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Review

The control of mtDNA replication during differentiation and development ☆

Justin St. John [1]

Mitochondrial Genetics Group, Centre for Genetic Diseases, Monash Institute of Medical Research, Monash University, 27-31 Wright Street, Clayton, Victoria 3168, Australia

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ABSTRACT

Background: Mitochondrial DNA (mtDNA) is important for energy production as it encodes some of the key genes of electron transfer chain, where the majority of cellular energy is generated through oxidative phosphorylation (OXPHOS). MtDNA replication is mediated by nuclear DNA-encoded proteins or enzymes, which translocate to the mitochondria, and is strictly regulated throughout development. It starts with approximately 200 copies in each primordial germ cell and these copies undergo expansion and restriction events at various stages of development.
Scope of review: I describe the patterns of mtDNA replication at key stages of development. I explain that it is essential to regulate mtDNA copy number and to establish the mtDNA set point in order that the mature, specialised cell acquires the appropriate numbers of mtDNA copy to generate sufficient adenosine triphosphate (ATP) through OXPHOS to undertake its specialised function. I discuss how these processes are dependent on the controlled expression of the nuclear-encoded mtDNA-specific replication factors and that this can be modulated by mtDNA haplotypes. I discuss how these events are altered by certain assisted reproductive technologies, some of which have been proposed to prevent the transmission of mutant mtDNA and others to overcome infertility. Furthermore, some of these technologies are predisposed to transmitting two or more populations of mtDNA, which can be extremely harmful.
Major conclusions: The failure to regulate mtDNA replication and mtDNA transmission during development is disadvantageous.
General significance: Manipulation of oocytes and embryos can lead to significant implications for the maternal-only transmission of mtDNA.
This article is part of a Special Issue entitled Frontiers of mitochondrial research.

1.1. The mitochondrial genome

The mitochondrial genome or mitochondrial DNA (mtDNA) is located at the inner membrane of the mitochondrion of eukaryotic cells. The size of the mitochondrial genome differs between species ranging from 16.2 kb in the mouse [1] to 16.7 kb in pigs [2] and, in humans, it is 16.6 kb in size [3]. The mitochondrial genome encodes 13 of the subunits of the electron transfer chain. These include 7 subunits of Complex I, 1 subunit of Complex III, 3 subunits of Complex IV and 2 subunits of Complex V. It also encodes 22 tRNAs and 2 rRNAs and has one non-coding region known as the displacement (D) loop. The D loop consists of 2 hypervariable regions and the control region [3]. The control region is the site of interaction for the nuclear-encoded transcription and replication factors that translocate to the mitochondria in order to initiate and drive the processes of transcription and replication. It also contains the origins of replication for the heavy strand whilst the origin of light strand replication is located two-thirds of the way round the genome [4]. Replication of the heavy and light strands requires interaction with their respective promoters, HSP and LSP, which are located within the D-loop region. The hypervariable regions can be used to determine an individual's genetic identity that is based on his or her maternal ancestry [5]. Indeed, they are commonly used in forensic science to determine the perpetrators of crime [6]; and following assisted reproductive technologies, to determine the genetic identity of, for example, cloned offspring and to establish whether the strict maternal patterns of inheritance have been maintained [7].

1.2. Replication of mtDNA

Mitochondrial transcription factor A (TFAM) initiates a process that enables the catalytic subunit of the mitochondrial-specific polymerase, Polymerase Gamma (POLG) A, to copy mtDNA [8]. This process is supported by three other factors: POLGB, which is the accessory subunit

Abbreviations: mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; TFAM, mitochondrial transcription factor A; PolgA, DNA Polymerase Gamma A; PolgB, DNA Polymerase Gamma B

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E-mail address: justin.stjohn@monash.edu.
[1] Tel.: +61399024749.

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of POLGA [9]; the mitochondrial helicase, Twinkle [10]; and the mitochondrial single stranded binding protein (mtSSB) [11]. These factors contribute to the core mtDNA nucleoid, responsible for the transcription and replication and packaging of the mitochondrial genome [12]. Other members of the core nucleoid include the key transcription factors, namely TFB1M, TFB2M, mtRNA polymerase and mTERF1 [12]. High energy requiring cells, such as cells from the heart, muscle, liver and brain cells possess high numbers of mtDNA copy [13]. This enables these cells to generate sufficient ATP through oxidative phosphorylation (OXPHOS) to undertake their complex and energy-demanding cellular functions [14]. For these cells, OXPHOS is the most appropriate process since it generates 32 molecules of ATP compared with two produced by the other key energy generating pathways, glycolysis [15]. Cells from tissues, such as the spleen, possess fewer copies of mtDNA [13] and numbers of mitochondria and are, therefore, much more dependent on glycolysis. Nevertheless, the use of BrdU pulse experiments has demonstrated that mtDNA is replicated in a semi-conservative manner [16] and further evidence has been provided in oocytes, where replication is indeed very focal in nature [17].

1.3. mtDNA ploidy

It is well documented that there is considerable variability in the number of mitochondria present in mature cells. This is perhaps best demonstrated by the very low numbers in mature human sperm, where 22 to 75 mitochondria are packaged in a helical fashion around the midpiece [18], and the high numbers distributed throughout the cytoplasm of fertilisable oocytes [19]. However, there has been much dispute over the number of mtDNA copies present within each mitochondrion. This can range from 1 to 15 copies [20] dependent on how the analyses were performed (discussed in detail in [21]). Early findings suggested that there were 2 to 8 copies of mtDNA per nucleoid [22]. Subsequent investigations determined that TFAM was not only the initiator of mtDNA transcription and replication but was also a highly abundant packaging protein, which mediates the formation of mtDNA molecules into nucleoids [23]. Based on the levels of TFAM present within cells, it has been proposed that there is one mtDNA molecule per nucleoid [24]. Others argue that this could, however, be as high as 1.4 to 3 molecules of mtDNA per nucleoid [25]. The presence of the number of mtDNA molecules per nucleoid per mitochondrion is important for our understanding of the segregation and transmission of mutant mtDNA.

1.4. Segregation of mtDNA

It is generally accepted that the mitochondrial genome is segregated and thus inherited in a non-Mendelian fashion. Segregation describes the partitioning and distribution of mtDNA genomes, which take place at various stages during development and in mature tissues. Mechanisms describing these processes have been very much influenced by the transmission of mutant mtDNA from one generation to the next and how mutant loading is acquired and maintained in affected cells and tissues. The mixing of mutant and wild type mtDNA within a tissue is described as heteroplasmy whilst the presence of wild type or mutant mtDNA is described as homoplasmy. Nevertheless, there are a number of mtDNA replication and reduction events during early development, which will influence how mitochondrial genomes are segregated [26]. These events will be discussed later in the context of mtDNA replication during development. However, I will first discuss some of the proposed molecular mechanisms that regulate mtDNA segregation, which are applicable to both fast-dividing proliferative cells and post-mitotic cells.

Each mitochondrion can undergo fission and fusion. Fission describes the budding off from one mitochondrion to generate two mitochondria, mediated by FIS1 and DRP1 (DLP1) [27]. As a result, there will be partitioning of mtDNA nucleoids into each newly divided mitochondrion. The complementation model proposes that nucleoids do not frequently exchange molecules of mtDNA and that heterologous copies of mtDNA are divided between mtDNA nucleoids allowing for wild type molecules to complement mutant copies within a cell [28]. Once fission has taken place, mtDNA ploidy will be restored by replication of the mitochondrial genome(s) present within each mitochondrion. This mechanism ensures that wild type molecules can compensate to ensure that cellular function is maintained unless the degree of mutant mtDNA exceeds a threshold level that would trigger the onset of the phenotype of the disease [29]. Equally so, fusion of mitochondria, mediated by factors such as Mitofusin 1 and 2 [30] and OPA1 [31] will likely result in an increase in the number of mtDNA nucleoids present, which may negate the need to replicate mtDNA, or coalesce barren and populated mitochondria.

Mathematical modelling of segregation suggests that heteroplasmic populations within a cell can differ significantly within a short timeframe and that a high copy number of mtDNA molecules results in delayed fixation of the allele harbouring the variant [32]. Others argue that random genetic drift, whether neutral or specific selection for mutant molecules, can account for the onset of disease [33] for which there is strong evidence for the tissue-specific and age-dependent selection of different mtDNA genotypes [34]. Most recently, it has been proposed that the transmission of mtDNA mutations through the germ line, which appear to be transmitted at a greater frequency than previously thought, results in the accumulative effect of somatically acquired mutations that result in the onset of pathological states earlier [35].

An alternative pattern of segregation involves Gimap3, which is a GTPase, located on the outer mitochondrial membrane that specifically regulates the segregation of mtDNA in leukocytes. Although the mechanisms are yet to be defined, it is thought that it is also involved in budding off of vesicles carrying pathogenic mtDNA [36], perhaps in a manner similar to FIS1 and DRP1, which are also GTPases. Others have shown a sex-specific genetic mechanism for the control of mtDNA levels. The strongest evidence comes from a single nucleotide polymorphism in the MRPL3 gene on chromosome 1, which is involved in mitochondrial protein translation and is male specific [37] and in many respects supports *Drosophila* models that suggest males are more likely to harbour deleterious mtDNA [38]. In many respects, these mechanisms may be present individually or collectively at any stage of cellular development.

1.5. Replication of mtDNA during development

Replication of the mitochondrial genome is essential to the maintenance of the maternal inheritance of mtDNA. The population of mtDNA that is inherited by an individual is present in the metaphase II oocyte just prior to fertilisation [26]. This originates from a population that is segregated to the primordial germ cells as embryonic differentiation (gastrulation) is initiated [17]. During the process of oogenesis, which takes place following the migration of the primordial germ cells to the ovary, replication ensues, however, significant increases are mainly confined to later stages [17,39,40]. Consequently, the increase in copy number from around 200 copies present in the primordial germ cells to >200,000 present in mature fertilisable metaphase II oocytes represents a thousand fold increase [17,39–41]. If the mitochondrial genomes are identical then there is clonal expansion of the 200 copies present in the primordial germ cells. Equally so, if mtDNA variants are present within some of those 200 copies, either molecule can preferentially be selected for or against (see Fig. 1). Consequently, mature metaphase II oocytes have variable representation of the population present in heteroplasmic primordial germ cells [17,39,40]. As mtDNA replication is focal in the developing oocyte [17], the selection of mutant or wild type mtDNA molecules for replication will be depended on their frequency and distribution within the oocyte's cytoplasm. However, oocyte maturation also involves mtDNA reduction events, as evidence during *in vitro* maturation experiments [41]. The persisting copies would then undergo a subsequent round of mtDNA replication as the

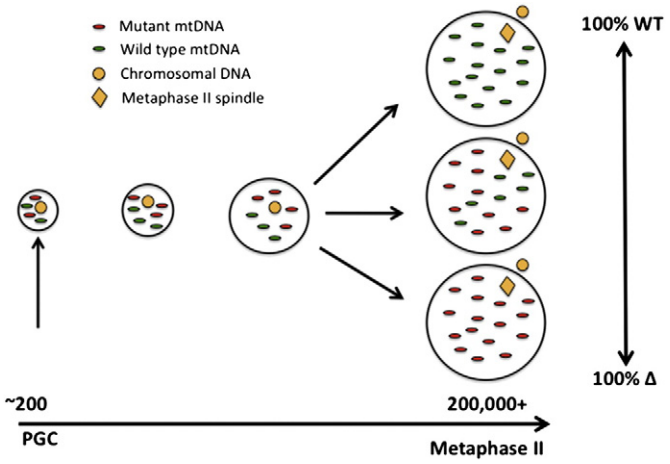

Fig. 1. Amplification of mutant mtDNA as primordial germ cells differentiate into mature oocytes. MtDNA randomly segregates to the primordial germ cells (PGCs) after they migrated to the ovary following gastrulation. As the primordial germ cells differentiate into mature oocytes during oogenesis, mtDNA copy number expands and mutant mtDNA appears to be randomly selected for, which accounts for the variability in the levels of heteroplasmy in each mature, fertilisable, metaphase II oocyte from one individual. WT = wild type; Δ = mutant.

metaphase II oocyte fixes its mtDNA composition prior to fertilisation, which would bias the oocyte's mtDNA content.

Whilst many of the mtDNA variants observed in oocytes may not be deleterious, some are and have significant consequences for the offspring's wellbeing [42,43]. Those variants that are not deleterious will often be *de novo* mutational events that have arisen through erroneous replication events mediated by proof-reading failure or base substitution arising from free radical activity [35]. Women who are carriers of mtDNA mutations that lead to mtDNA disease normally possess one of a range of the deleterious mutations in each of her oocytes [44,45]. For example, neuropathy, ataxia, and retinitis pigmentosa (NARP) is a well-characterised mtDNA disease that primarily affects the nervous system and is defined by the levels of the mutational event at position 8993 bp of the mitochondrial genome [46]. The level of the mutational rate at position 8993 bp can range from 0 to 97% of an oocyte's total mtDNA content amongst a cohort of oocytes from the same individual [47]. Consequently, for a woman who is aware that she is a carrier of a severe mtDNA mutation, it is a significant risk for her to become pregnant [26,48]. She has no clear indication of whether her child will be affected by the disease. In those cases where the offspring is not affected, the female offspring maintains the potential of being a carrier of mtDNA disease, which ensures the continued transmission of the disease to subsequent generations.

Equally so, it has recently been demonstrated that the number of women carrying mtDNA mutations is far more frequent than previously thought. It is now estimated that 1 in 200 females are carriers [49–51], however, this only translates into an incidence of mtDNA disease of between 1 in 1000 and 1 in 5000 individuals. It is highly likely that the most deleterious mutations do not necessarily result in live offspring but either there is failure earlier on during development as the embryo seeks to implant into the endometrium of the female reproductive tract, or the embryo or the more advanced foetus undergoes spontaneous abortion [52,53]. Indeed, it is evident from studies conducted on developmental rate in humans that, in many cases of high mutant loading, spontaneous abortion occurs at later stages of development [48].

Whilst it is commonly accepted that OXPHOS and the Krebs cycle are the main metabolic pathways used during the early stages of preimplantation development, and this is to some extent species specific, there is increased glucose uptake at the blastocyst stage along with increased oxygen consumption [54]. However, these outcomes are based on *in vitro* analyses and little is known about *in vivo* development [55]. Nevertheless, the fact that embryos carrying pathological mutations progress during *in vivo* development supports the hypothesis that rapidly dividing progenitor cells, as with tumour-initiating cells, favour an environment where there is reduced generation of ATP through OXPHOS and increased utilisation of anaerobic production of ATP through glycolysis [56–59]. To this extent, it is more beneficial for an embryo to grow in a low oxygen environment and therefore rely on substrates contributing to glycolysis as the main fuel for the process of cell proliferation and early development [60–62]. Indeed, the inhibition of all or some of the ETC complexes results in an improved blastocyst rate suggesting that many *in vitro* grown embryos are generated in inappropriate metabolic environments [63]. This would ensure a rapid turnover of cells and proliferation of cell number, which is essential to early development and tumorigenesis. During the later stages of development, when organogenesis takes place, there is a greater demand for oxygen consumption and thus increased utilisation of ATP through OXPHOS. If there is high mutant load at the stage, then decreased OXPHOS capacity could lead to foetal mortality.

In many respects, the early embryo facilitates the propagation of mutant mtDNA. Once fertilisation has been initiated, mtDNA copy number reduces significantly during preimplantation development [41,64]. This is a two-fold mechanism where, firstly, each newly divided blastomere reduces its mtDNA copy number following division. Secondly, there appears to be an active reduction process throughout preimplantation development in large mammals but not the mouse [41,64,65]. It remains to be fully determined whether there is uniform distribution amongst the blastomeres for mtDNA copy and for any mutations that would be present. Studies in the mouse [66] suggest that indeed this is the case whilst studies in larger animals [67], including human [48,68], suggest otherwise. Nevertheless, in larger mammals, the reduction in mtDNA copy number persists through preimplantation development until the blastocyst stage [41,64].

The reduction in mtDNA copy number is matched by decreased levels of expression for POLGA and TFAM in porcine embryos and non-existent levels for TFAM in bovine embryos until the morula/blastocyst stages of development [64]. In the mouse, there is upregulation of *Tfam* at the 8-cell stage with the highest levels of expression at the morula stage whilst *PolgA* expression is first upregulated at the morula stage [69]. In zebrafish, mtDNA copy number is downregulated at equivalent stages with lower levels of PolgA but higher levels of Twinkle and TFAM [70]. This is matched by the highest levels of non-mitochondrially generated ATP during development [71]. The persistence of TFAM suggests that it is either involved in transcription, as evidenced by the expression of the mtDNA-encoded genes in bovine, mouse and zebrafish embryos or that it is involved as a packaging factor but not as a replication factor. These mtDNA reduction and replication events may also facilitate the replication of mutant or wild type mtDNA dependent on the foci of the embryo that will undergo mtDNA replication at any one given time point, as described in mouse oogenesis [17]. Consequently, reduction and replication could result in a neutral or skewed distribution of mtDNA content that persists through to gastrulation and may be dependent on whether complementation of mtDNA nucleoids is maintained or is biased by the region within the embryo that undergoes replication.

The formation of the blastocyst is a key developmental process, which marks the first stage of cellular differentiation within the embryo [72]. This is signified by the development of a trophectoderm, which consists of an outer layer of cells that is closely surrounded by the zona pellucida (the egg's shell). The trophectoderm is characterised by enlarged cells, which contain a greater number of mitochondria and copies of mtDNA. The trophectoderm is transcriptionally characterised by the expression of Cdx2 and Gata 3 amongst other factors, which are genes indicative of the early differentiation of the extra embryonic lineages [72,73]. The blastocyst also contains a blastocoele, which provides growth factors and other pre-requisites to promote blastocyst development [74]. Also within the blastocyst is a mass consisting of a smaller number of cells approximately one third of those that are associated with the trophectoderm. These cells constitute the inner cell

mass cells, which will later give rise to the embryo proper and the foetus [75]. Isolation of the inner cell mass cells and their culture in vitro gives rise to embryonic stem cells [75], which are used to study development and disease and may provide new cellular therapies [76].

The inner cell mass cells have reduced mtDNA copy number and continue to suppress mtDNA replication [39,41]. This suppression is maintained as embryonic stem cells are established [56]. In pluripotent embryonic stem cells, which have the potential to differentiate into all somatic cell types, mtDNA copy number is reduced to between 30 and 200 copies [13,56]. This low level of mtDNA copy number is maintained in the pluripotent stem cells until they commit to differentiation. In heteroplasmic embryos, there may be skewing of mutant nucleoids to either the inner cell mass cells or the trophectoderm. In either case, the copies present in the trophectoderm will be present for replication whilst the inner cell mass cell copies will undergo further reduction and affect the level of mutant molecules present for segregation at gastrulation.

The mtDNA reduction event in the inner cell mass cells establishes the mtDNA set point, which ensures that each newly divided cell has a similar number of mtDNA copies that are clonally expanded as undifferentiated cells commit to a specific lineage fate [13,56,58,77] (see Fig. 2). This mechanism is very similar to the amplification of mtDNA as primordial germ cells undergo differentiation into mature oocytes during oogenesis [39]. As with trophectodermal differentiation, the maintenance of pluripotency is also regulated by expression of specific genes. The key regulators of pluripotency are Oct4, commonly described as the 'gatekeeper' of pluripotency [78,79], Sox2 [80] and Nanog [81,82]. These 3 factors form the pluripotent network where they interact with each other and suppress differentiation [83]. However, either down- or up-regulation in expression of one of these genes triggers the process of differentiation.

It has become increasingly clear that the control of mtDNA copy number plays an important role in maintaining pluripotency. Along with the key genes associated with pluripotency, the nuclear-encoded PolgA is expressed at low levels [56]. Disruption to *PolgA* expression, as demonstrated by siRNA knockdown experiments, results in the loss of pluripotency in undifferentiated embryonic stem cells and triggers the expression of the earliest markers of lineage specification, such as Brachyury [56]. The onset of spontaneous differentiation is marked by variable levels of expression for Oct4, Sox2 and Nanog and also changes in mtDNA copy number. In some mouse embryonic stem cell lines, there is an initial surge (day 1 of differentiation) in mtDNA replication that is then suppressed and levels return to steady state levels, as is the case for all mouse embryonic stem cell lines. Nevertheless, for those lines already analysed, there is a significant increase in mtDNA copy number at day 6 of differentiation [56]. Day 6 equates to embryonic day 6.5 in mouse embryonic development [84] and marks the stage of development when PolgA homozygous knockout mice die in utero [85] suggesting that this is a key developmental regulatory checkpoint to determine whether mtDNA replication is functioning effectively [57]. Again, after day 6 of differentiation, there is a significant decrease in mtDNA copy number, with a return to higher levels on day 11 onwards, which is then maintained [56]. Consequently, in embryos and foetuses harbouring heteroplasmic mtDNA nucleoids, there is the potential to select for or against mutant mtDNA.

These mtDNA replication events during differentiation are regulated in a cell specific manner by DNA methylation of exon 2 of PolgA [13]. Initially, it was proposed that mtDNA copy number was regulated during spermatogenesis in a stage-specific manner by DNA methylation to this intragenic region [86]. In the mouse, oocytes and blastocysts have very little DNA methylation of PolgA [13]. This is modulated at various stages of differentiation. However, fully mature cells have very different levels of DNA methylation within exon 2 dependent upon their requirement for mtDNA copy number and thus ATP generated through OXPHOS. As a result, cells such as cardiomyocytes, muscle cells and neuronal cells have high levels of mtDNA copy number whilst cells of the spleen have very few copies of mtDNA [13,87].

As with pluripotent and tumorigenic cells, reduced mtDNA copy number in mature cells is likely to be associated with their proliferative capabilities. There is little proliferation in neuronal cells [88], whilst the spleen, which generates many of the cells associated with immune response would be recruited quickly necessitating faster cell turnover [89]. Furthermore, their life expectancy is likely to be short as they primarily phagocytose the 'foreign' body in order to negate the effect of the insult and would require the faster-generating ATP process of glycolysis. The life expectancy of post-mitotic neuronal cells appears to be significantly longer. They mediate numerous cellular and intercellular activities including the generation and trafficking of neuronal transmitters. Consequently, the cell-specific requirement for the generation of energy is determined by the chromosomes of that particular cell, mediated by various epigenetic mechanisms that regulate the expression of the chromosomal genes [90,91]. This represents an interesting scenario, especially as the chromosomal genome goes to extraordinary lengths to actively DNA methylate the mitochondrial genome and then erase these marks, as demonstrated by recent studies where the process of DNA demethylation counters the process of DNA methylation [13].

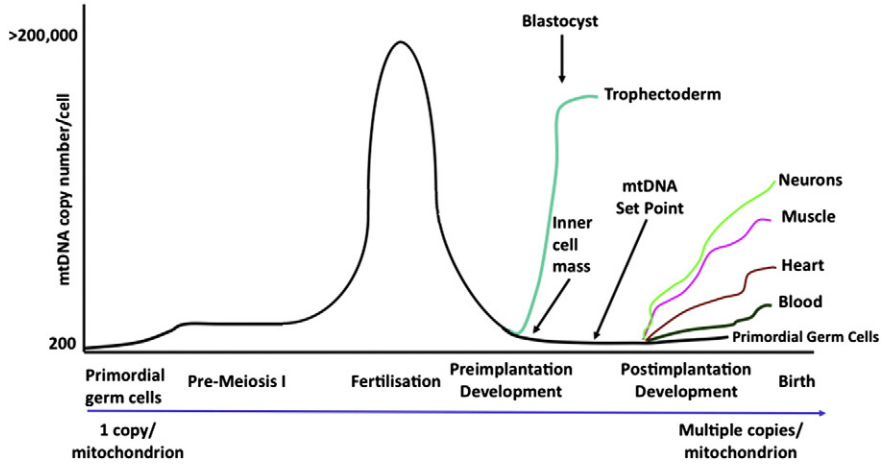

Fig. 2. The control of mtDNA copy number at key stages during development. During oogenesis, mtDNA copy number increases up to the metaphase II stage. In larger mammals, it then undergoes significant reduction during preimplantation development. At the blastocyst stage, replication is initiated at the blastocyst stage but this is confined to the trophectoderm. MtDNA copy number undergoes continual reduction in the inner cell mass cells to establish the 'mtDNA set point'. This ensures that specialised cell types, once they have differentiated, acquire the appropriate mtDNA copy number to meet their needs for ATP production in a cell type specific manner.

1.6. MtDNA transmission and assisted reproductive technologies

Within the last 30 years, a series of new assisted reproductive technologies has appeared in both human fertility clinics and the animal livestock industry [26]. In the fertility clinics, these have been introduced in order to enhance infertility outcomes for couples that have persistently failed to achieve a pregnancy. In this case, infertility is defined as a couple being unable to achieve a pregnancy after a year of attempting to do so without the use of any contraceptive aids [92]. In the livestock industry, assisted reproductive technologies have been introduced to enhance breeding potential and to select for specific genetic traits that the industry wishes to propagate, in order to produce sufficient food to overcome food security issues worldwide [93]. These social practices, however, impact on the transmission and inheritance of mtDNA. This is because some of these technologies transfer genetic material from additional sources to that of the mother and father. The process of *in vitro* fertilisation, where sperm is introduced to the oocyte in a culture dish, mimics the process of natural fertilisation more than the 'invasive' procedures do [94]. In this case, the processes of natural selection are allowed to take place although these sperm will not have transited the hostile environments of the cervical mucus or the female reproductive tract in order to meet the oocyte in the fallopian tube just after ovulation.

Furthermore, the recently introduced technology, intracytoplasmic sperm injection, where the sperm is immobilised and introduced into the egg via a microinjection pipette, does not result in the transmission of sperm or exogenous mtDNA [95]. However, its protégé, cytoplasmic transfer, which introduces cytoplasm taken from a donor oocyte along with a sperm into the egg [96], often results in the transmission of 2 populations of oocyte mtDNA to the offspring [97,98]. This approach was introduced into clinical medicine for those women whose embryos underwent repeated developmental arrest at or around the 8-cell stage of preimplantation development. It was argued that, by introducing 'extra energy-generating units', the resultant embryos would be more viable and progress through to blastocyst, implantation and term [96]. Although the actual contribution from the donor oocyte is relatively low at the time of transfer, initial reports, which were never followed up, demonstrated that donor oocyte mtDNA contributed to approximately 40% of the offspring's total mtDNA genetic content [97,98].

A further problem with cytoplasmic transfer is that when heteroplasmy consists of 2 genetically divergent populations of mtDNA, as opposed to heteroplasmy arising from a specific mutation that leads to a severe mtDNA disease, the probability of the offspring inheriting a disorder is increased. From the few attempts of clinical cytoplasmic transfer, there were significant abnormalities. There was one case of autism and two cases of Turner's syndrome (XO) where one pregnancy was aborted spontaneously and one elected abortion procedure was performed [97]. In mouse experiments using such approaches, the offspring presented with numerous physiological abnormalities including systemic and pulmonary hypertension, increased body mass and abnormalities associated with electrolytes and haematological parameters [99]. In other studies of heteroplasmic mice, there are similar effects, including reduced activity, food intake, respiratory exchange ratio; accentuated stress response; and cognitive impairment, which render the offspring at a significant disadvantage [100]. The authors rightly argue that these findings demonstrate the advantage that the uniparental inheritance of mtDNA confers. One approach for circumnavigating the effects of heteroplasmy is to introduce mitochondria containing genetically identical mtDNA [67]. This can be achieved by subjecting women to superovulation protocols, as is the practice in many IVF clinics, but instead of fertilising all of the metaphase II oocytes, some would be sacrificed and used as mitochondrial donors. Such an approach has been demonstrated to enhance pig fertilisation outcomes [67]. Others have proposed isolating mitochondria from oocyte precursor cells which appear to be present in the ovary and can be isolated using a simple FACS protocol [101]. However, these approaches need robust testing before introduction into the clinic.

The assisted reproductive technology, somatic cell nuclear transfer (SCNT; cloning; Fig. 3A) was primarily designed to generate genetically identical offspring for biomedical science [102]. SCNT requires the transfer of a somatic cell into an oocyte that has had its chromosomes removed (enucleated). The somatic cell is accompanied by mitochondria, and thus mtDNA, as it is transferred into the recipient oocyte [103]. Often, the somatic cell is a fibroblast and, commonly, they possess between 2000 and 3000 copies of mtDNA [104]. In the reconstructed oocyte, this represents a contribution of between 1 and 1.5% of the oocyte's total mtDNA content. In many respects, this would appear minimal and many cloners took this view. Indeed, the first report demonstrated that 'Dolly the Sheep' and the other sheep clones appeared to eliminate their somatic donor cell mtDNA at some stage during development [105]. Whilst screening of the clones at the chromosomal and mtDNA levels ensures that the process of SCNT has taken place, it also demonstrates that the clones are not genetically identical and they possessed 'foreign' mtDNA. However, studies on the levels of mtDNA heteroplasmy in cloned offspring produced some remarkable outcomes. In cattle clones generated through a donor embryonic cell, there were variable levels of reported transmission, with the suggestion that the level of inherited donor mtDNA was equivalent to the initial contribution at oocyte reconstruction [106]. However, it is now evident from a range of reports in livestock (sheep, cattle and pigs) that the transferred mtDNA can contribute from 0% to 59% of the total mtDNA content of the offspring [107] and there appears to be no correlation with the starting population of donor mtDNA. More importantly, many of these offspring suffer from a series of abnormalities, which include cardiomyopathy, lung disorders, and anatomical and epigenetic abnormalities [108]. It now needs to be determined whether these diseases, which to some extent mimic those associated with mtDNA disorders, are mtDNA in origin or arise from the incomplete reprogramming of the somatic cell by the oocyte's cytoplasm.

Based on the assumptions made for cytoplasmic transfer, it is essential that mtDNA accompanying the donor cell should be eliminated prior to SCNT. Indeed, this has been achieved by depleting the donor cell of its mtDNA content prior to SCNT [104,109] (see Fig. 3B). As a result, the resultant offspring thus inherited their mtDNA content from only the recipient oocyte [110], as this is the case following natural

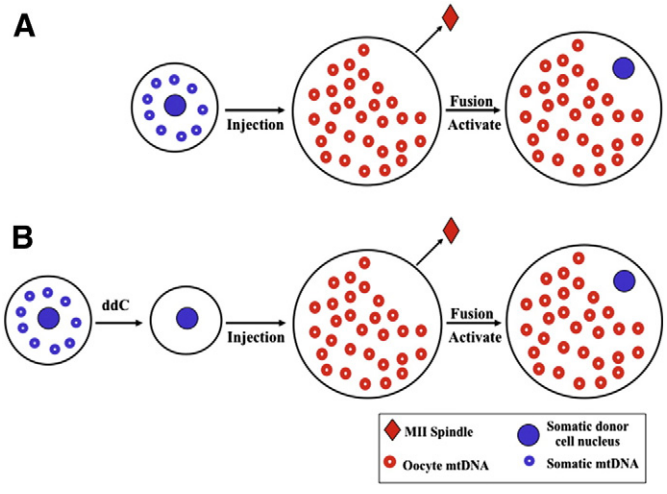

Fig. 3. Somatic cell nuclear transfer (SCNT). (A) The conventional approach involves the enucleation of the chromosomes from a mature recipient oocyte and the introduction of a donor somatic cell, containing chromosomes and mtDNA, into the enucleated oocyte followed by fusion of the two entities. The reconstructed oocyte is then activated, cultured *in vitro* and transferred to the uterus of a surrogate. (B) Donor cell mtDNA can be eliminated using a mtDNA depletion agent, e.g. 2′,3′-dideoxycytidine (ddC). The donor cell is transferred into an enucleated recipient oocyte, as in (A). This ensures that mtDNA is transmitted from the oocyte only.

fertilisation. However, reduction of donor cell mtDNA content to very low levels still results in it persistence throughout preimplantation development [109] meaning that it is present at gastrulation where it will be segregated to some or all lineages including the germ line. Indeed, whatever mtDNA is introduced into the recipient oocyte, it has the potential to be transmitted. In the one report of cloned non-human primates, there was also leakage of sperm mtDNA that contributed to the embryonic cell that constituted the donor cell [111]. This arose as the donor cell was generated from gametes that were from interspecific crosses, which were not eliminated by the process of ubiquitination and subsequent proteolysis, as is the case for intraspecific crosses [112].

1.7. Why does donor cell mtDNA persist following SCNT?

SCNT has significantly enhanced our understanding of the importance of controlling mtDNA replication events during early development. Following the transfer of the somatic cell into the recipient oocyte, the somatic cell continues to actively express the mtDNA-specific replication factors Tfam, PolgA and PolgB [104,109]. Consequently, the failure to silence these genes during preimplantation, as is the case following *in vitro* fertilisation, enables donor cell mtDNA to be selected for replication during preimplantation development rather than being diluted out. However, the variable rates of donor cell mtDNA transmitted to the offspring remain a quandary.

Nevertheless, the outcome of these studies demonstrates that the control of mtDNA replication during preimplantation development is an important developmental process. As fertilisation takes place, the sperm enters the egg and brings with it a small population of mtDNA [113]. In abnormal human embryos, this population of mtDNA can persist to the blastocyst stage [114] and would therefore be present in the cells progressing through to gastrulation. By preventing mtDNA replication taking place, this ensures that sperm mtDNA is not replicated prior to its elimination once fertilisation has taken place and before the embryonic genome is activated [77]. Interestingly, there is one reported clinical case of a male suffering from a mitochondrial myopathy due to the transmission of his father's sperm mtDNA demonstrating the importance of eliminating sperm mtDNA [115]. Importantly, sperm mtDNA contributes 0.0001% of a fertilised oocyte's total mtDNA content. However, for the mutated sperm mtDNA to trigger a phenotypic effect, it would need to be present at between 80 and 90%, as was the case with the male patient. This represents a significant selective replication event for the mutant mtDNA. Consequently, the introduction of any mtDNA into an egg at the time of fertilisation has the potential to be amplified.

1.8. The risks of using assistive reproductive technologies to treat women who are carriers of mtDNA disease and to prevent the transmission from one generation to the next

For women who are carriers of mtDNA disease, it is a real challenge to prevent the transmission of mutant mtDNA from one generation to the next. For some carriers, there will be no history of mtDNA disease in the family or from maternal ancestors because the disease is only observed in patients who present with high mutant loading that affects tissue or organ function [116]. Consequently, the birth of a baby or a child that develops mtDNA disease will be completely unexpected. Others, who know that they are carriers, may have sought genetic counselling and chosen to use assisted reproduction followed by preimplantation genetic diagnosis to select for oocytes that are least at risk of affecting the offspring [117]. However, for those who wish to have children that would not be at risk of harbouring mutant mtDNA, there are currently two candidate assisted reproductive technologies under experimental investigation. These are metaphase II spindle transfer [118] (Fig. 4A) and pronuclear transfer [119,120] (Fig. 4B).

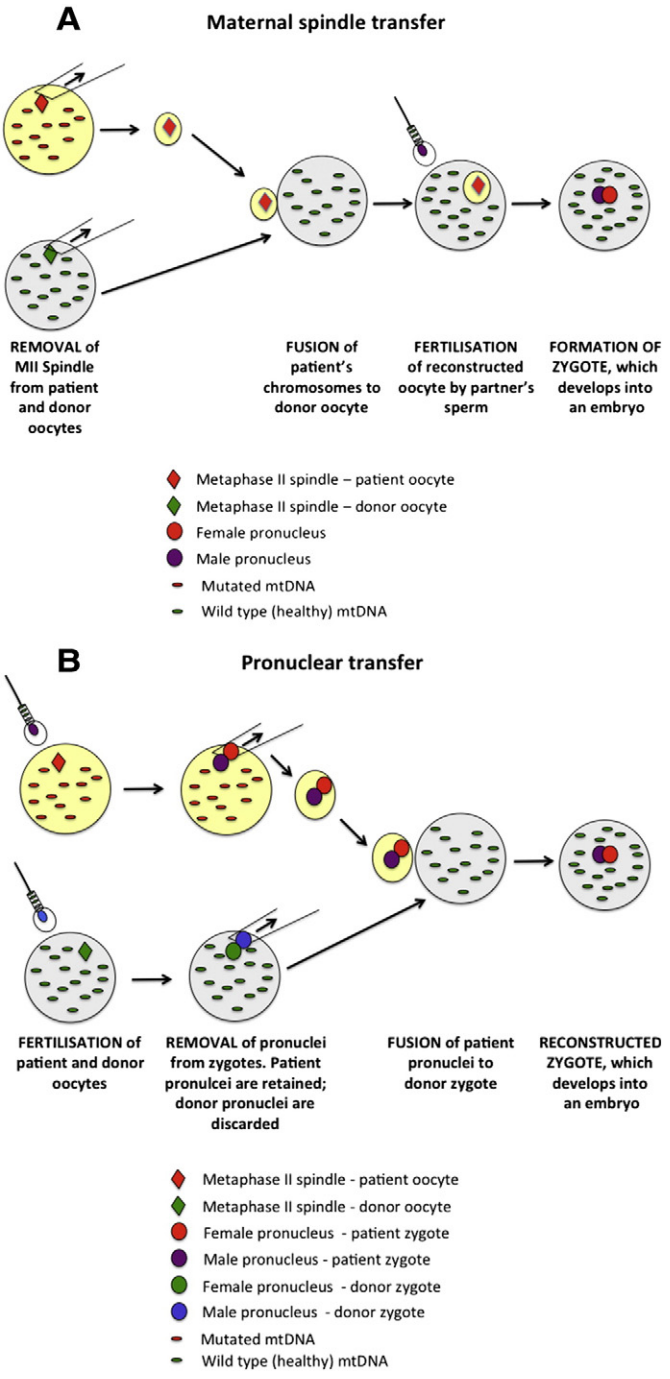

Fig. 4. The candidate approaches to prevent the transmission of mutant mtDNA. (A) Metaphase II spindle transfer. The metaphase II-spindle containing the mother's chromosomes is removed from the mature oocyte of a carrier of mtDNA disease (yellow oocyte). It is then transferred into an enucleated oocyte donated by a non-carrier of mtDNA disease (grey oocyte) and fused. The reconstructed oocyte is fertilised, cultured *in vitro* and transferred to the uterus of the 'chromosomal' mother. (B) Pronuclear transfer. The male and female pronuclei of a fertilised oocyte are removed from a fertilised oocyte (yellow), transferred to and fused to a 'non-affected' zygote (grey), which is then cultured *in vitro*, as for metaphase II-spindle transfer. In each case, mtDNA accompanying the karyoplast can be transferred and transmitted.

Normally, a metaphase II oocyte is a mature oocyte that has completed its growth and development, is haploid and has extruded the first polar body and is thus described as being developmentally competent [121]. Once ovulated, it is fertilised by sperm that has transited the female reproductive tract as far as the oviduct. As part of an assisted reproduction regime using hyperstimulation protocols, a number of oocytes are ovulated. This provides embryologists with a greater

number of embryos from which to generate embryos for couples with subfertility. As demonstrated in a monkey model, metaphase II spindle transfer involves the removal of the metaphase II spindle from an ovulated mature haploid oocyte carrying mtDNA mutations in its cytoplasm [118]. It is transferred into an ovulated metaphase II enucleated recipient oocyte from a donor who would have been screened to ensure that she was not a carrier of mutant mtDNA (Fig. 4A). Once the transfer has taken place, the reconstructed oocyte is fertilised with the partner's sperm, as is the case for *in vitro* fertilisation, the second polar body is extruded and the resultant zygote develops into an embryo. Once blastocysts have developed, one or possibly two are transferred to the chromosomal mother's uterus ready for implantation. It is pertinent that as few embryos as possible are transferred to prevent multiple births occurring.

Pronuclear transfer is similar, except that the partner's sperm first fertilises the superovulated oocytes [119,120] (Fig. 4B). Once fertilisation has occurred and zygotes have formed where the sperm and oocyte chromosomes oppose each other, prior to syngamy, the pronuclei are removed from the zygotes and transferred into enucleated zygotes. The embryos are cultured to blastocyst, and transferred to the chromosomal mother, as for metaphase II spindle transfer.

The problem faced by both these technologies is that mitochondria surround the metaphase II spindle and the pronuclei prior to transfer and it is unfeasible to remove the karyoplast housing the chromosomes without removing some mitochondria as well. Studies in mouse and monkey have shown using both technologies that the contaminating mtDNA can persist [120,122,123], where, for example, the transferred mtDNA can contribute from 6% to 69% of the total mtDNA content of a particular tissue [120]. Other studies using pronuclear transfer in human have also shown that the transferred mtDNA can persist to the blastocyst stage [119] indicating that it is viable at the time of gastrulation for selective amplification and inclusion into the genome of the offspring. Studies in monkeys using metaphase II transfer have shown that the accompanying mtDNA is not necessarily transmitted to the offspring's somatic tissues but it is transmitted to the germ line of these offspring [123]. Consequently, to ensure that no mutant mtDNA persists, it is essential that the transferred karyoplast does not carry any accompanying mtDNA into the recipient oocyte.

1.9. How do mtDNA haplotypes affect early development?

MtDNA haplotypes have evolved over billions of years. MtDNA haplotypes are a series of polymorphic variants found within the mitochondrial genome that are indicative of populations from specific regions or continents around the world [124]. In humans, they are subdivided according to letter (A–Z). Each human mtDNA haplogroup originates from the most recent common maternal ancestor for all currently living humans, namely Mitochondrial Eve. They can confer a positive or negative advantage to the individual [125]. For example, they influence sperm motility in humans, whereby haplotype T is associated with decreased sperm motility in men suffering from infertility whilst haplotype H is indicative of normal sperm motility [126]. They are indicative of adaptation to warm and cold climates [127], longevity [128] and predisposition to various age-associated disorders [125], such as cancer [129] and diabetes [130]. They also predispose individuals to and protect against Alzheimer's [131,132] and Parkinson's disease [133–135]. In livestock, mtDNA haplotypes influence milk quality [136] and fertility [67,137]. In mice, they regulate growth and physical performance [138].

It is well described that each of mtDNA transcription, replication and translation are entirely or mostly regulated by the chromosomal genome. However, the presence of different mtDNA haplotypes has a phenotypic effect on an individual. That being the case, it is essential to know whether those haplotypes can influence chromosomal gene expression patterns. Recently, it has been demonstrated using an embryonic stem cell model, containing the same set of chromosomes but different mtDNA backgrounds, that different mtDNA haplotypes can indeed influence chromosomal gene expression [139]. As with many outcomes associated with the phenotype of an individual, these effects are established during earlier development. In pluripotent embryonic stem cells, those cells yet to undergo differentiation, there are slight differences in the levels of expression of the three key genes associated with the pluripotent network, Oct4, Sox2 and Nanog and also Rex1, which is the first gene to usually be effected as differentiation is initiated. The changes in chromosomal gene expression then manifest as the cells differentiate into all lineages of the body. Consequently, there are haplotype biases during differentiation towards either the mesodermal or the ectodermal lineages. There are discrete changes in mtDNA copy number, ATP production and lactate levels based on mtDNA haplotype. Furthermore, there are variable levels of DNA methylation for the pluripotent genes and within exon 2 of PolgA suggesting that mtDNA haplotypes can affect the epigenetic status of a cell and its mtDNA copy number, especially during neural differentiation. Changes in epigenetic status have also been observed in tumour cells harbouring different mtDNA haplotypes but the same chromosomal genotype [140].

Taken a step further, those oocyte reconstruction protocols used by assisted reproductive technologies also produce interesting outcomes in terms of the relationship between the chromosomes and the mitochondrial genomes. In this particular case, a number of studies have indicated that successful nuclear transfer is dependent upon an appropriate set of chromosomes being matched with a compatible population of mtDNA [141]. Interestingly, the choice of mtDNA background is not that of the donor cells own mtDNA background but a population of mtDNA that is slightly more genetically divergent [142]. Indeed, it appears to be disadvantageous for the chromosomes to interact with a mtDNA haplotype that is the same as its own and that the window of opportunity lies with a slightly more divergent population but, as divergence increases towards the species barrier, or in other words, a more incompatible population of mtDNA, then successful development to term of any cloning procedure is significantly decreased. Likewise, the levels of DNA methylation within exon 2 of PolgA tend to be different for different mtDNA haplotypes [13], which suggests a mtDNA haplotype specific relationship is formed with the chromosomal genome to regulate mtDNA copy number.

2.1. Conclusion

The regulation of mtDNA copy number during development is a tightly regulated process. This ensures that the individual is able to regulate the number of mtDNA copies that are present in mature cells to achieve the appropriate levels of energy metabolism through OXPHOS that are required by each cell type. Some stem cell technologies fail to regulate these processes effectively, such as induced pluripotent stem cells and those derived through somatic cell nuclear transfer. As a consequence, these approaches may not result in fully functional cells. Equally so, the process of regulation of mtDNA copy number is mediated in a cell-specific manner by DNA methylation of PolgA at each of the key stages of development. However, the mtDNA haplotype will regulate this process at various stages during development, which ensures that the haplotype is indicative of the phenotypic behaviour associated with a group of individuals of specific origin.

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