were flushed from the uterus with a 30 gauge needle. Blastocysts were collected.

4.2.3 TMRM and MitoTracker Green (MTG) labelling

Dendra or C57BL/6 embryos at the desired stage were incubated in M2 medium containing 25 nM or 50 nM TMRM or 200 nM MTG for 30 minutes (or as indicated) at 37°C in the dark. Then embryos were washed in fresh M2 medium 3 times before imaging.

4.2.4 Confocal imaging

Live embryos labelled with TMRM were imaged at 37°C in a glass-bottomed dish using a laser-scanning confocal system (SP8, Leica). A 488 nm laser line and a 495-523 nm bandpass filter were used to image Dendra signal. A 552 nm laser line and a 563-627 nm bandpass filter were used to image TMRM signal. For sequential scanning, the Hybrid detector (HyD) was used to acquire Dendra and TMRM signal sequentially. For simultaneous scanning, one PMT detector and one HyD were used to capture Dendra and TMRM signal simultaneously. Line average was set at 4 times for high quality images.

For time lapse imaging of 8-cell embryos, embryos were plated in 5µl droplets of SAGE One Step embryo culture medium (CooperSurgical Fertility & Genomic Solutions) on a glass bottom dish covered by mineral oil. Embryos were imaged using a laser-scanning confocal system (SP8, Leica) with a stable incubation chamber set at 37°C in 5% CO_2. A 552 nm laser line was used to image TMRM signal. Images were acquired every 30 minutes.

4.2.5 Ratiometric analysis

Ratiometric analysis was performed using ImageJ software. Briefly, Dendra (green) and TMRM (red) channels of the confocal images were separated and converted to 32-bit images, and the background of each channel was set to Not a Number (NaN) using thresholding, which was determined visually. The same threshold was applied to other images from the same experiment. Then TMRM fluorescence value was divided by Dendra fluorescence value pixel by pixel using Image Calculation function in ImageJ. Calculated ratio image is shown by a preset lookup table in ImageJ called 16_colors, which shows higher value with warmer colour.

4.2.6 Statistical analysis

Data analysis was carried out using unpaired t-test. Error bars on column graphs represent standard error of the mean (SEM). * represents a p value of < 0.05, **

represents a p value of < 0.01, *** represents a p value of < 0.001, **** represents a p value of < 0.0001.

4.3 Results

4.3.1 TE of *in vivo* grown blastocyst shows higher TMRM/Dendra ratio than ICM regardless of TMRM loading time

To investigate if there is any mitochondrial activity-based sorting during early embryo development, the MMP of *in vivo* blastocysts were first measured. Fluorescence images showed similar Dendra fluorescence across the entire blastocyst but TMRM of TE was higher than that of ICM. Ratiometric analysis suggested that MMP of ICM was significantly lower than that of TE cells (Figure 4.1A&B). This result is consistent with previous finding using JC-1 [150]. However, MTG, a MMP independent mitochondrial marker, also showed low intensity in ICM (Figure 4.1C), which suggests that these mitochondrial targeted dyes might have equilibration problem while labelling mitochondria in blastocyst. To examine this, blastocysts were loaded with TMRM for 1 hour to see if doubling the loading time can affect TMRM equilibration. As shown in Figure 4.1D, the TMRM fluorescence intensity remained lower in ICM than TE cells (Figure 4.1D), so was the TMRM/Dendra ratio (Figure 4.1E).

As TMRM penetrates gradually into cells while direct loading, it may be that the reduced labelling in the ICM reflects limited access as it first needs to traverse the trophectoderm. To address this potential issue, TMRM (at an increased dose of 'concentration') was injected into the blastocoel in an effort to increase access to

the ICM, and the blastocysts were placed into TMRM drops, for loading as normal (Figure 4.2A). Microinjection caused the blastocysts to collapse, however, the TMRM intensity of the inside cells was still lower than the outer cells (Figure 4.2B). Considering blastocoel fluid containing TMRM may be flushed and diluted into the drop while the blastocyst was collapsing, suggesting this method may not solve the equilibration problem, as the ICM may still not access the same concentration of TMRM as the TE cells.

A second approach involved breaching the TE in three locations and then incubating the blastocyst in a TMRM drop (Figure 4.2C), which was intended to leave a few channels across TE, so that the inside and outside of the blastocyst can exchange fluids and reach an equilibration of TMRM concentration. The blastocyst also collapsed with this manipulation; however relatively low TMRM intensity was again found in ICM (Figure 4.2D).

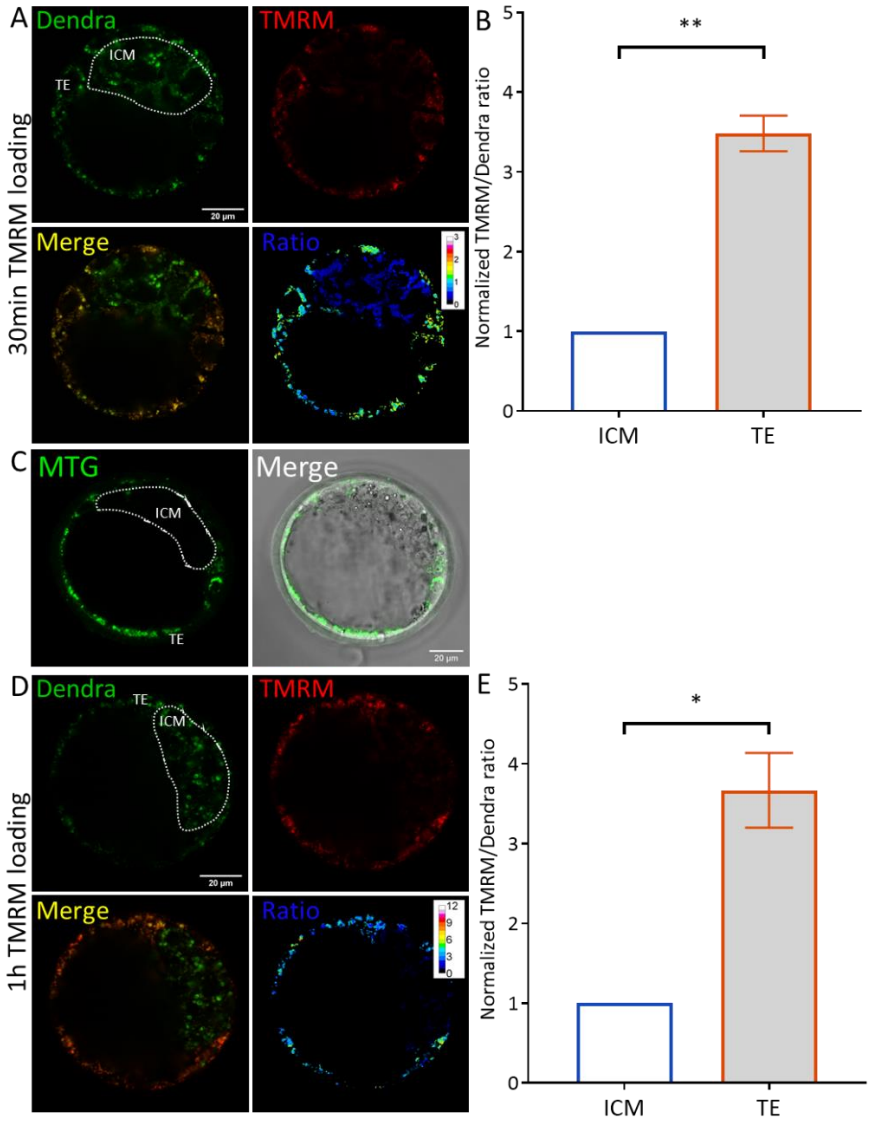

Figure 4.1. TE shows higher TMRM/Dendra ratio than ICM regardless of TMRM loading time. A) *In vivo* derived blastocyst (d3.5) from Dendra mouse was labelled with TMRM (25nM) (red) for 30 min. Ratio image was produced by ratiometric analysis and shown in pseudo colour. B) Bar graph shows mean normalized TMRM/Dendra ratio of two groups. Data shown as Mean ± SEM. One experiment. n = 4 embryos from 1 mouse. Paired Two-tailed T-test. **, $p < 0.01$. C) *In vivo* blastocyst labelled with MTG. D) *In vivo* grown blastocyst from Dendra mouse was labelled with TMRM (25nM) (red) for 1h. Ratio image was produced by ratiometric analysis and shown in pseudo colour. E) Bar graph shows mean normalized TMRM/Dendra ratio of two groups. Data shown as Mean ± SEM. One experiment. n = 4 embryos from one mouse. Paired Two-tailed T-test. *, $p < 0.05$.

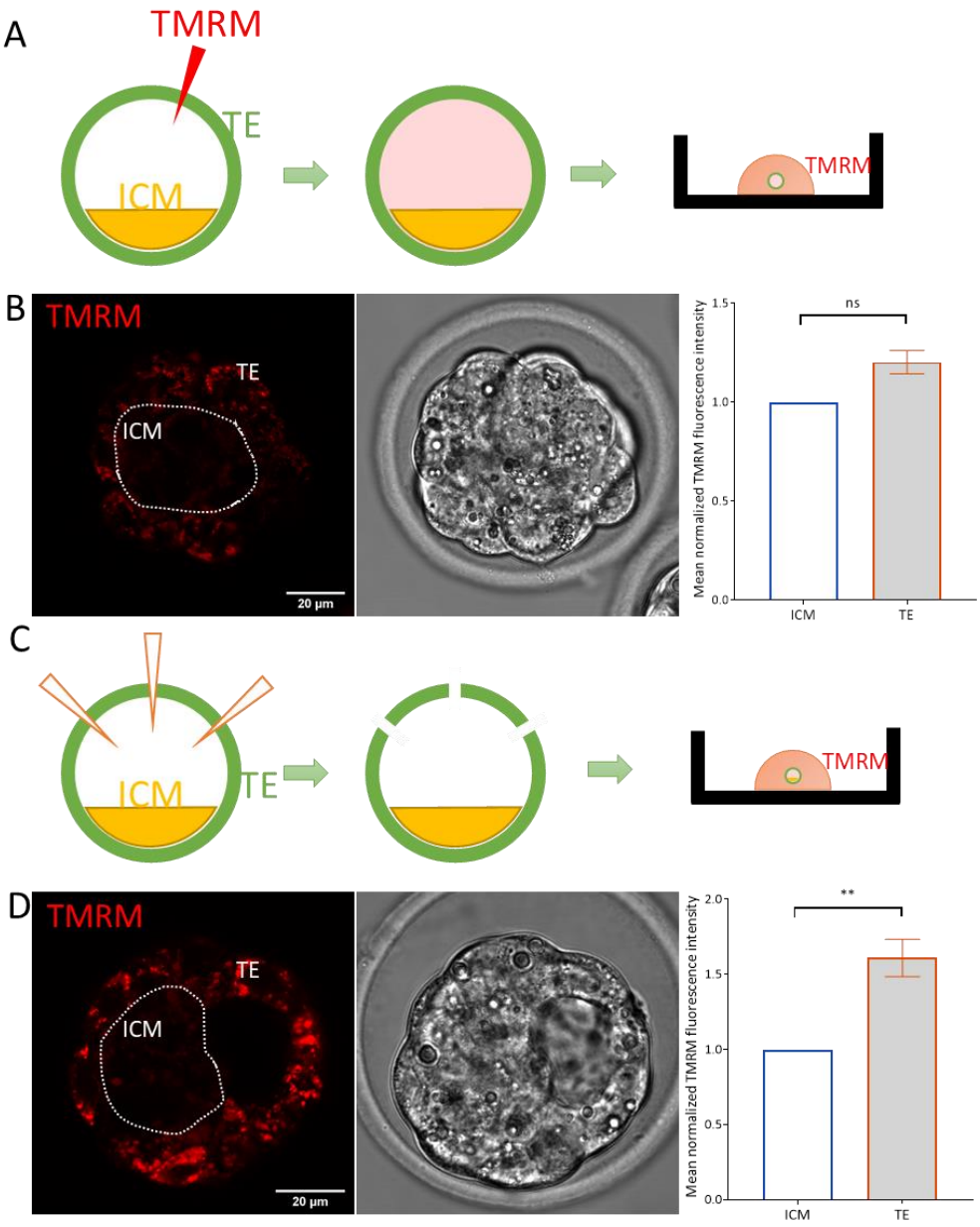

Figure 4.2. TMRM injection into blastocyst cavity or TMRM loading after breaking TE of the blastocyst both show low TMRM intensity in ICM. A) Schematic of TMRM (50mM) injection. B) Blastocyst was injected with concentrated TMRM and imaged 30 min later. Bar graph shows mean normalized TMRM fluorescence intensity of ICM and TE. Data shown as Mean ± SEM. One experiment. n = 3. T-test. C) Schematic of TMRM loading. D) Blastocyst was labelled with TMRM (25nM) for 30 min after breaking the TE at 3 different positions. Bar graph shows mean normalized TMRM fluorescence intensity of ICM and TE. Data shown as Mean ± SEM. One experiment. n = 4. Two-tailed T-test.

4.3.2 Isolated ICM by immunosurgery shows higher TMRM intensity than TE of intact blastocyst.

The above attempts were conducted on whole blastocyst, which could not convincingly resolve the potential equilibration issue because it remains possible that TMRM was excluded from the inner cells as a consequence of blastocyst collapse. Therefore, isolation of ICM and TE was conducted as a means to overcome this issue. Mechanical isolation of ICM from TE has been successful previously on human blastocyst [318]. Thus, traditional immunosurgery was utilized to isolate the ICM. In this case, because TE cells will be lysed and mitochondria in TE cells will be depolarized, TMRM intensity of isolated ICM was compared to TE of a different intact blastocyst (Figure 4.3A). As shown in Figure 4.3B, under same loading conditions, TMRM fluorescence intensity of isolated ICM was significantly higher than that of the TE of intact blastocyst. Thus, this approach indicates that ICM cells may indeed have an MMP that is similar to TE cells and that MMP measurements in intact blastocysts may underestimate the MMP of ICM cells due to access issues. However, an alternative explanation may be that, the manipulation of immunosurgery and isolation of ICM cells, may lead to an increased MMP.

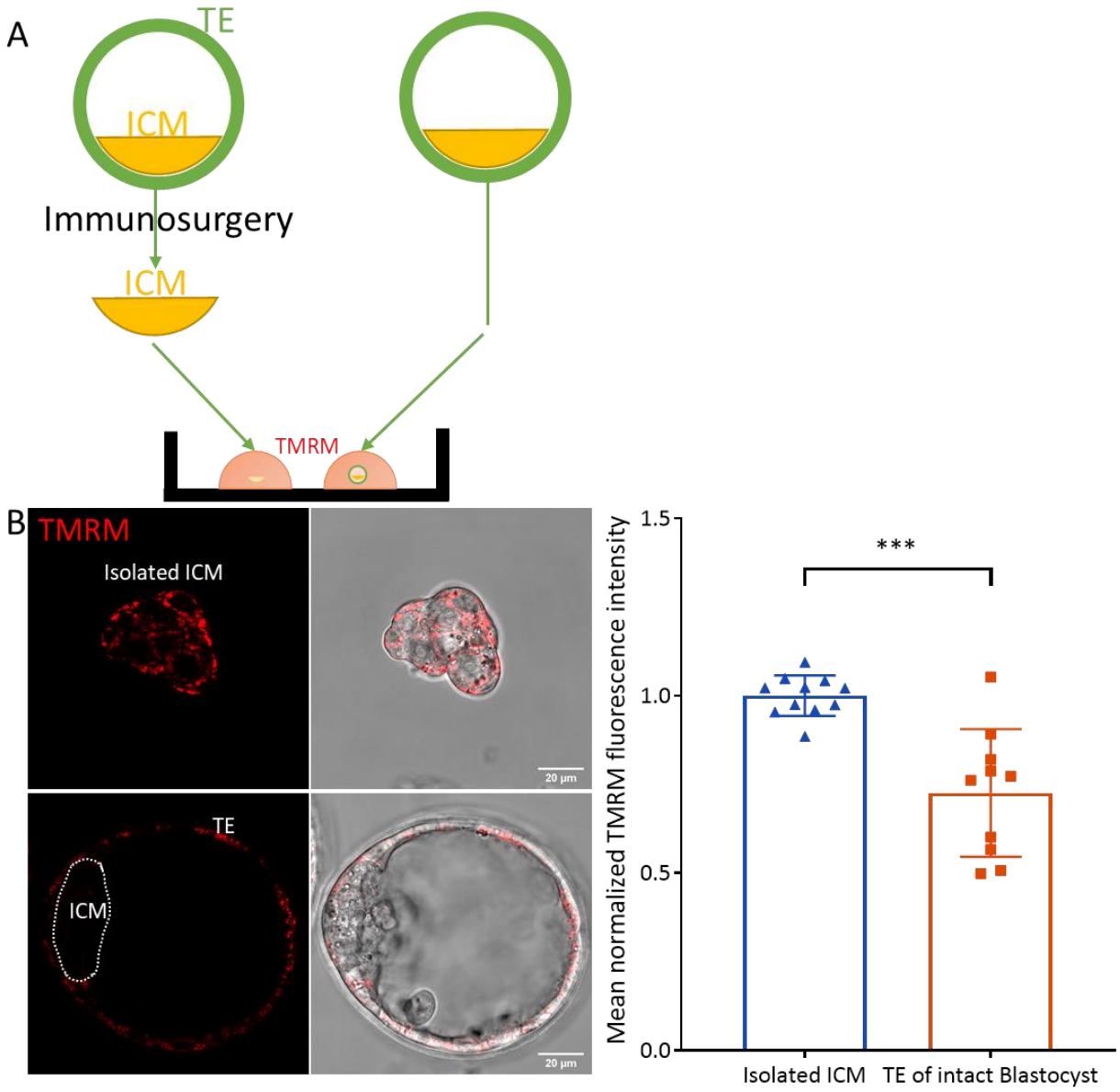

Figure 4.3. Isolated ICM by immunosurgery shows higher TMRM intensity than TE of intact blastocyst. A) Schematic of TMRM comparison between isolated ICM and TE of intact blastocyst. B) Isolated ICM (n = 11) and whole blastocyst (n = 10) were labelled with TMRM (25nM). Bar graph shows mean normalized TMRM fluorescence intensity of the two groups. Data shown as Mean ± SEM. Two experiments. Two-tailed T-test. ***, $p < 0.001$.

4.3.3 TMRM (high concentration) injection from ICM side shows higher MMP in TE

The blastocoel is bordered by the TE cells and as well as ICM cells, thus it will be ideal if TMRM was loaded from inside of the blastocyst. The previous method used to inject TMRM into the blastocoel was via insertion of the microinjection pipette through the TE, opposite to the ICM. The damage caused by the microinjection pipette led to the collapse of the blastocyst. In an effort to avoid the collapse, I injected TMRM into the blastocyst through the ICM side (Figure 4.4A). This approach prevented blastocyst collapse with the result that TMRM could access both TE and ICM from the blastocoel. Importantly, no TMRM was present in the drop so all loading occured via the injected TMRM. As shown in Figure 4.4B, TE cells showed higher TMRM/Dendra ratio than ICM cells, suggesting TE cells have higher mitochondrial activity than ICM cells. However, access of TMRM to the ICM relative to TE remains in question because the ICM is covered by a layer of primitive endoderm which on close inspection has a higher level of MMP. To date my experimental approaches have not definitively elucidated if the apparent low MMP in ICM of intact blastocysts reflects the actual physiology, or is simply caused by the ICM having reduced access to TMRM relative to the TE than the neighbouring ICM.

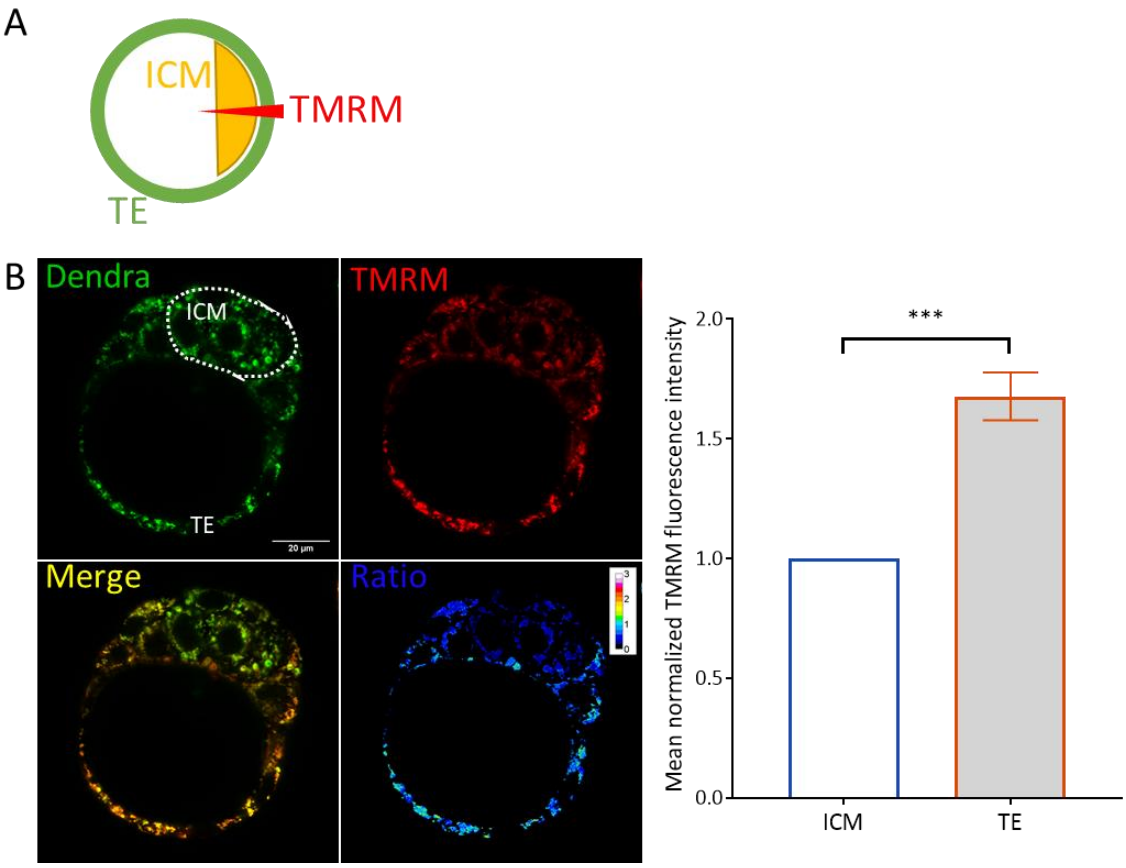

Figure 4.4. TMRM injection from ICM side into blastocoel shows higher TMRM/Dendra ratio in TE. A) Schematic of how TMRM was injected into blastocoel. B) Blastocyst from mito-Dendra mice was injected with TMRM from ICM side. Data shown as Mean ± SEM. One experiment. n = 5 embryos from one mouse. Paired T-test. ***, $p < 0.001$.

4.3.4 Blastocysts matured *in vitro* from 8-cell embryos show no difference in TMRM intensity between ICM and IE

To further optimize the methodology for comparing mitochondrial activity between ICM and TE of blastocysts, *in vivo* 8-cell embryos were collected at day E2.5 and labelled with 50nM TMRM. Then embryos underwent time lapse confocal imaging with 10nM TMRM in the medium (Figure 4.5A). In this scenario, all the blastomeres have similar access to TMRM at 8-cell stage and the presence of TMRM in the medium will ensure equilibrium will persist through the duration of the transition from 8-cell to blastocyst. It is anticipated that loading the blastomeres prior to blastocyst formation will avoid the question of reduced TMRM access to the ICM, as well as the disrupted physiological environment experienced by isolated ICM cells. As such it may provide the best possible effort to determine if there is a difference in the MMP of ICM and TE. As shown in Figure 4.5B, the 8-cell shows similar TMRM intensity among blastomeres. After 24 h *in vitro* culture, embryos that reached blastocyst stage exhibit comparable TMRM intensity between ICM and TE (Figure 4.5C&D). This result provides the best evidence to date that ICM and TE MMP are similar and that prior measurements in this Chapter with TMRM, and in previous studies with JC-1[150], may be compromised by issues of indicator access to the ICM cells. Although this approach avoids the TMRM access question, I cannot definitively conclude from

this experiment that ICM cells have a lower MMP than TE because it remains a formal possibility *in vitro* culture from the 8-cell stage to blastocysts causes an increase MMP in the ICM relative to TE. Further approaches down this line using a ratiometric approach and improved culture conditions may help to alleviate these concerns and allow a more definitive answer to the question of MMP in the early embryonic lineages.

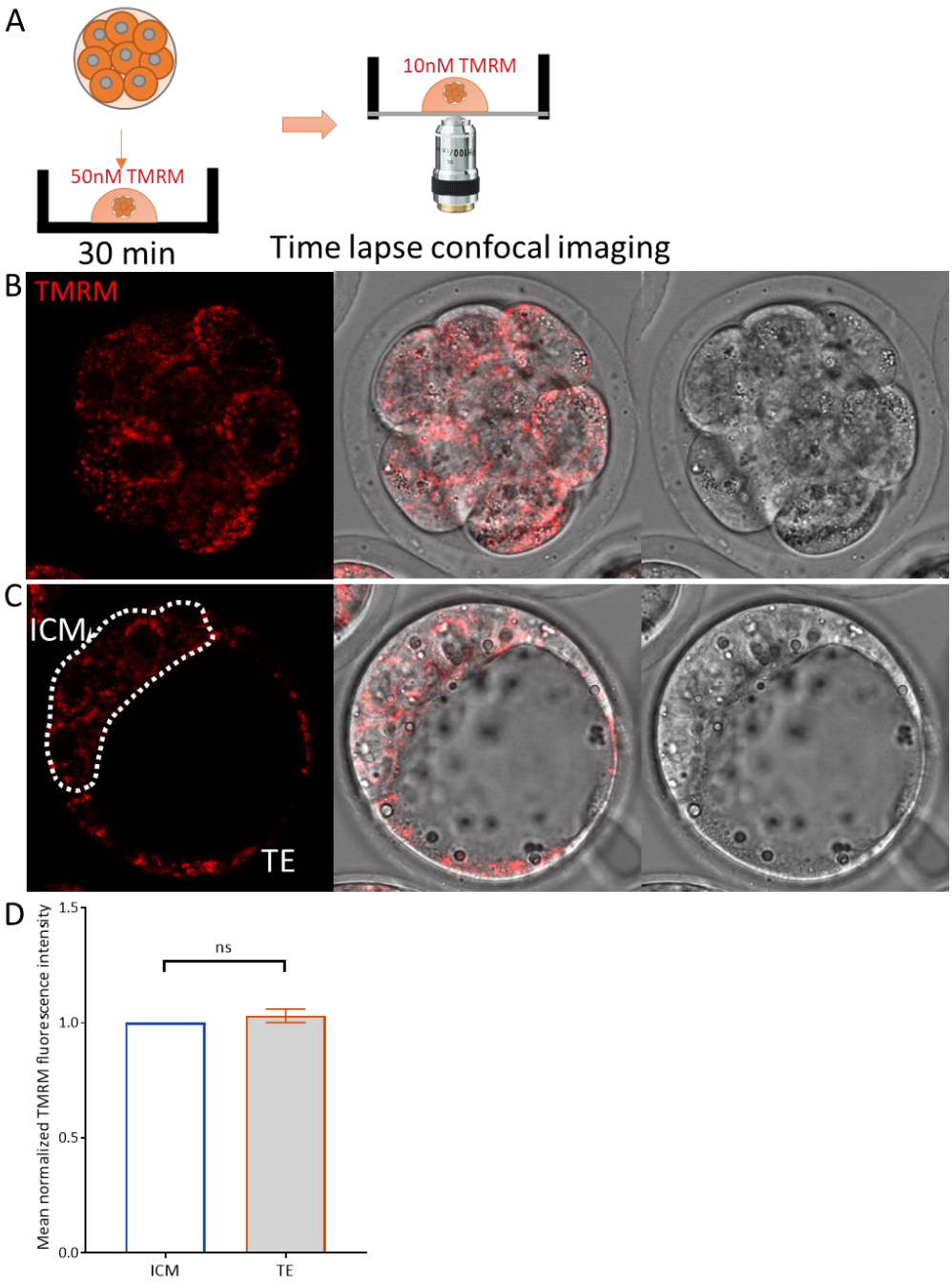

Figure 4.5. Blastocyst matured *in vitro* from 8-cell embryos shows no difference in MMP between ICM and TE. A) Schematic of TMRM loading. B) Eight-cell embryo labelled with TMRM. C) Blastocyst derived from pre-TMRM labelled 8-cell embryo *in vitro*. D) Mean normalized TMRM intensity of ICM and TE. n = 21, paired T-Test.

4.3.5 Competent 8-cells show lower TMRM intensity after compaction than abnormal 8-cells

In experiments monitoring MMP during the 8-cell to blastocyst transition, it was noted that, similar to oocytes, where a small number fail to mature, some of the 8-cell embryos do not develop into a normal blastocyst during time lapse confocal imaging. Based on 3 repeated experiments, in total 23 out of 45 8-cells formed normal blastocysts, whereas the rest 22 embryos were arrested at 8-cell or morula stage or formed a morphologically abnormal blastocoel (Figure 4.1A). Interestingly, normal and abnormal embryos showed different pattern of TMRM intensity during the *in vitro* development. As shown in Figure 4.2B&C, both embryos show an increase in TMRM intensity in the first few hours. However, the TMRM intensity of normal 8-cells peaks prior to those abnormal embryos and starts to decrease at around 9 h after imaging, whereas TMRM intensity of abnormal embryos peaks around 8 h and is always higher than that of normal embryos. These results show that mitochondria of competent embryos are less active than those of abnormal embryos, suggesting that a relatively quiet mitochondrial state might be ideal for subsequent development.

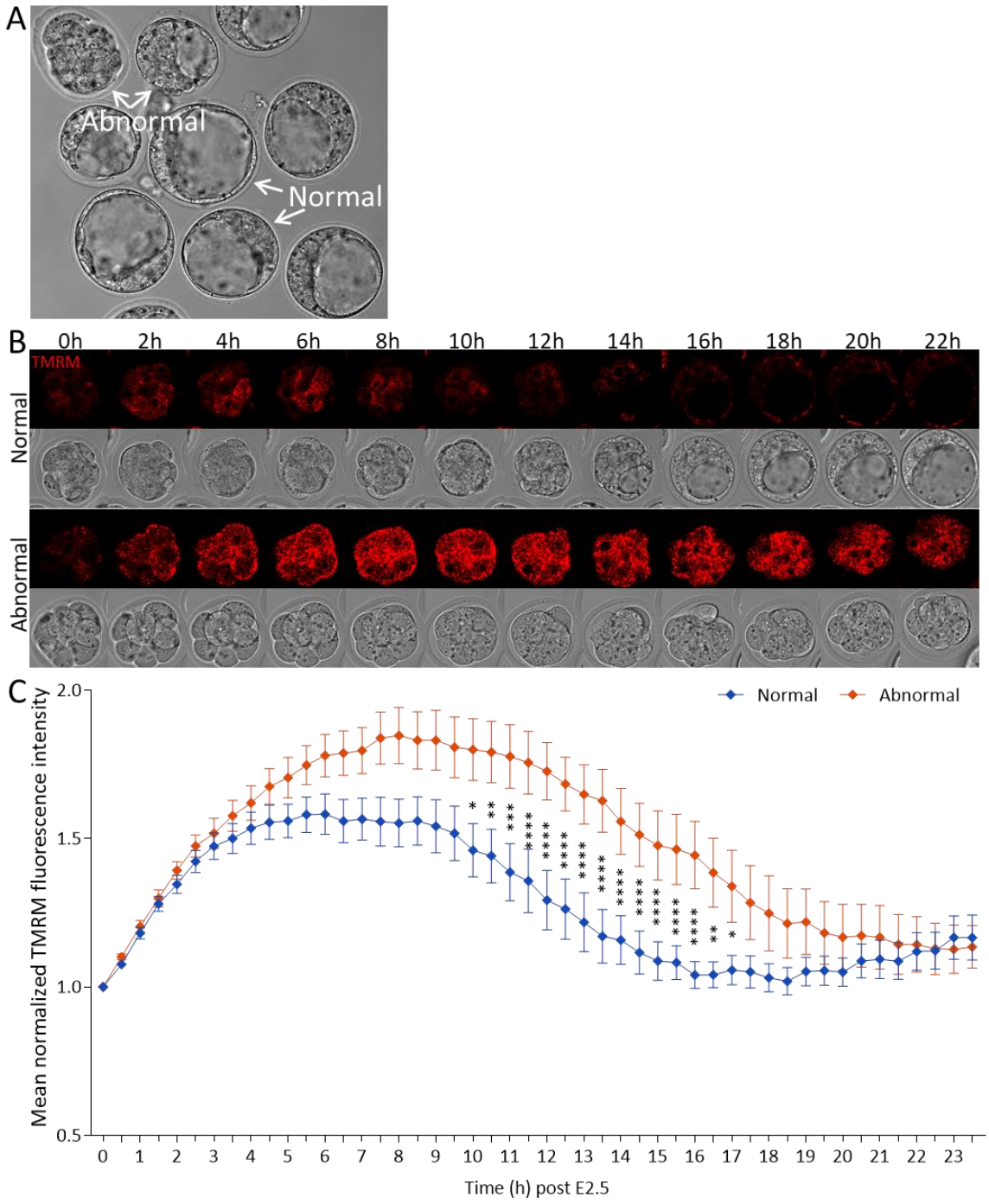

Figure 4.6. Mitochondrial activity from 8-cell to blastocyst stage. A) Bright field image showing embryos after 24 h time lapse confocal imaging. B) Representative images of morphologically normal and abnormal embryos. C) Mean normalized TMRM intensity of normal (n = 23) and abnormal embryos (n = 22). Two-tailed T-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

4.4 Discussion

This chapter focuses on mitochondrial activity heterogeneity during early embryonic development, a phenomenon described in the first experimental chapter, where mitochondria in a single oocyte can be different in their activity according to their location. Here a number of methods have been attempted to compare the difference in mitochondrial activity between ICM and TE and each method has their own drawbacks and ultimately provide different results. Labelling TMRM from outside or inside of the blastocoel shows higher TMRM intensity in TE than ICM where labelling TMRM in the blastomeres at 8-cell stage shows similar TMRM level.

With the development of early embryo, blastomeres start to differentiate from 8-cell stage, after which they will develop into two distinct cell types, ICM and TE. Starting with direct labelling of TMRM on Dendra oocytes to enable ratiometric analysis, the first trial gives consistent results with the JC-1 study, however, less staining of MTG in the ICM than TE raised the concern about the penetration of TMRM. Because ICM is completely surrounded by TE and TMRM is in the medium drop, TMRM equilibrates into TE before the ICM. It is not clear if it is the structure of blastocyst that causes the difference in TMRM and MTG signal between the two cell types, but loading TMRM from the medium drop may not label both cell groups at the same time, or to the same level.

To determine an optimal approach to label blastocysts with TMRM, a number of methods were attempted, including. injection of a relatively high concentration of TMRM from the ICM side into the blastocoel without causing collapse of the blastocyst. A similar method has been applied to injection of embryonic stem cells (ESCs) to create genetically modified mice, where injection through the ICM side leads to higher chimerism rates than the traditional method, which injects ESCs through the opposite side to ICM [319]. This method leaves TMRM in the blastocoel fluid and both ICM and TE can access to TMRM. The result is consistent with the direct loading method, but the difference in TMRM/Dendra ratio between ICM and TE is smaller via the injection method, which indicates this method does optimize the equilibration of TMRM to both cell types. However, it is observed that a layer of cells, the primitive endoderm, between the blastocoel and ICM also show higher MMP than ICM. It is still unknown whether the cell membrane of ICM or endoderm are the same. If they have different permeability to TMRM, if so, this indicator-based loading may not be appropriate for mitochondrial comparison across multiple cell types in the blastocyst.

To avoid potential permeability difference to TMRM between ICM and TE, TMRM was loaded at 8-cell stage, when each blastomere is identical to others. They show similar TMRM intensity and mitochondrial distribution, and most importantly, the dye is retained in the mitochondria of blastomeres and follows the

division of blastomeres until blastocyst stage. Following live cell imaging on the embryos, the TMRM intensity between ICM and TE of those normal blastocysts was compared. Different from the previous study [150] and results from this chapter, which suggest that TE has higher mitochondrial activity than ICM, here I found that the TMRM intensity is similar between ICM and TE. However, this method also has its disadvantages. Previous study shows that *in vitro* produced blastocysts have higher mtDNA copy number and ATP content than those derived *in vivo* [320]. Thus, the blastocysts derived from *in vitro* culture may have different mitochondrial states with those grown *in vivo* and heterogeneity in mitochondria activity could also be different. The actual physiological difference in mitochondrial activity between ICM and TE will need more investigation. Future study may employ genetically encoded fluorescent sensors to investigate the difference in mitochondria activity more precisely, for example, ATeams, a series of fluorescence resonance energy transfer (FRET)-based indicators for ATP, which enables the visualization of ATP levels within single living cells [321]. A similar biosensor has been successfully applied to oocytes but not early embryos [234]. If the ATP level between the two cell types shows similar difference with the TMRM intensity, then the next question is when and how the difference is formed. One possibility is that the metabolism is different in ICM and TE cells. It has been shown that TE cells have higher oxygen consumption, ATP production

and total amino acid turnover than ICM cells [322], suggesting that mitochondria in TE cells are more active than those in ICM. This upregulation of metabolism in TE might be powered up by TEAD4, a transcription factor, which promotes mitochondrial transcription in TE cells [323]. Another possibility could be mitochondrial sorting, a process by which highly polarized mitochondrial are translocated to blastomeres which will form the TE. It is challenging to test this possibility as *in vivo* imaging of the early embryonic development is nearly impossible and *in vitro* imaging may cause side effects on embryos as mentioned above.

Interestingly, during time lapse confocal imaging of 8-cell embryos, I found that competent embryos experienced an increase in TMRM intensity as soon the imaging started and the intensity reached the highest after 6 h. Since the embryos were imaged in a drop containing 10nM TMRM, the increase in TMRM intensity might be contributed to the continuous uptake of TMRM into the embryos and it reached saturation after around 6 h. Test using the same condition on GV oocytes also showed increase in TMRM intensity, but the signal peaked after only 3 h (data not shown), suggesting that the increase observed in the embryos may correlate with an elevation of mitochondrial activity. Considering the embryos were cultured *in vitro* and underwent intermittent confocal imaging, they might be stressed and mitochondria were boosted to meet extra energy requirement. Similar

high mitochondrial activity was also found in *in vitro* matured oocytes compared to *in vivo* matured ones as shown in Figure 2.5 in Chapter 2. To further investigate the reason why the TMRM intensity increases, future study may compare the MMP between *in vivo* and *in vitro* grown embryos at the same time point. After reaching the peak, the TMRM intensity of those competent embryos underwent a dramatic drop which happened a few hours before the cavitation. This might be due to the switch of ATP production from OXPHOS to glycolysis [324], which results in the decrease in mitochondrial activity. Compared to the competent embryos which developed to blastocyst normally, those embryos ended up arrested or forming abnormal blastocysts exhibited higher TMRM intensity, which is opposite to the findings in oocyte *in vitro* development, where competent oocytes that reach maturation show lower mitochondria activity than those arrested at metaphase stage. This might be an evidence to the "quiet embryo hypothesis" [279] and this quiescence may be important for the subsequent development. Interestingly, the TMRM intensity of those competent embryos dropped to a similar level to the initial TMRM intensity when the embryos were first imaged. One explanation could be that mitochondria are adapting themselves to the new environment and recovered their activity to the initial level after a certain time. It is observed that those abnormal embryos that formed the blastocoel quite late or formed a smaller blastocoel also displayed a decrease in TMRM

intensity at some time point before the blastocoel formed, suggesting that there might be a correlation between mitochondrial quiescence and blastocoel formation.

4.5 Summary

The transition from the 8-16 cells to blastocyst stage of preimplantation development is the earliest stages of mammalian cellular differentiation. Considering the fate of ICM and TE, this transition could be an opportunity for the embryo to exclude detrimental cellular components out of the fetus. In this chapter, differences in mitochondria activity between ICM and TE was investigated. However, due to the controversial results derived from different methods, it is hard to draw a definitive conclusion. Further optimizations need to be done on the methodology for comparing the mitochondrial activity between the two cell types. Embryo *in vitro* competences may be linked to mitochondrial activity, which could be a criterion for accessing embryo quality in assisted reproductive technology.

Chapter 5. Conclusion

Mitochondria have been shown to be involved in oocyte and embryo developmental competence [305, 325, 326] and their distribution, activity and structure are related to cellular functions. Mitochondrial heterogeneity has been reported in many cell types and this feature is important for transcript elongation rate and protein noise, and also prediction of cell cycle duration [199-201], but it is not well studied in oocytes and embryos. In this thesis, heterogeneity in mitochondria activity in single oocyte and blastocyst was investigated.

To precisely measure mitochondrial heterogeneity in activity in single oocytes, TMRM/Dendra ratiometric analysis was developed, which can eliminate the inaccuracy introduced by mitochondrial clusters. It can successfully distinguish the difference in mitochondrial activity at both inter- and intracellular level. The observation of the difference in MMP among oocytes that matured from different methods has led to the further investigation into the relation between mitochondrial activity and oocyte *in vitro* competence. The results showed that oocytes that can mature at the end of the *in vitro* culture have higher mitochondrial activity at their GV stage and this high level of MMP needs to be maintained during the *in vitro* growth. This means MMP is linked to oocyte competence during *in vitro* maturation and to overcome the difference between *in vitro* and *in vivo* environment, only oocytes with more robust mitochondria can successfully

complete maturation. These results may provide guidance for developing novel markers to distinguish highly competent oocytes during ART. For example, developing an indicator that is not toxic to the oocyte and also enables visual comparison on MMP between competent and non-competent oocytes would be a significant benefit for selecting optimal oocytes in ART. Also, for those oocytes which failed IVM, treating them with antioxidants that can boost mitochondrial activity might provide a novel approach to improving oocyte quality.

It is also observed that mitochondria undergo dramatic redistribution during *in vitro* maturation, specifically they form a mitochondria envelope around the MI spindle, which is consistent with previous studies [155, 156]. Interestingly, during *in vivo* maturation, mitochondria also form clusters but no such envelope was found around the MI spindle. Comparison in the proportion of mitochondria around MI spindle between MII competent and MI arrested oocytes indicates the importance of such mitochondrial enveloping around the spindle. One explanation could be that spindle formation and migration demands relatively high levels of ATP, which recruits a large proportion of mitochondria around the spindle area, especially in the absence of support from cumulus cells. With the ratiometric analysis, mitochondrial heterogeneity in activity was examined in oocytes at different stages. Results suggest that mitochondria around nuclear DNA area are more active than those more distant from the nuclear DNA, which might be an

adaptation to the high energy demand required by nuclear events. However, the mechanism behind this spatial dependent distribution of mitochondria with different activity is unknown. Whether it results from an upregulation of mitochondrial activity after accumulating around the spindle or is due to highly polarized mitochondria being relocated to the spindle area along microtubules will need more investigation. The importance of this potentiometric activity based mitochondrial distribution is also demonstrated during *in vitro* maturation, whereby competent oocytes usually exhibit such MMP-based mitochondrial localization but MI arrested oocytes do not. This is the first time that this mitochondrial heterogeneity in activity and its relationship with oocyte competence was reported. With the development of non-invasive MMP detection, this feature could become a criterion for selection of highly competent oocytes in IVF procedures.

The second experimental chapter of this thesis established an NGS protocol on mtDNA from oocytes and used this technique to investigate the basis of aging-associated mtDNA mutations in oocytes. Sequencing was first done on single oocytes from both young and old mice. As reported previously, old oocytes carry more mtDNA mutations and higher mutation frequency, but a synonymous mutation which was never reported before, was found in old oocytes. Indels are also found in both groups. Although there is no difference in the number of Indels,

old oocytes do have higher frequency, which suggests aging might deteriorate the existing mtDNA insertions or deletions. Other research in a mouse model which has the same genetic background as these old mice did not find any deletions using NGS on oocyte mtDNA [135], hence further experiments are needed to confirm our novel mtDNA Indels and investigate their potential effects on oocyte competency. Since aging has been shown to be related to mitochondrial dysfunctions, including disrupted mitochondrial fission [143] and reduced mtDNA copy number [209-211], the hypothesis was that these aging related mitochondria dysfunction might cause mtDNA mutations. However, by sequencing oocytes from two mouse models – Drp1 KO and Tfam KO mouse, which simulate mitochondrial fission deficiency and copy number reduction, respectively, no elevated mtDNA mutation load was detected, suggesting that aging induced mtDNA mutations may not be the consequence of defective in mitochondrial fission or decrease in mtDNA copy number. To further study the correlation between mtDNA mutation and aging, mutator mouse, in which the mtDNA proofreading function is silenced, was utilized. The facts that $Polg^{mut/mut}$ mice have been demonstrated to have a premature aging syndrome [134, 316] and physiological aging can cause mtDNA mutations have led to the hypothesis that mice with advanced age and $Polg^{mut/mut}$ mice have in common a spectrum of mtDNA mutations that contribute to poor oocyte quality. Results show that

deficiency in mtDNA proofreading can not only cause de novo mtDNA mutations, but also deteriorate the pre-existing mtDNA deletion at position 4051, which is a non-synonymous variant and has 'HIGH' effect on protein coding. The other pre-existing mtDNA deletion at position 5171 is not elevated in $Polg^{mut/mut}$ oocytes but was observed to be aggravated in old oocytes. These results suggest that there is no similarity in mtDNA mutation spectrum between the old and mutator mice, which means the premature phenotype found in the mutator mouse may not be a good model for aging studies. But the high mutation load in mutator oocytes can be utilized to investigate how mtDNA mutations are inherited and what is the mechanism behind the purifying selection that was reported previously [111]. This chapter allowed me to learn the complete mtDNA sequencing procedure, including some useful software packages for bioinformatics, which can be applied to future projects.

Finally, utilizing the ratiometric analysis combined with a few different methods to label MMP, the difference in mitochondrial activity between ICM and TE was studied. Although this question has been addressed around two decades before, the dye it employed has been recently proven to have some limitations [202]. A different dye was used in the last chapter and multiple labelling methods were attempted. However, the results varied depending on how TMRM label was applied. Loading TMRM to the blastocyst directly from the drop or from the

blastocoel shows higher MMP in TE than ICM; Staining ICM isolated by immunosurgery with TMRM shows higher MMP in ICM than TE of intact blastocyst; Labelling TMRM from 8-cell stage followed by live cell confocal imaging shows similar MMP between ICM and TE. Each method has its disadvantages as discussed, thus, to confirm the heterogeneity pattern of mitochondrial activity in blastocyst, further optimization or alternative markers are needed to tackle this issue. One solution could be endogenously tagged fluorescence markers that can reflect mitochondrial activity, for example, ATeams, which enables the visualization of ATP levels within single living cells [321]. It will also be interesting to see if embryos are able to sort bad mitochondria during the first differentiation to the TE rather than ICM, including depolarized mitochondria and mtDNA mutations. This could act as a quality control system in excluding detrimental mitochondria from the offspring. Time lapse confocal imaging also revealed that there is a correlation between mitochondrial activity and embryo competence where quiescence in mitochondria would benefit embryo development *in vitro*. Again, with innovations in visualizing mitochondrial activity, this could become one of the aspects included in assessments of embryo competency in ART.

In summary, based on the findings in this project, a number of follow up studies are indicated. For example:

1. Development of an indicator of mitochondrial activity which can be readily visualised and has no effect on oocyte in vitro development.
2. Modify culture medium to adjust mitochondrial activity to examine if it leads to improved in vitro maturation.
3. Utilize mutator mice as a model and introduce mtDNA mutations to WT zygotes by cytoplasm transfer, and compare the mtDNA mutation load between ICM and TE at the blastocyst stage. This will test if there is a sorting based quality control system on mtDNA integrity during early embryonic development.
4. Study the correlation between MMP and early embryo development and test if MMP can act as a proxy for embryo competence.

In conclusion, mitochondrial activity is adaptive to oocyte maturation and early embryo development. The new approach of measuring MMP allows comparison of mitochondrial activity at an intracellular level. Mitochondrial heterogeneity in activity revealed by this method has uncovered the spatial-temporal regulation of mitochondrial function in mammalian oocytes and possibly early embryos. The correlation between mitochondrial activity and development competency may benefit the clinical selection of high-quality oocyte and embryo. Mitochondrial DNA mutations were found in oocytes from old or mutator mice but not mice with mitochondrial fission deficiency or mtDNA amplification defect. The mutation

spectrum is completely different between old and mutator mice, suggesting mutator mouse may not be a good model of aging, but the high mutation load in mutator mice could be a good model for studying mitochondrial heterogeneity in mtDNA integrity and mtDNA sorting during early development.

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