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Review

The Role of Mitochondria in Oocyte and Early Embryo Health

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Abstract

The mitochondria of the oocyte are a prominent source of energy metabolism as well as mitochondrial DNA that will later populate the cells of the offspring. Recent discoveries provided new insight into the physiology of the mitochondria and its unique genetics. The concept of heteroplasmy defined as the presence of more than one type of mitochondrial genome, is gaining increasing recognition as an important contributor to several complex morbidities, age-related reproductive dysfunction and aging. Understanding the changes caused by pathogenic mutations as well as identifying defects occurring during reproductive aging will enhance our knowledge of the role of mitochondria as organelles in germ cell biology. In this review, we summarize the current state of knowledge about the role of mitochondria in embryo and fetal development.

Keywords
Mitochondria; mtDNA; mitochondrial dysfunction; oxidative phosphorylation; heteroplasmy; mitochondrial transfer

1. Introduction

The oocyte has the largest number of mitochondria and mitochondrial DNA (mtDNA) copies per cell, $2x10^5$, which is significantly more than other cell types with high energy requirements such as muscle cells and neurons that possess several thousand copies [1]. The mitochondria in the oocyte have two major roles; one is the maintenance of the oocyte's metabolic needs coupled to regulation of cell death, and the other is to serve as a reservoir of intact mtDNA for the future developing offspring.

Oogenesis, the process of oocyte development, takes several stages and can span over a few decades [2]. Oogenesis starts in fetal life with the transition of the primordial germinal cells (PGCs) into dividing oogonia that later produces the primary oocyte. It is estimated that the oogonial ovarian pool is derived from as little as 50 PGCs that migrate from the base of the allantois through the primitive streak and into the female genital ridge. The migration and concomitant rapid mitotic divisions of the PGCs lead to an increase in the number of oocyte precursors – "oogonia". In humans, this process results in formation of 6-7 million primary oocytes [3, 4]. During these divisions, the original mitochondrial pool is partitioned between the oogonia; subsequent replication of the mtDNA then stabilizes the mtDNA pool [5-7]. These oocytes then remain arrested in the prophase of the first meiotic division until ovulation, which can occur up to five decades after original entry to meiosis. The supporting somatic component of the follicle that surrounds the oocyte, known as granulosa cells, originate from the ovarian surface epithelial cells [8]. These cells form the inner layer of the follicle and play an important role in the development and maturation of the oocyte as well as in the endocrine signalling function of the follicle. Like oocytes, the pre-granulosa cells are formed during fetal life and remain relatively inactive for many years until the recruitment of the follicle into the growing follicle pool commences. The innermost layer of granulosa cells that surrounds the oocyte, the cumulus oophorus, maintain direct cytoplasmic communication with the oocyte until the time of ovulation. These serve as a conduit for the exchange of nutrients and signalling molecules essential for the oocyte growth [9]. The recruitment of the dormant follicles into the growing cohort is marked by a rapid oocyte growth spurt with a dramatic increase in its size. This process is accompanied by a profound increase in the mitochondrial mass, to an average of ~ 200,000 copies at the time of ovulation[10]. This large number of mitochondria is reached gradually during oocyte maturation and involves a strictly regulated and coordinated replication of the mitochondria and its mtDNA.

Oxidative phosphorylation (OXPHOS) is the main source of ATP in the oocyte, similarly to other high energy consuming cells such as neurons and cardiomyocytes. The cumulus cells (CC) surrounding the oocyte uptake and metabolize glucose to provide the oocytes with products of glycolysis [11, 12], namely lactate and pyruvate, as substrates for mitochondrial ATP generation via the citric acid (TCA) cycle [13, 14]. Glycolysis in CCs is stimulated by paracrine factors secreted by the oocyte; BMP15 and GDF9[15]. OXPHOS involves the action of the mitochondrial respiratory

electron transport chain (ETC) consisting of five complexes which are located on the inner mitochondrial membrane (see Figure 1). An alternative fuel source for TCA cycle in fully grown oocytes is breakdown of lipids, which generate ATP within mitochondria using oxidation [16].

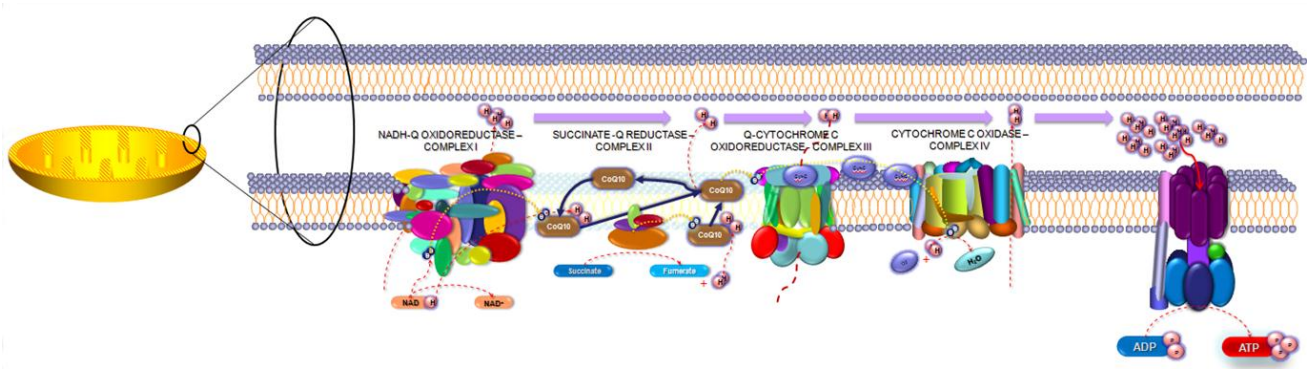

Figure 1 The mitochondrial electron transport chain. The mitochondrial respiratory chain is organized in five complexes situated in the inner mitochondrial membrane. Complex I: NADH-Q oxidoreductase, complex II: Succinate-Q reductase, complex III: Q-cytochrome c oxidoreductase, complex IV: Cytochrome c oxidase, and complex V: ATP synthase. The central role of coenzyme Q 10 (CoQ10) in both electron and proton transport is highlighted (This figure is reproduced with permission of the publisher, [17]).

During oocyte maturation, the respiration rate is low with only a small percentage of the mitochondria active, yielding a low steady state generation of ATP as well as low but detectable production of mitochondrial ROS [18]. During metaphase II arrest and with the resumption of meiosis, ATP generation and consumption is dramatically elevated, which is reflected in ROS accumulation [19].

In the last two decades, mtDNA has become a focus of intense research related to cell signaling, survival and senescence. The interaction of mtDNA with nuclear DNA is among the most important examples of genome synergism and co-evolution. Also, mtDNA was proven to be one of the most useful tools in population genetics and molecular phylogenetics. In this article we review the current knowledge on mtDNA and discuss briefly how its structure, function and transmission effect oocyte function and embryo development in human and in other species. While major advances have been made in better understanding the function of mitochondria, important questions remain regarding interventions to improve mitochondrial function and their role in the optimization of reproductive outcome.

2. Mitochondrial DNA and the Uniparental Inheritance of the Mitochondrial Genome

Mitochondria are believed to originate from a prokaryotic ancestor that became an eukaryotic symbiote and has lost most of its genetic information during the course of evolution, keeping only a small fraction of the genes separate from the nuclear DNA [20, 21]. The human mitochondrial genome is a double-stranded, circular DNA containing 16,569 base pairs of organized nucleotides which, unlike nuclear DNA, is not bound by histones [22]. Another major difference is the lack of introns, non-coding intergenic sequences, and significant 5’ and 3’ untranslated regions (UTRs) [23]. Figure 2 depicts the organization of 37 mtDNA genes; 13 encoding protein components of

ETC, 22 transfer RNAs and 2 ribosomal RNAs [17, 24]. Out of the 1500 proteins that are required for mitochondrial function, only 13 are encoded by the mitochondrial genome. These mitochondrial genes likely persisted in the mitochondria as a separate genome due to the highly hydrophobic nature of their protein products, limiting the ability to transport them to the mitochondrial matri. Furthermore, this will allow local immediate regulation [25].

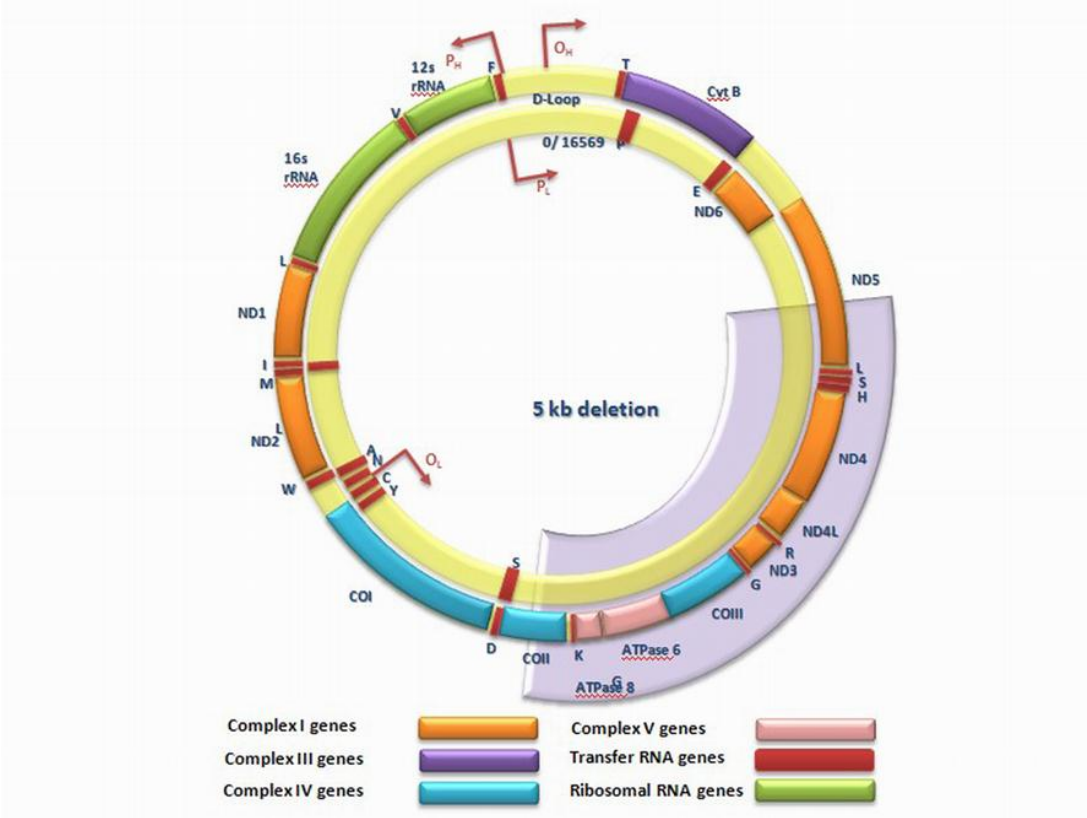

Figure 2 Mitochondrial DNA. The double-stranded circular mitochondrial genome. Genes locations is color-coded. The site of the common deletion is demonstrated by the arch on the lower right side of the drawing. The colours of the mtDNA genes notes their function as detailed above the drawing (This figure is reproduced with permission of the publisher, [17]).

An alternative explanation for the migration of mitochondrial genes to the nucleus may be an attempt to limit damage of vulnerable genes. Whereas the genes retained in the mitochondria would have the ability to immediately sense and respond to the change in redox state and bioenergetics, being near the site of OXPHOS may increase the likelihood or damage by reactive oxygen species (ROS), a byproduct of OXPHOS [26, 27].

The inheritance of mtDNA is almost strictly maternal as sperm mitochondria are ubiquitinated and degraded via proteasomes combined with autophagy following their entrance to the oocyte [28]. Sato & Sato, in their elegant work on a C. elegans model, showed that localized and specific autophagy of paternal mitochondria is triggered by the penetration of sperm into the egg and independent of onset of anaphase. They also showed that inhibition of paternal mitochondria autophagy resulted in retained paternal mitochondria that were proliferation and fusion inactive, thereby adding another mechanism for their selective elimination by dilution [29]. Recently

several reports described rare paternal mtDNA transmission (termed paternal leakage in both human and non-human organisms, however, its potential contribution to a pathophysiology is yet to be determined [30, 31]. The uniparental inheritance of the mitochondrial genome protects the embryo from the deleterious mutations in sperm mtDNA that result from exposure to high oxidative stress during spermatogenesis and ensures homoplasmic offspring that have a single type of mtDNA [32, 33]. However, due to the uniparental pattern of mtDNA inheritance, mtDNA lacks the benefits of recombination and the presence of two alleles that control the phenotype. As a result, several mechanisms evolved to improve the quality of mitochondria and minimize the transmission of pathogenic mtDNA to the offspring. These include the bottleneck, fusion/fission, as well as mitophagy which will be discussed later. Pre- and a post- fertilization mechanism exist to reinforce the homoplasmic state both in the male and female germline. The elimination pathways responsible for clearance of paternal mitochondria upon fertilization are still unclear, but likely involve a combination of lysosomal activity and digestion of mtDNA by mitochondrial endonucleases prior to fertilization and sequestration in specific blastomeres [34]. The paternal mitochondria are ubiquitinated during spermatogenesis and are eliminated during early embryogenesis [35]. This mechanism is believed to prevent the transmission of paternal mitochondria which often contain a severely damaged mtDNA due to the extensive use of the mitochondria to produce the energy needed for sperm motility. The elimination of the paternal mitochondria should create a state of relative maternal homoplasmy in which most or all the mitochondria share the same mtDNA unless mutations in mtDNA arose during oogenesis or escaped selective elimination during germline commitment and expansion. However, variation in the contribution of paternal lineage to the mitochondrial pool has been reported in some mammalian species and interspecies hybrids [36, 37].

There is a large inter-oocyte variability in mtDNA content [1] with either too few or too many mitochondria implicated as hallmarks of poor embryo viability [38, 39]. Wei et al. presented convincing “threshold data” on the minimal mtDNA copy number requirements. This data clearly shows the need for ~ 50,000 copies to support the post-implantation embryo development [40]. However, oocytes with as few as 4,000 copies can survive until ovulation and might even be fertilized. Upon ovulation, the mtDNA replication pauses and organelles are partitioned through several rounds of cell divisions into individual blastomeres. The total number of mtDNA copies per embryo does not change until the blastocyst stage (day 5-6 embryo) despite numerous cell divisions, resulting in a progressively declining mtDNA content in each of the blastomeres [41].

3. Mechanism to Improve Mitochondrial Integrity and Purity

3.1 Elimination of Impaired Mitochondria - The Bottleneck Theory

The mitochondrial bottleneck theory was developed in order to explain the observations of intergenerational changes in the diversity of mtDNA and is currently perceived as one of the mechanisms to improve mtDNA integrity and purity (see Figure 3). The pre-fertilization bottleneck occurs during oogenesis, where the number of mitochondria in the germline is severely reduced before maturation of the oocyte. The post-fertilization bottleneck occurs between the zygote formation and the blastocyst stage, during which there is intense cell division but suppression of mitochondrial proliferation, a mechanism that leads to a reduced number of mitochondria per cell [5, 42-44].

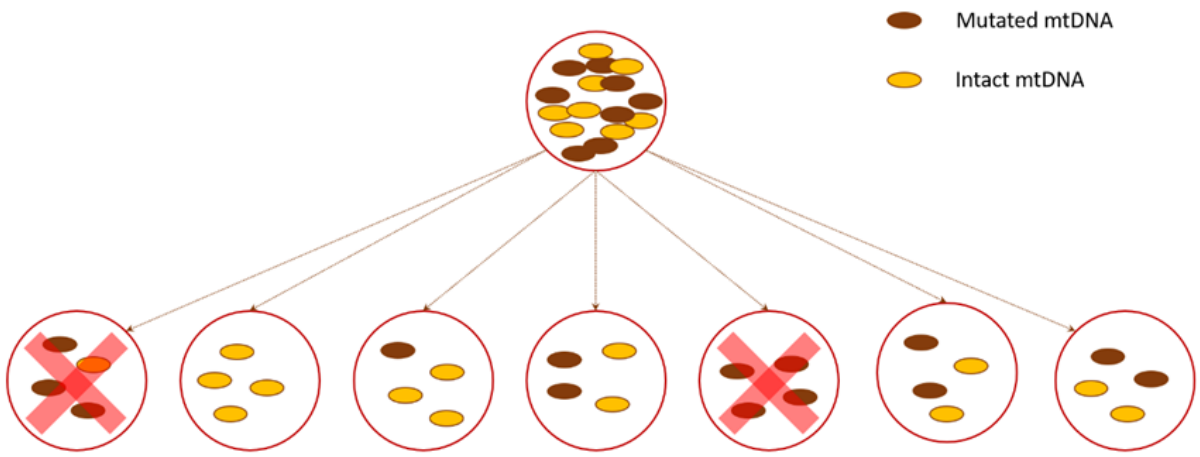

Figure 3 The bottleneck theory. The theory describes the process of elimination of germ-cell precursor cells containing a large ratio of mitochondria containing mutated mtDNA by an extreme reduction in the number of mitochondria to offspring cells leaving a minimal number of mitochondria that is needed to support the metabolic need of the cell. However, if the cell contains a large percentage of dysfunctional mitochondria containing deleterious mtDNA mutation it does not survive thus eliminating the cell and its mtDNA content from the pool of oocytes [45].

The mtDNA is vulnerable to mutations due to its structure and the lack of efficient repair mechanisms. Some mutations may be pathogenic and are associated with maternally inherited complex diseases. The expression of symptoms related to mitochondrial dysfunction depends on the severity of the point mutation, the percentage of the heteroplasmy and the level of energy consumption of the tissues. Therefore, since the brain and muscle tissues have the highest energy consumption, they are often the first to be affected. However, it is not uncommon to find individuals who carry a small percentage of mtDNA that differs from the rest of their mtDNA pool; these are termed low-heteroplasmy variants [46]. Since mtDNA is almost exclusively maternally inherited, there was no clear explanation for the short-term alteration in the mtDNA variants.

The proof that low-heteroplasmy mutations were maternally transmitted through the germline was described initially in bovine and later documented in other animals including humans. These studies showed that mitochondria harbouring one of two mtDNA variants appeared alternately in successive generations; it took two maternal generations to completely switch from one haplotype to the other [47]. Another study in a bovine model showed that cows carrying one of four different variants of mtDNA switched between the four different haplotypes over an eight pedigree period [48]. Interestingly, when the mtDNA of each of the offspring was tested, they were found to be homoplasmic for one of the variants, suggesting that the genotype switching occurred during oogenesis via differential germline transmission of low-heteroplasmic genotypes.

To validate the findings observed in the bovine model, a follow-up study was designed to examine the hypothesis of maternal germline segregation of mtDNA in a murine model [49]. In this study, heteroplasmic embryos were created by transferring cytoplasm from one inbred strain

(NZB) that had mtDNA containing 108 neutral base substitutions to a zygote of another inbred strain (BALB/c) via electrofusion. The heteroplasmic embryos were then implanted into a foster mother that gave birth to F1 embryos. These embryos carried 3%-7% of the donor mtDNA. The F1 females were mated with BALB/c males. The progeny (F2) showed a similar rate of mtDNA heteroplasmy to F1, but in some cases, it was as high as 29.6% [44]. These findings had led to the confirmation of the "bottleneck" theory. According to this theory, the primordial germ cell contains only a few copies of the founder mitochondrial genome selected to populate the organism. The drastic reduction in the number of mitochondria with a mixed population of mtDNA may result in a relative overproduction of one of the haplotypes.

There were several conflicting reports regarding the number of mitochondria in each of the stages of oocyte development [44, 49]. Recent studies, using GFP reporter specific to PGCs, show that in mice the number of mitochondria in PGCs rapidly declines, reaching its lowest number at 7.5 days post conception with an average number of 40 mitochondria per cell containing a total of 150-200 copies of mtDNA per cell. The mtDNA copy number increases as PGCs develop into oogonia (to 1500 copies), and further expand with entry to meiosis (~ 6000 per cell) [44]. It is commonly believed that the purpose of the 700-fold reduction in the number of mitochondria during PGC formation, is to select against cells with a high percentage of deleterious mtDNA mutations. The PGCs containing a minimal number of mitochondria out of which a large percentage are dysfunctional will not be able to survive and develop into oocytes, thereby removing these cells from the potentially heritable pool (See figure 3) [45]. In fact, it was shown that murine models heteroplasmic for lethal mtDNA mutations produced pups with a lower percentage of mutant mtDNA with successive generations [50, 51]. Stewart et al., used mtDNA mutator mice expressing a proofreading-deficient mitochondrial DNA polymerase model to study the transmission of different types of mtDNA mutations through the germline bottleneck. The proof-reading deficiency led to the formation of multiple random mtDNA mutations. When authors examined the spectrum of the mtDNA mutations in female germ cells, they found that mutations causing amino acid changes were strongly under-represented in comparison with "silent" changes in the protein-coding genes. These results support the notion that mtDNA is subject to strong purifying selection in the maternal germ line (see Figure 3) [45]. Recent work on cytoplasmic sharing between oocytes prior formation of follicles, further complicates the bottle neck theory . The central and most connected oocytes in oogonial cysts will become enriched in organelles including mitochondria [52]. Those oocytes which transfer their mitochondria (nurse cells) will subsequently die and those that become enriched will form primordial follicles [52]. It is not clear how this enrichment affects heteroplasmy states and if enrichment only occurs after pre-selection of viable mtDNA molecules. Additional selection of "good quality oocytes" during follicular growth may depend on the presence of functional mitochondrial pool. Atresia may remove those oocytes which happen to expand at a certain threshold level of abnormal (e.g. mutated) mitochondria [53]. However, not all mutations are created equal. Several reports describe the segregation pattern of mtDNA with severe pathologic forms that either does not show a selection bias [54] or even present a pattern of enrichment [45, 55]. The unclear pattern of heteroplasmic mtDNA segregation during oogenesis gave rise to multiple theories on potential mechanism as well as the timing of execution [10]. A recent large-scale human study examined the transmission of several different pathogenic mtDNA mutations in 577 mother-child pairs [56]. Patients harbouring one of the mtDNA mutation (m.8993T>G/C) showed a much more rapid

segregation of heteroplasmy levels than any of the other examined mutations, suggesting that the size of the bottleneck may differ according to the type of the mutation and its effect on energy supply and cell viability. Mitochondria containing mtDNA mutation with a more detrimental effect on its function will not be able to support the metabolic needs of the PGC during the drastic reduction in the number of mitochondria thus leading to their elimination. However, in mtDNA mutations that show transgenerational expansion, the resulting mitochondrial dysfunction may affect the process of mitochondrial elimination via mitophagy and thereby lead to widening of the bottleneck.

3.2 Mitochondrial Dynamics - Fusion and Fission

Mitochondria are dynamic organelles that undergo cycles of fission and fusion which is critical for cell health [57]. As mitochondrial fusion and fission are necessary to respond to the changing metabolic needs of the cell, it is not surprising that alterations in the cellular concentration of mitochondrial fusion and fission proteins have been found to negatively affect both oocyte quality and embryo development.

Morris et al., in their study on single mitochondrion mtDNA deep sequencing originating from neural tissue in both mouse and human, were able to demonstrate several populations of mtDNA within the same cell. They showed extensive intracellular heteroplasmy with a significant percentage of mtDNA harbouring single nucleotide variance with potentially detrimental effects on mitochondrial function [58].

Mitochondrial fusion is a process in which two discrete mitochondria fuse in order to merge their genetic information. In this way, two mitochondrial genomes with different defects in their genome are able to complement one another, so that one genome encodes for what the other lacks [59]. Fusion requires the action of transmembrane GTPases mitofusin-1 and 2 (MFN1/2) and OPA1; the mitofusins are located at the outer mitochondrial membrane and regulate its fusion, while OPA1 resides at the inner mitochondrial membrane and regulates the shape of mitochondrial cristae [60]. All three GTPases were found to be important for embryonic development. Homozygous deletion of *Mfn1* and *Mfn2* in mice are embryonic lethal; lack of either mitofusins led to resorptions of the embryos around E12, presumably due to placental dysfunction [61]. Interestingly, while global *Mfn2*-knockout embryos had a severe disruption in the placental trophoblast giant cell layer, *Mfn1*-deficient giant cells were normal [61]. Levels of MFN2 were also decreased in placental villous tissue of human patients with unexplained miscarriage and were also associated with apoptosis and abnormal mitochondrial morphology [62], suggesting that MFN2 is crucial for placental development. Global *Opa1*-knockout embryos were also nonviable and were resorbed at E11.5, similar to *Mfn1* and *Mfn2* deficient embryos [63].

Mitochondrial fission describes the budding off from one mitochondrion to generate two organelles [64]. During this process the mtDNA is partitioned between the two daughter mitochondria. The mtDNA will replicate in newly formed organelles, leading to a net increase in the cellular amount of mtDNA as well as the organelle itself. However, mitochondrial fission also exists as a protective mechanism to separate damaged mtDNA and other dysfunctional components of mitochondrial structure from the core, to ensure their subsequent elimination by mitophagy (see below). Thus, it is not surprising that mitochondrial fission is also essential for proper embryonic development. Mdivi-1, an inhibitor of Drp1, a GTPase involved in mitochondrial

fission, was shown to decrease the rate of blastocyst formation of porcine embryos when added to a culture medium [65]. Whole animal knockout of the *Drp1* gene resulted in embryo demise at ~E12 [66, 67], similar to a phenotype caused by *Mfn2*-deficiency as these embryos also lacked a proper trophoblast giant cell layer [67]. These phenotypes indicate that regulation of mitochondrial fission and fusion is crucial for proper trophoblast cell differentiation or maintenance. As giant cells are important for hormonal signalling required for proper maintenance of pregnancy [68], their absence may be a likely cause of mid-gestation embryo loss. Furthermore, females with oocyte-specific deletion of *Drp1* have impaired follicular maturation and meiotic resumption. Mitochondria in these oocytes are aggregated and fail to segregate properly from secretory vesicles and endoplasmic reticulum, causing impaired calcium signalling and intercellular communication [69]. Interestingly, maternal age was associated with reduced Drp1-dependent mitochondrial fission and defective organelle morphogenesis.

In addition to the mitochondrial fusion and fission proteins mentioned above, two proteins, named Miga1 and Miga2, were recently shown to promote mitochondrial fusion and contribute to female fertility [70]. Female mice with either whole animal single or double knockout of *Miga1* or *Miga2* were sub-fertile and had poor oocyte quality. Ovulated oocytes from these mice had aggregated mitochondria, increased ROS concentration, and decreased mitochondrial membrane potential. Furthermore, the developmental potential of *Miga1/2* deficient oocytes after fertilization was poor, as only 30% were able to develop into a blastocyst, compared to ~73% of embryos conceived from wildtype oocytes [70].

In contrast, overexpression of *Mfn1* led to perinuclear aggregation of mitochondria that disrupted the organelle compartments including the chromosomal spindle resulting in a loss of asymmetric division of the MII oocyte, which decreased its potential to be fertilized and develop into an embryo [71]. Thus, neither overexpression nor depletion of the proteins involved in mitochondrial biogenesis seems to be beneficial to oocyte and embryo health. The results of these studies involving manipulation of mitochondrial fusion and fission proteins seem to clearly demonstrate that a balance of both fusion and fission is necessary for proper oocyte and embryo development.

3.3 Programmed Removal of Impaired Mitochondria

The ability of cells to rid of unwanted or dysfunctional mitochondria is a key cellular defence against the accumulation of altered mtDNA or organellar damage. Programmed removal of these organelles is used by specialized cells such as red blood cells which contain neither nucleus nor mitochondria. Selective removal of a subpopulation of mitochondria is used by almost all cell types that depend on mitochondria for survival. Several key recognition pathways have been identified and appear to be cell type and trigger specific; for example, mitochondrial membrane depolarization is a trigger for mitophagy in some cell types (for reviews see [72, 73]). However, mitochondrial membrane depolarization does not activate the elimination of mitochondria in oocytes [74]. Therefore, it was proposed that metabolically compromised oocytes cannot eliminate defective mitochondria, and this leads to a compromised oxidative capacity in muscle of their offspring [75].

As already mentioned in the introduction, elimination of paternal mitochondria represents a barrier towards the transmission of potentially deleterious mitochondrial genomes to the next

generation. The molecular pathways responsible for the elimination of paternal mitochondria have only been recently elucidated and include ubiquitination and clearance by autophagy in embryos shortly after fertilization as well as inhibition of paternal mitochondrial proliferation and fusion [29]. In a porcine model, microtubule-associated protein 1 light chain 3α (LC3), ubiquitin recognition adaptor SQSTM1 and GABA type A receptor-associated protein (GABARAP) were found to be involved in recognition of ubiquitinated paternal mitochondria and their removal by autophagy [76]. Similarly in mouse, shortly after fertilization paternal mitochondria depolarize and are eliminated via the mitophagy pathway, which depends on maternally/embryonically derived mitochondrial outer membrane protein FIS1, kinase PINK1, and partially redundant roles executed by ubiquitin ligases PARKIN and MUL1 [77]. While the process of paternal mtDNA elimination is very efficient in most cases, there are several reports of paternal mitochondria detected in human embryos, both in embryos arrested at the cleavage stage as well as embryos that reached the blastocyst stage [78]. A recent report describing the existence of several unusual families with biparental inheritance of mtDNA across several generations raises a possibility that in some cases paternal transmission mode can be tolerated but may have negative physiological consequences [31].

4. Embryo and Oocyte Quality Determined by Mitochondria Integrity

In several mammals, including humans, the oocytes remain arrested for several decades at the entry to the first meiotic division. Upon release from the arrest, chromosome segregation is facilitated by the action of the meiotic spindle. Recently several publications examining the association between mtDNA copy number, maternal age, aneuploidy and implantation showed conflicting results ranging from a strong negative association to no effect [38, 79, 80]. Aging and age-related somatic pathologies are frequently associated with loss of mitochondrial function at least in part due to the accumulation of mtDNA mutations and deletions [81]. Several theories have been proposed to explain the formation of the common mtDNA deletion. The mtDNA has two coding strands both containing identical replication initiation sites at the two ends of the deleted section [82].It is possible that as oocytes age, they acquire multiple mtDNA mutations and deletions that result in declining mitochondrial energy production [83, 84]. As a result, oocyte maturation processes especially nuclear spindle activity and chromosomal segregation may become impaired, resulting in an increased rate of aneuploidy, especially trisomies observed in older women. This hypothesis is supported by data demonstrating that mitochondrial mutations in follicular cells increase with age, suggesting that oxidative phosphorylation and ATP production in the follicle may be impaired in older women [85]. Therefore, it is reasonable to suggest that the documented relationship between maternal age and chromosomal abnormalities in human embryos could be a result of the diminished mitochondrial activity.

One of the main reasons for the poor performance of embryos from older patients is the increased rate of chromosomal aberrations [83]. It was suggested that dysfunctional cytoplasmic factors are responsible for abnormalities of the spindle architecture, which in turn may contribute to chromosomal mal-segregation and delays in embryo development [86]. Mitochondrial inner membrane potential (ΔΨm) is used as an indicator of mitochondrial health as a deviation in the potential reflects abnormal mitochondrial metabolic activity. Previously, the membrane potential was correlated with day-3 human embryos ploidy using Fluorescence In Situ Hybridization (FISH)

and analysis of the meiotic apparatus [87]. Results of this analysis revealed a strong association between low ΔΨm and chaotic mosaicism with defective segregation of chromosomes between blastomeres triggered by abnormal meiotic apparatus assembly. Embryos with chaotic mosaicism exhibited a slower rate of cleavage and were more frequently conceived by the older group of patients [87]. Conversely, we observed elevated membrane potential in arrested fragmented murine and human embryos, where mitochondria hyperpolarize and produce excessive levels of ROS [88-90].

Increased ROS and mitochondrial dysfunction accompanied by architectural defects in meiotic apparatus are also found in the high-fat diet (HFD) mouse model [70] causing a series of infertility phenotypes [91, 92]. Exact molecular targets of ROS initiated damage in oocytes are not well characterized. Recent work revealed decreased expression of *Sirt3*, mitochondrial sequestered NAD+-dependent deacetylase, in oocytes of HFD mice and identified Superoxide Dismutase (SOD) as its deacetylation target [93]. Overexpression of *Sirt3* corrected ROS levels in HFD oocytes, improved meiotic spindle and chromosomal positioning, while knockdown of *Sirt3* triggered these defects [93]. *Sirt3* is also required for preimplantation embryo survival particularly when facing conditions of stress. Maternal *Sirt3* is required for handling ROS response in p53 gene-dependent manner [94].

Historically, the process of cumulative DNA damage was attributed to the continuous exposure to ROS generated through normal metabolism by the mitochondria themselves [95]. However, this view has been recently challenged by intriguing observations of prolonged lifespan attributed to increased mitochondrially derived ROS [96]. Decades of long constant exposure of mtDNA to ROS, a by-product of the OXPHOS system, together with the lack of protection from histone proteins, and the lack of non-coding introns, make the mtDNA more prone to functional mutations [97]. The maintenance and repair of mtDNA rely upon the same enzymes that maintain the nuclear genome. However, some of those enzymes are not available for the mtDNA, making the repair process less effective, with a high rate of mutation in mtDNA. This, in fact, may be the result of the faulty action of the DNA repair mechanism on the mtDNA [82]. One of the more frequent mtDNA deletions is the “common deletion” of 4977 base pairs, almost a third of the whole mtDNA genome. This deletion was shown to have a high prevalence in unfertilized oocytes and oocytes from older patients [98, 99] as well as in aged cows [100]. Interestingly, work in invertebrates had shown maternal germ cells and oocytes to harbour a much higher rate of mtDNA deletions in comparison to somatic cells [101]. Since the mtDNA contains genes encoding proteins of the respiratory chain, a significant loss of this DNA will ultimately result in dysfunctional OXPHOS characterized by the absence of cytochrome c oxidase activity. Evidently, mutations in mitochondrial oxidase C1 have been recently linked to premature ovarian insufficiency [102].

5. Factors Involved in Mitochondrial Function are Vital to Oogenesis and Embryogenesis.

A large number of proteins (>1000), encoded either in the nuclear or mitochondrial genomes, contribute to the function and maintenance of mitochondria. For example, mtDNA encodes several subunits of the mitochondrial respiratory chain complexes, while the majority of proteins encoded by nuclear DNA are assembled in the cytosol and are later transported to mitochondrial compartments appropriate for their function. Several of these proteins are essential for proper

oocyte and embryo development, and alterations in their expression have been found in mouse models with decreased oocyte quality.

5.1 Genes Involved in the Replication and Maintenance of Mitochondrial Genomes

The replication and translation processes of the mtDNA are continuous, cell cycle independent and controlled by several nuclear-encoded transcription factors. Depletion of genes that encode for the mtDNA replication machinery was found to be involved in embryo development. Mitochondrial transcription factor A (encoded by the *TFAM* gene) enables the action of the catalytic subunit of the mitochondrial specific DNA polymerase; Polymerase Gamma A (encoded by the *POLGA* gene) and Polymerase Gamma 2 (encoded by *POLG2*) that possesses DNA polymerase, 3'-5' exonuclease and 5'dRP lyase activities [103]. This process is supported by the mitochondrial helicase, Twinkle (TWNK) [104] and the mitochondrial single strand binding protein (mtSSB) [104]. Murine embryos with a homozygous knockout of *Tfam* die after gastrulation between embryonic day E8.5-10.5 [105]. When examined, none to an extremely low copy number of mtDNA was detected in these embryos, confirming that *T*fam is necessary for mtDNA replication during embryogenesis. A study by Wai et al. [40] showed that while *Tfam* deficient oocytes with very low mtDNA copy numbers (~4000 mtDNA copies) were able to be fertilized and survive during the preimplantation stages, a much higher threshold amount of mitochondria was needed for survival post-implantation (40,000-50,000 copies). The homozygous deletion of *PolgA* in mice led to embryo lethality at E7.5 dpc [106], while deletion of *Polg2* led to death at E8-8.5 dpc [107]. Despite the proofreading activity, most of the age-related point mutations and deletions in mtDNA can be attributed to spontaneous replication errors of this polymerase [108-110] and the rate of age-related mtDNA mutations is significantly amplified in carriers of *POLG* mutations causing premature aging [111, 112]. The homozygous deletion of PolgA in mice led to embryo lethality at E7.5 dpc [106], while homozygous deletion of Polg2 led to death at E8-8.5 dpc [107]. Knockout of a ribonuclease gene *Rnaseh1*, with function in both the nucleus and mitochondria [113], had similar consequences, with the embryos failing to survive past E8.5 [114]. This lack of embryo survival was attributed to the depletion of its mitochondrial fraction, as mtDNA copy number was significantly decreased at E7.5 while total chromosomal content remained unchanged between the genotypes.

Interestingly, while the day at which demise of the various knockout embryos occurs was variable, there was a clear correlation with the function of these genes [106, 107, 113, 114]. The ability of the embryo to implant yet die mid-gestation seems to be characteristic of depletion of the mitochondrial pool caused by disruption of mtDNA replication. Robust replication of mitochondrial genome has not been observed during preimplantation embryo development in various species and it is generally believed that there is either none or very little mtDNA replication until after implantation [60]. Therefore, it seems embryos deficient in components of mtDNA replication machinery only exhibit the results of this deficiency when mitochondrial replication needs to restart and support the growing metabolic needs.

An interesting relationship between mitochondria and fertility is seen in Perrault syndrome. This disorder is characterized by sensorineural hearing loss (SNHL) in both males and females. It is also accompanied by ovarian dysfunction ranging from gonadal dysgenesis that manifests as primary amenorrhea to primary ovarian insufficiency (POI) [115, 116]. The diagnosis of Perrault

syndrome is molecularly confirmed by the presence of biallelic pathogenic variants in one of six genes, all involved in mitochondria functions: caseinolytic mitochondrial matrix peptidase proteolytic subunit (*CLPP*), Era like 12S mitochondrial rRNA chaperone 1 (*ERAL1*), histidyl-tRNA synthetase 2, mitochondrial (HARS2), hydroxysteroid 17-beta dehydrogenase 4 (*HSD17B4*), mitochondrial leucyl-tRNA synthetase 2 (*LARS2*), and twinkle mtDNA helicase (*TWNK*). Role of *Clpp* in oocyte quality was recently confirmed by analysis of ovarian phenotype in the mouse model, confirming defects in oocyte mitochondrial function and upregulated ovarian mTOR signalling [117].

5.2 Genes Involved in Oxidative Phosphorylation are Vital for Proper Oocyte Maturation and Embryogenesis

The proper completion of oocytes maturation and progression through preimplantation embryo development requires tremendous amounts of energy. Supply of ATP and other metabolic by-products of the citric acid cycle such as fumarate as well as acetyl and methyl groups are dependent on the ability of the mitochondrial pool to properly execute metabolic pathways needed to support numerous cellular processes. Thus, it is not surprising that disruption of multiple genes that regulate mitochondrial metabolism lead to meiotic defects often followed by disrupted embryo development.

5.2.1 Pyruvate Metabolism

Pyruvate is a major source of oocyte energy [13]. PDHE1, encoded by *Pdha1* gene, is an enzymatic subunit of pyruvate dehydrogenase complex necessary for meiotic progression. Due to CC support, oocytes deficient on *Pdha1* can survive, grow, and few can even become fertilized; however, they fail to progress beyond the zygote stage [118]. Furthermore, most of these oocytes fail to execute meiosis properly resulting in numerous chromosomal defects and abnormal spindle positioning. Pyruvate dehydrogenase kinase (PDKs) which modulate cellular response to metabolic needs via phosphorylation of PDHE1 is also involved in regulation of meiotic resumption. Four PDK isoforms are expressed in the oocytes albeit at variable level, however their role in meiotic progression is poorly understood. A recent report identified PDK3-mediated phosphorylation of Ser293-PDHE1α as a driver of meiotic spindle disruption accompanied by decreased ATP levels [119]. Interestingly, PDK1 and PDK2 were also found to promote meiotic maturation, as their knockdown disturbs the meiotic apparatus albeit without altering the ATP content [119]. More work is needed in order to determine which phenotypes are caused by ATP deficiency and which by the absence of other by-products of pyruvate metabolism.

5.2.2 Ubiquinone (CoQ10) Metabolism

CoEnzyme Q10, also known as ubiquinone, is an essential component in oxidative phosphorylation. It facilitates the electron transfer from complexes I and II to complex III which provides the energy needed to transfer protons to the intermembrane space [120]. The CoQ10 molecule is composed of a benzoquinone head and an isoprenyl tail, CoQ10 is produced by the combined action of at least 10 different enzymes. Some of these enzymes have been studied in the context of oocyte and embryo development. One of these enzymes is Coq7 (clk-1; demethyl-Q

7) which hydroxylates demethoxyubiquinone (DMQ), a CoQ10 precursor molecule [121]. The Coq7 deficient embryos arrest at mid-gestation, although the mitochondrial activity of Coq7 deficient cells was only mildly affected [121, 122]. Because of this finding it was proposed that CoQ10 may have other extra-mitochondrial roles, and the lack of these other, yet unknown, processes, is the cause for the impairment in embryogenesis. An alternative explanation might be that even mild impairment in oxidative phosphorylation in developing embryos may be enough to halt their development.

Pdss2 (prenyl diphosphate synthase, subunit 2), another CoQ10 biosynthetic enzyme which produces the isoprenyl tail specific to the CoQ10 molecules, has been shown in our laboratory to contribute to oocyte health [123]. Protein levels of Pdss2 decline in aged oocytes, implying that Pdss2 insufficiency may contribute to the decline in oocyte quality seen with increased maternal age. Additionally, oocyte-specific knockout of *Pdss2* leads to many common age-associated deficiencies, such as reduced ovarian reserve and mitochondrial metabolic dysfunction [123] demonstrating the importance of this enzyme in oocyte health. Transcript levels of other CoQ10 biosynthetic enzymes were found to be decreased in oocytes from old female mice compared to the oocytes from young mice, but whether these enzymes have a significant influence on oocyte and embryo health remains to be established.

5.2.3 Cytochrome C Oxidase

Cytochrome c oxidase (COX), complex IV, is the last complex in the mitochondrial electron transport chain. It transfers electrons to a dioxygen molecule and pumps protons into the intermembrane space [124]. It is composed of 14 subunits that are encoded by both nuclear and mitochondrial DNA, and it is assembled by various assembly proteins [125]. Mutations of genes encoding the COX subunits as well as the assembly units have been studied extensively in the context of mitochondrial diseases and been implicated in female fertility. Examples include patients with primary ovarian insufficiency that show an increased incidence of missense mutations in *MT-CO1*, a gene that encodes the main subunit of COX [102]. Also, a mutation in SCO2, a gene that encodes a COX assembly protein, was found in a female patient with a history of recurrent early pregnancy loss[126], suggesting that COX stability is important for sustaining pregnancies.

5.2.4 Bcl-2 Proteins

In addition to their well known role in metabolism, mitochondria also regulate heat production, detoxify ammonia, produce steroid hormones and even regulate cell death. The major regulators of cell death pathways that localize to mitochondria are members of the Bcl2 protein family. While the role of the members of the Bcl-2 family in the regulation of mitochondrial function during death has been extensively researched, their metabolic involvement (direct or indirect) is less understood.

Bcl-x (Bcl2-like-1) is one of the founding members of Bcl-2 protein family, responsible for survival and expansion of early hematopoietic and neuronal lineages. Numerous splicing isoforms are produced from Bcl-x immature transcript. Two major isoforms of Bcl-x, the full-length isoform (Bcl-xL), protects cells from stress-induced suicide while the shorter isoform (Bcl-xS), generated by alternative splicing, promotes cell death. While the majority of Bcl-xL resides in the outer

mitochondrial membrane, a small fraction of this protein can be found at the mitochondrial inner membrane where it interacts with the subunit of the F1F0 ATP synthase [127]. This and many other interactions of Bcl-xL protein modulate the efficiency of mitochondrial bioenergetics [128]. Both isoforms are expressed by oocytes, particularly when exposed to doxorubicin [129]. In addition, human preimplantation embryos with poor developmental potential activate Bcl-xS expression [130], and modulation of Bcl-xS splicing is sufficient to activate cell death in preimplantation embryos [131]. Murine embryos susceptible to 2-cell arrest due to exposure to suboptimal culture medium respond by re-distribution of their mitochondria, increase mitochondrial membrane potential [88] and decrease Bcl-xL expression. Microinjection of recombinant Bcl-xL protein restores developmental potential under arresting conditions, in addition to adjusting the redox state and restoring mitochondrial subcellular distribution [89].

MCL1 (Myeloid Cell Leukemia 1) is an antiapoptotic member with strong cell survival role in numerous cell types. In addition to interfering with cell death pathways via sequestering activity of proapoptotic members, e.g. BAK, BOK and BAX and sensitizers such as NOXA, MCL1 also regulates mitochondrial architecture and function [132]. A unique protein isoform generated by N-terminal cleavage of MCL1 localizes to mitochondrial matrix and facilitates assembly of electron transport chain (ETC) complexes [133]. MCL1 is abundantly expressed in human and murine oocytes, and its disruption causes premature ovarian insufficiency. *Mcl1* deficient oocytes that survive folliculogenesis and are ovulated exhibit a decrease in mitochondrial membrane potential increased ROS production and decreased metabolic substrates malate and fumarate; however no change in citrate or total ATP level was observed [134]. While cell death of *Mcl1* deficient oocytes can be prevented by *Bax* inactivation, mitochondrial phenotypes and meiotic abnormalities cannot be reverted by *Bax* deficiency.

6. The Role of Cumulus Cells

CCs are a subpopulation of granulosa cells (GCs) that surround the oocyte and are physically connect the oocyte to the mural granulosa cells within the follicles. The CCs are directly responsible for nutrient supply to the oocyte and its metabolism. These cells are also essential for proper oocyte maturation. In fact, the bidirectional cross-talk between the oocyte and CC is crucial for proper oocyte development as well as the function [133, 135].

Mitochondria, isolated from GCs of older patients show an age-dependent decline in oxidative phosphorylation, likely associated with declining intracellular concentrations of CoQ10 [136]. This is consistent with diminished expression of several CoQ biosynthetic enzymes in with increased maternal age[137]. In addition, Boucret et al. [138], measured the transcript levels of several genes involved in mitochondrial biogenesis and found that the expression levels of TFAM, OPA1, and POLG in human CCs were positively correlated with oocyte mitochondrial mass. .

Premutation in Fragile X gene, caused by intermediate expansion of repeats in 5’ regulatory region of FMR1gene, had been linked to POI [139]; however, the exact mechanism and affected cell types in the ovary remain elusive. While mitochondrial defects were shown in somatic cells of premutation carries [140], whether these abnormalities also occur in oocytes or in GCs is unknown. Recent work [141], using a transgenic mouse model with 130 CGG repeats, reported that both oocytes and GCs exhibit mitochondrial structural defects, accompanied by mitochondrial

depletion in a subset of CCs and deregulated expression of *Opa1* and *Mfn1/2* in a gene dose-dependent manner.

Thus, numerous results support the idea that mitochondrial dysfunction within the CCs may affect oocyte health, therefore measuring various mitochondrial parameters in CCs can serve as a non-invasive method to indirectly assess the fitness of oocytes and embryos.

7. Mitochondria-Related Morbidity

Mitochondrial diseases are a group of genetic diseases that may encompass a wide spectrum of disruption of cellular processes, including bioenergetics, cofactor and macromolecule precursor synthesis, redox balance, immunity, proteostasis, calcium handling and ROS homeostasis [142]. Initially, mitochondrial diseases were thought to be rare childhood diseases, caused by recessive nuclear genes, that presented with severe and progressive neuro-muscular degeneration. The prevalence of children born with severe mitochondrial disease is estimated to be 6/100,000 [143]. Lately, however, it became increasingly clear that mitochondrial dysfunction involves a wide range of common adult metabolic, degenerative and malignant diseases as well as aging [144]. These are usually complex syndromes with a marked variation in clinical presentation among different patients, involving several organ systems and a vague heritability pattern. The discovery of a multitude of pathogenic mtDNA mutations and the establishment of the concept of heteroplasmy helped clarify the pathogenesis of these conditions.

The mtDNA has an exceptionally high mutation rate, estimated to be a 10-fold higher than nuclear genome [145]. This is likely the result of the higher vulnerability of the mtDNA, being exposed to high ROS concentration while lacking histones, introns and a robust DNA repair mechanisms. A recent study by Kennedy et al., employed highly sensitive Duplex Sequencing to examine the prevalence and type of mtDNA mutations in human neural tissue. The authors showed the frequency of mtDNA mutations increase 5 fold over an average life span. Interestingly, they found no age related change in the prevalence of the G->T conversions, commonly associated with oxidative damage , implying that most of the age-related increase in mtDNA mutation load originates from DNA polymerase dysfunction [146]. These mtDNA mutations are gained over the years and are therefore more abundant with age but can also be inherited by fertilization of a heteroplasmic oocyte. In both cases, the relative amount of mutated mtDNA may drift with replicative segregation creating diverse levels of heteroplasmy between adjacent cells or sibling embryos. To add to the complexity, there are differing tissue sensitivities to the resulting mitochondrial dysfunction. Tissues with high energy demand such as the brain, heart, muscle and endocrine system show a much higher sensitivity to the bio-energetic defects. Furthermore, the type of mtDNA mutation and whether it was inherited or gained during adult life, affects the level of mitochondrial dysfunction and the broadness of its distribution.

A maternally inherited low level heteroplasmy that initially only causes a mild subclinical mitochondrial dysfunction may be further aggravated by all of the following; the introduction of a newly acquired deleterious mtDNA mutation, the nature and availability of calories, an increase energy demands, oxidative stress, the age-related decline in the cellular concentration of CoQ10 in addition to medications and environmental toxins that reduce the efficacy of the OXPHOS. Also, heteroplasmy of pathogenic mtDNA mutations that affect the complex -I in particular as well as the mutations in mitochondrial tRNA and rRNA were shown to promote ROS production and to be

strongly linked to cancers, neurodegenerative and metabolic diseases [142]. These mutations were also associated with premature ovarian senescence and aging [147]. The alteration in mitochondrial energetics further accelerates mtDNA damage and the acquired mutations disrupt mtDNA replication and the process of removal of defective mitochondria. This leads to an accumulation of somatic mtDNA mutations and reduced mitochondrial energy output . Once the mitochondria fail to meet the energy demands of the tissue, functional and metabolic deficiencies start to show [142, 148].

8. Treatment and Prevention of Mitochondrial Disease

Diagnosis and treatment of mitochondrial disease remain a major challenge. For most patients, treatment is aimed at symptoms relief and prevention of complications. However, there are some conditions which are amenable to medical treatment such as patients with primary or secondary CoQ10 deficiencies Some conditions, especially those associated with musle and renal dysfunction improve with exogeneous high dose CoQ10 administration, however neurological defects often remain refractory to treatment (reviewed in [137]). Treatments aimed at relieving oxidative stress and optimization of mitochondrial biogenesis, such as resveratrol and agonists that activate peroxisome proliferator-activated receptor gamma, may be beneficial for some patients. The efficacy and the role of these interventions in the treatment of mitochondrial disease is still controversial, however [149]. Recently, much progress was made in developing curative treatments to several mitochondrial conditions. These treatments are based on discoveries such as mitochondria-specific nanocarrier molecules that allow mitochondria-specific drug delivery, medications that stabilize mitochondrial tRNA, as well as the resolving specific mitochondrial enzyme deficiencies [149-151].

Very significant progress, both scientifically and legislatively, was made with regards to the prevention of maternal transmission of mutated mtDNA via mitochondrial replacement therapy (MRT). Since mitochondria are almost exclusively maternally inherited, a mother homoplasmic for a pathogenic mtDNA mutation will very likely transmit the deficiency to her offspring. Tachibana *et al.* [152], had recently described a technique in which the nuclear genome was transferred from an affected unfertilized oocyte to nuclear DNA free donor oocyte via spindle-chromosomal complex transfer (ST). After the transfer of the karyoplast, the oocytes were fertilized with ICSI. The result was a euploid embryo containing nuclear DNA of both parents, while almost of the mtDNA originated from the healthy donor oocyte. Another option is to wait after fertilization and then transfer the two pronuclei (PN) from the patient's zygote to a donor zygote that had its pronuclei removed. This PN transfer is however criticized for the significant carryover of the original mtDNA to the healthy zygote creating an average heteroplasmy level of 24% likely the result of the largest cytoplasm amount transferred between the zygotes [153]. One more option for MRT is the transfer of a polar body. During the first meiotic division, the corresponding bivalent chromosomes separate to oocyte and the first polar body while in the second, with fertilization, sister chromatids segregate to form a zygote and the second polar body. Both polar bodies contain a complement of chromosomes with a very small amount of cytoplasm and mitochondria. Transfer of a first polar body to a recipient MII oocyte that had its spindle removed or a second polar body to a zygote that was fertilized with the father's sperm and had its maternal

pronuclei removed was done successfully in mice, however a similar result could not yet be replicated in other mammals [154, 155].

In February 2015, The British House of Commons voted overwhelmingly approved the use of MRT for women suffering from a mitochondrial disease in order to prevent transmission of the pathology to their children [156]. First successful birth after mitochondrial transfer has been reported [157], yet the efficacy and safety of this approach remain to be determined.

At the end of the 1990s, innovative attempts were made of improving embryo quality by injecting mitochondria-rich cytoplasm from young donor eggs into the eggs of women with recurrent reproductive failures [158-160]. This procedure resulted in the birth of healthy babies [158-160]. In 2004, an autologous source of germline mitochondria was proposed by Johnson et al. [161]. These cells reside in the avascular outer lining of the ovarian cortex [162] and can beisolated from the ovarian cortical tissue of reproductive-age women using antibodies to the VASA analogue, found on the surface of egg precursor cells [162]. A few studies have demonstrated that mitochondria isolated from these cells are of high quality and therefore may serve as an autologous source of mitochondria to improve the embryo quality[163].

Recently, a triple blinded randomized clinical trial conducted by Labarta et al., compared the outcome of IVF treatment among patients with recurrent IVF failure that were either treated with autologous mitochondrial transfer and ICSI vs. standard care. Results failed to show any benefit of the modified mitochondrial function over blastocyst formation rate, euploid embryo per mature oocyte rate, embryo morphokinetics as well as cumulative live birth rate [164].

9. Conclusions

Recent years brought about major advances in the understanding of mitochondrial physiology and genetics as well as their role in multiple complex morbidities, poor reproductive outcome and aging. This provided the basis to development of specific treatments for relief of symptoms, prevention of complications and cure. MRT had also paved the way for circumvention of maternal inheritance of mitochondrial disease that previously was possible only with using donor oocytes or adoption. The role of AMT in improving age-related reproductive outcome is not as clear and requires further rigorous scietific studies.

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Competing Interests

The authors have declared that no competing interests exist.

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