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Transmission of mitochondrial DNA following assisted reproduction and nuclear transfer

E.C.Spikings, J.Alderson and J.C.St. John[1]

The Mitochondrial and Reproductive Genetics Group, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

[1]To whom correspondence should be addressed at: The Medical School, The University of Birmingham, Birmingham B15 2TT, UK. E-mail: j.stjohn.1@bham.ac.uk.

Mitochondria are the organelles responsible for producing the majority of a cell's ATP and also play an essential role in gamete maturation and embryo development. ATP production within the mitochondria is dependent on proteins encoded by both the nuclear and the mitochondrial genomes, therefore co-ordination between the two genomes is vital for cell survival. To assist with this co-ordination, cells normally contain only one type of mitochondrial DNA (mtDNA) termed homoplasmy. Occasionally, however, two or more types of mtDNA are present termed heteroplasmy. This can result from a combination of mutant and wild-type mtDNA molecules or from a combination of wild-type mtDNA variants. As heteroplasmy can result in mitochondrial disease, various mechanisms exist in the natural fertilization process to ensure the maternal-only transmission of mtDNA and the maintenance of homoplasmy in future generations. However, there is now an increasing use of invasive oocyte reconstruction protocols, which tend to bypass mechanisms for the maintenance of homoplasmy, potentially resulting in the transmission of either form of mtDNA heteroplasmy. Indeed, heteroplasmy caused by combinations of wild-type variants has been reported following cytoplasmic transfer (CT) in the human and following nuclear transfer (NT) in various animal species. Other techniques, such as germinal vesicle transfer and pronuclei transfer, have been proposed as methods of preventing transmission of mitochondrial diseases to future generations. However, resulting embryos and offspring may contain mtDNA heteroplasmy, which itself could result in mitochondrial disease. It is therefore essential that uniparental transmission of mtDNA is ensured before these techniques are used therapeutically.

Key words: assisted reproduction technology/mitochondrial DNA/nuclear transfer

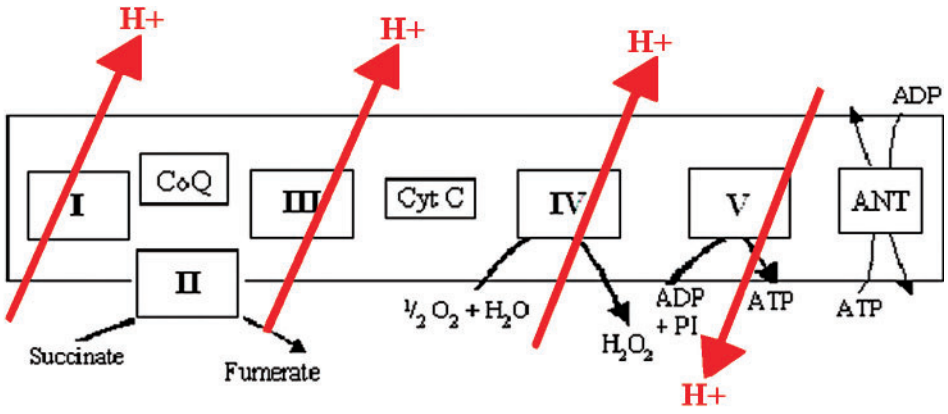
Introduction

Mitochondria are found in all eukaryotic cells and are essential to cellular function because of their ability to generate ATP, the cell's major source of energy. They comprise a double membrane with the outer membrane separating the mitochondrial matrix from the cytoplasm of the cell, whereas the inner membrane forms the cristae. Mitochondrial cristae are the site of the electron transfer chain (ETC), the final stage in cellular respiration where oxidative phosphorylation (OX-PHOS) takes place (Sherratt, 1991). This aerobic process allows further metabolism of the products of anaerobic glycolysis and the citric acid cycle to produce carbon dioxide and water, with the subsequent release of 32 molecules of ATP. This is a far more economical return than provided by anaerobic generation of ATP (Pfeiffer *et al*., 2001). The ETC consists of five enzyme complexes, each containing multiple polypeptide subunits (Figure 1). Electrons flow through the first four complexes of the ETC releasing protons from the mitochondria which set up the mitochondrial membrane potential. Pumping these protons back into the mitochondria through the fifth complex results in the generation of ATP.

The importance of ATP in oocytes and early embryos has been demonstrated in cattle, as assessed by morphology. Here, the higher quality oocytes contained significantly higher levels of ATP than poorer quality oocytes (Stojkovic *et al*., 2001). Those containing the highest ATP levels also progressed to produce a significantly higher proportion of blastocysts. A similar pattern has been demonstrated in human oocytes, with those containing at least 2ρmol ATP at fertilization being more capable of embryonic development and implantation (Van Blerkom *et al*., 1995). The correlation between mitochondrial distribution in individual blastomeres and blastomere ATP content further emphasizes the importance of OX-PHOS for early preimplantation development (Van Blerkom *et al*., 2000).

Mitochondrial DNA

Components of the ETC are encoded by both chromosomal and mitochondrial DNA (mtDNA), with all components being required for efficient function of the ETC. The human mitochondrial genome is a double-stranded circular DNA consisting of



Complex	I	II	III	IV	V
Inhibitor	Rotenone		Antimycin	KCN	Oligomycin
nDNA subunits	>18	4	8	10	10
mtDNA subunits	7	0	1	3	2

Figure 1. The electron transfer chain (ETC) is the final stage in cellular respiration where oxidative phosphorylation (OX-PHOS) takes place. The ETC consists of five enzyme complexes which, with the exception of complex II, all consist of polypeptides encoded by both the nuclear and the mitochondrial genomes. Electrons flow through the first four complexes of the ETC releasing protons (H+) from the mitochondria and thereby setting up the mitochondrial membrane potential. Pumping these protons back into the mitochondria through the fifth complex results in ATP generation. CoQ, Coenzyme Q; Cyt C, Cytochrome C; ANT, adenosine nucleoside transporter; KCN, potassium cyanide; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

16 569 base pairs (Anderson *et al*., 1981). Using a slightly different genetic code to that of nuclear DNA, mtDNA encodes 13 polypeptides involved in the ETC, 22 transfer (t)RNAs and 2 ribosomal (r)RNAs (Anderson *et al*., 1981). MtDNA codes for seven subunits of nicotinamide adenine dinucleotide (NADH) dehydrogenase (ND; complex I), one component of complex III, three subunits of cytochrome C oxidase (complex IV) and two subunits of ATP synthase (complex V). All other components of the ETC are encoded by the nuclear genome (Figure 1). The mitochondrial genome is highly compact, with no introns contained between the coding regions (Anderson *et al*., 1981) and some of the coding regions even overlap (ATPase 6 and ATPase 8, ND4 and ND4L; Anderson *et al*., 1981). Others lack termination codons, with these sequences being provided by post-transcriptional polyadenylation (Ojala *et al*., 1981). Despite its small size in comparison with the nuclear genome, the host cell depends on the mitochondrial genome for aerobic respiration as much as the mitochondria rely on proteins encoded by the host cell's nuclear DNA. In fact, as much as one third of the total DNA content of an oocyte consists of mtDNA (Piko and Matsumoto, 1976). Somatic cells have between 10^3 and 10^4 copies of mtDNA, with 2–10 copies per organelle (Satoh and Kuroiwa, 1991), correlating to each particular cell's requirement for OX-PHOS. For example, it has been reported that human subcutaneous fat cells contain more mtDNA copies than peripheral blood mononuclear cells (Gahan *et al*., 2001) and that liver tissue contains more mtDNA than various muscle tissues (Wiesner *et al*., 1992).

MtDNA transcription and replication

Transcription of mtDNA occurs following interaction between nuclear-encoded regulatory proteins and regions within the D-loop of mtDNA (Figure 2). Requirements for transcription include the mitochondrial RNA polymerase (Tiranti *et al*., 1997), mitochondrial transcription factor A (TFAM; Fisher and Clayton, 1985; 1988), and one of the recently identified TFB1M and TFB2M (Falkenberg *et al*., 2002; Table I). Once initiated, transcription generates a polycistronic precursor RNA transcript, allowing coordinated transcription of all genes on the same strand. Excision of the polycistronic precursor by endonucleases produces precursor rRNAs and tRNAs, which are then processed further to allow them to translate the precursor mRNAs (Ojala *et al*., 1981).

The replication of mtDNA is also dependent on nuclear-encoded regulatory proteins (Table I). The replication process begins with light strand transcription from the light strand promoter region to the second conserved sequence block (CSB) within the D-loop region, producing an RNA sequence complementary to the light strand DNA (Xu and Clayton, 1995). Binding of the mtDNA-specific polymerase gamma (PolG) to the resulting RNA–DNA hybrid allows replication of the heavy strand to begin from the origin of heavy strand replication (O_H) contained within the D-Loop (Figure 2). After the replication of approximately two thirds of the genome, the formation of the new heavy strand exposes the origin of light strand replication (O_L) on the heavy strand parent. Light strand replication is then able to commence in the opposite direction.

As mtDNA transcription and replication, and therefore cellular ATP-generating capacity, are so dependent on proteins encoded by the nuclear genome, mutations in these genes can result in mtDNA depletion syndromes (reviewed by Suomalainen and Kaukonen, 2001). Unlike the vast majority of mtDNA diseases, these diseases can be transmitted in a Mendelian fashion and include chronic progressive external ophthalmoplegia (CPEO; Van Goethem *et al*., 2001; Graziewicz *et al*., 2004) and Alpers syndrome (Naviaux *et al*., 1999). This effect is also mimicked by

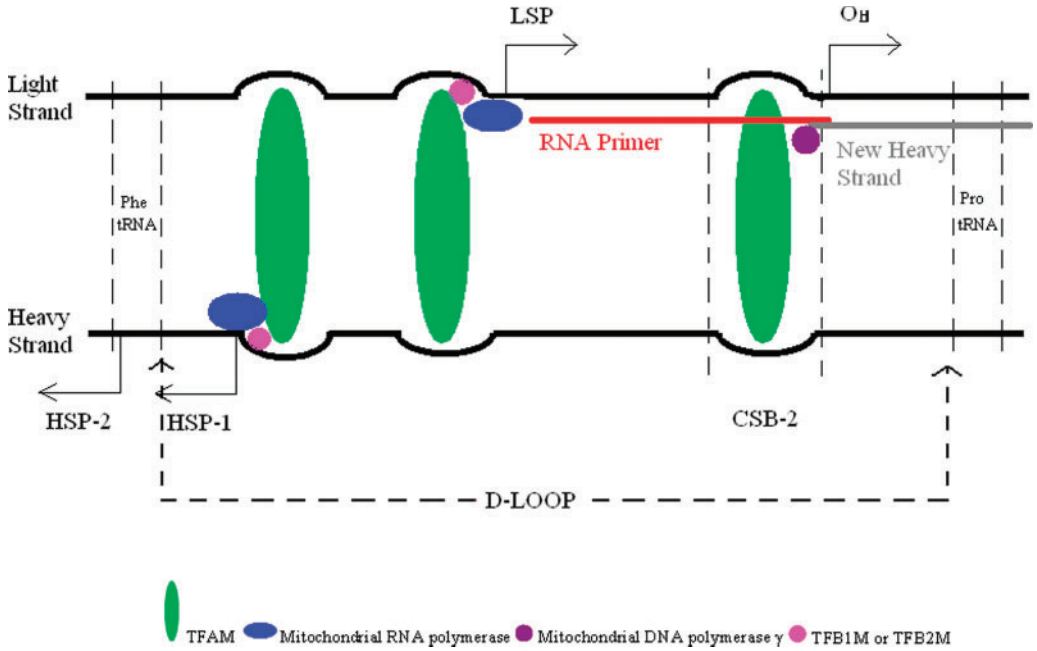


Figure 2. Mitochondrial DNA (mtDNA) transcription and replication origins in the D-loop. Mitochondrial transcription factor A (TFAM) binds and separates the two DNA strands. The accessory transcription factor, either TFB1M or TFB2M, allows mitochondrial RNA polymerase (mtRNAPol) to bind to the correct part of the DNA to produce the transcripts and the RNA primer for replication. Mitochondrial DNA polymerase gamma (PolG) is then able to use the primer to produce the daughter DNA strand. HSP, heavy strand promoter; LSP, light strand promoter; CSB, conserved sequence block; O_H, origin of heavy strand replication; D-loop, displacement loop, the non-coding region that is the binding site for nuclear-encoded transcription and replication factors; Phe tRNA, phenylalanine transfer RNA; Pro tRNA, proline transfer RNA.

Table I. Major nuclear-encoded regulatory factors required for mitochondrial DNA (mtDNA) transcription and replication

Protein	Binding site	Role
TFAM	Enhancer region	Causes unwinding of mtDNA, exposing the binding site for mtRNA polymerase
TFB1M or TFB2M	RNA polymerase	Form heterodimer with RNA polymerase allowing specific transcription initiation
MtRNAPol	Transcription initiation region	Produces RNA copy of mtDNA sequence
PolG	RNA–DNA hybrid	Produces replicate copy of mtDNA
MtSSB	Single-stranded mtDNA	Prevents re-annealing of single-stranded mtDNA allowing PolG polymerization
MTERF	RNA polymerase	Termination of transcription
RNase MRP	MtDNA primary light strand transcript	Processing of primary light strand transcripts to produce RNA primer for replication

MTERF, mitochondrial transcription termination factor; mtRNAPol, mitochondrial RNA polymerase; mtSSB, mitochondrial single-stranded binding protein; PolG, polymerase gamma; RNase MRP, mitochondrial RNA processing endoribonuclease; TFAM, mitochondrial transcription factor A; TFB1M/TFB2M, mitochondrial transcription factor B1 or B2.

nucleoside antiretroviral analogues such as 3′-azido-3′-deoxythymidine (AZT) and 2′3′-dideoxycytidine (ddC). For example, men treated with these drugs for 12 months had increased numbers of deleted mtDNA molecules in their sperm samples (White *et al*., 2001). Furthermore, although heterozygous PolG knockout mice develop normally, despite slightly reduced mtDNA levels, homozygous knockouts have severe mtDNA depletion and do not survive beyond embryonic day 8.5 (Hance *et al*., 2005).

TFAM plays an important role in both transcription and replication of the mitochondrial genome. It is therefore not surprising that low levels of TFAM have been associated with a variety of mitochondrial depletion syndromes, such as infantile mitochondrial myopathy (Poulton *et al*., 1994), familial mtDNA-associated liver disease (Spelbrink *et al*., 1998), fatal childhood myopathy (Larsson *et al*., 1994), skeletal muscle and mitochondrial encephalomyopathy disorders (Siciliano *et al*., 2000) and ocular myopathy, exercise intolerance and muscle wasting (Tessa *et al*., 2000). In contrast, there is a decrease in TFAM expression localized to the mitochondria during spermatogenesis. This has been proposed as a functional mechanism for specifically reducing mtDNA copy number in maturing sperm and therefore paternal mtDNA transmission to future generations, in both the human (Larsson *et al*., 1997) and the mouse (Larsson *et al*., 1996). This arises at the late spermatocyte/early spermatid stage where an isoform is expressed lacking the mitochondrial targeting sequence. In the mouse, TFAM was also detected in the nucleus of developing sperm cells, indicating a possible further role as a nuclear transcription factor (Larsson *et al*., 1996). The significance of TFAM expression as a major regulator of mtDNA copy number is exemplified in homozygous knockout mouse embryos that are completely depleted of mtDNA and do not survive after embryonic day 10.5 (Larsson *et al*., 1998).

The replication of embryonic mtDNA, at least in the mouse, does not occur during early preimplantation development (Piko

and Taylor, 1987). However, a small window of replication at the two-cell stage was recently reported (McConnell and Petrie, 2004). The mtDNA molecules present in the mature oocyte at fertilization are therefore largely relied upon to provide sufficient ETC components to allow OX-PHOS activity during the preimplantation phase of embryonic development. In order to meet this demand, mtDNA is amplified during oocyte maturation within the ovary, with a 45-fold increase being reported in cattle from the primordial germ cells stage to pre-ovulating oocytes (Smith and Alcivar, 1993), resulting in a mean of 260 000 copies being present in mature bovine oocytes (Michaels *et al*., 1982). Similar amplifications have been reported in other species. For example, human premigratory primordial germ cells possess approximately 10 mitochondria per cell (Jansen and De Boer, 1998). Mature mouse oocytes contain mean mtDNA copy number values between 119 000 (Piko and Taylor, 1987) and 159 000 (Steuerwald *et al*., 2000). In the human, copy number ranges from 10 000 to 700 000 (Almeida-Santos *et al*., 2006), with other reported mean values of 314 000 (Steuerwald *et al*., 2000) and 193 000 (Reynier *et al*., 2001).

Oocytes with higher copy numbers of mtDNA are known to be associated with improved fertilization rates. For example, human oocytes failing to fertilize because of poor sperm quality, and therefore assumed to be of good quality, had significantly higher mtDNA copy number than poor quality oocytes which were fertilized by good quality sperm (mean 255 000 versus 152 000 copies; Reynier *et al*., 2001). Furthermore, low-quality oocytes from patients with a variety of ovarian disorders contained an average of 100 000 mtDNA molecules compared with an average of 256 000 copies in oocytes from healthy individuals (May-Panloup *et al*., 2005). We have also recently reported similar results in porcine (El Shourbagy *et al*., 2006) and human (Almeida-Santos *et al*., 2006) oocytes. However, we also saw significantly different copy number between arrested two-cell human embryos and those that developed further (Almeida-Santos *et al*., 2006). A putative threshold number of mtDNA molecules has been proposed for the mature unfertilized oocyte to allow embryo development to take place after fertilization. In the mouse, this has been predicted at approximately 100 000 copies per oocyte (Piko and Taylor, 1987), with a similar value being appropriate for the pig (El Shourbagy *et al*., 2006).

The relevance of mtDNA copy number threshold to fertilization outcome and embryo development

The number of mtDNA copies per organelle is indicative of the number of mitochondria in an oocyte. In the mouse, each mitochondrion has just one copy (Piko and Taylor, 1987). Consequently, the minimum number of mtDNA copies would indicate the minimum number of mitochondria required for fertilization to take place. These mitochondria would support embryonic development to cavitation, after which glycolysis becomes increasingly important in many species (human – Leese *et al*., 1993; pig – Sturmey and Leese, 2003; cow – Thompson *et al*., 1996; rat – Brison and Leese, 1991; mouse – Houghton *et al*., 1996). Furthermore, this minimum number may be essential as it has been hypothesized that a threshold level of ATP is required to energize cell division (Brenner *et al*., 2000) and may also synchronize mitochondrial dynamics. The distribution of mitochondrial networks throughout the cell varies with different stages of the cell cycle (Margineantu *et al*., 2002). Mature and immature oocytes also have distinct patterns of mitochondrial distribution (Stojkovic *et al*., 2001; Nishi *et al*., 2003). Those oocytes with mtDNA copy number lower than the required threshold may be unable to form the networks required for developmental competence. In support of this, developmental competence appears to be related to the numbers of mitochondria present (El Shourbagy *et al*., 2006).

In sea urchin oocytes, mitochondria act as calcium sinks (Eisen and Reynolds, 1985). The Ca^{2+} wave pacemaker that is necessary for the completion of meiosis at fertilization is maintained by mitochondrial uptake of Ca^{2+} (Dumollard *et al*., 2003). Failure to maintain the Ca^{2+} wave pacemaker results in apoptotic cell death of the oocyte (Liu *et al*., 2001). Therefore, oocytes with low levels of ATP production, because of low levels of mitochondria and mtDNA, may be unable to maintain the Ca^{2+} wave pacemaker and complete meiosis, and instead, undergo apoptosis. We have previously hypothesized that oocytes without sufficient wild-type mtDNA, and therefore the capacity to generate ATP would not normally be ovulated (St. John, 2002). This is reflected in the low numbers of mtDNA observed in degenerate oocytes (mean = 44 629 ± 40 729; Almeida-Santos *et al*., 2006). Consequently, the use of superovulation protocols during IVF allows these low-quality oocytes to undergo the maturation process and the inadequacies of insufficient mtDNA molecules to be observed (St. John, 2002).

Mitochondrial morphology changes after oocyte maturation to the inactive state (Bavister and Squirrell, 2000). This may be because of the lack of mtDNA replication and the import of nuclear-encoded regulatory factors from the cytoplasm. However OX-PHOS is still functional during early embryo development, and mitochondria are dynamic organelles, moving to provide ATP where it is most needed (Stojkovic *et al*., 2001). Staining of mitochondria has shown that during embryo development, they cluster around the nucleus of blastomeres during the cleavage stages, providing the energy required for cell division processes such as spindle formation. However, when cell division is not taking place, they tend to spread throughout the cytoplasm of the cell (Stojkovic *et al*., 2001; Sun *et al*., 2001; Wilding *et al*., 2001).

Equal segregation of mitochondria following cell division reduces the possibility of resulting tissues of the offspring lacking the ability to produce sufficient ATP through OX-PHOS. However, disproportionate numbers of mitochondria among blastomeres has been reported in the pig (El Shourbagy *et al*., 2006) and human (Van Blerkom *et al*., 2000; Lin *et al*., 2004). This may result in some blastomeres with reduced ATP-generating capacity. If this occurs early on in development, blastomeres may fragment, resulting in embryo arrest or less competent blastocysts with fewer cell numbers. In the human, mitochondrial morphology changes back to the active state at the eight-cell stage, coinciding with the activation of the embryonic genome (Sathananthan and Trounson, 2000). Furthermore, during cavitation, shortly after embryonic genome activation, there is an increased dependence on glycolysis in most mammalian species (see Van Blerkom *et al*., 2002 for discussion). This would suggest that nuclear embryonic genome activation provides a signal to the mitochondria to reduce OX-PHOS activity. Increased use of glycolysis may allow embryonic cells that are no longer able to generate sufficient ATP through OX-PHOS to survive for long enough to enable production and import of nuclear-encoded regulatory factors necessary for the onset of mtDNA replication post-implantation (El Shourbagy *et al*., 2006). Indeed,

studies in pigs using OX-PHOS inhibitors have indicated that the inhibition of OX-PHOS function at compaction can improve embryo viability (Machaty *et al*., 2001). This hypothesis is supported by reports of glycolysis rather than OX-PHOS metabolism occurring in other cell types with a functioning nuclear genome but low mtDNA copy number, including somatic cells being depleted of their mtDNA (King and Attardi, 1989). However, ATP production through glycolysis is far less efficient than through OX-PHOS. It is therefore vital that sufficient mtDNA molecules are present at fertilization to maintain embryo survival until mtDNA replication resumes.

The relevance of mtDNA replication to maternal transmission of mtDNA

MtDNA is further unlike nuclear DNA as it does not follow the normal Mendelian pattern of inheritance but is maternally inherited (Giles *et al*., 1980). Owing to its size, a mature oocyte can contain far more mitochondria in its cytoplasm than the fertilizing sperm cell. Coupled with replication of the mitochondrial genome during oocyte maturation in the ovary, this results in an oocyte : sperm mtDNA ratio of 1000:1 (Ankel-Simons and Cummins, 1996), with sperm mtDNA contributing approximately only 100 molecules at fertilization (Hecht and Liem, 1984).

MtDNA is usually homoplasmically transmitted, i.e. only one mtDNA genotype being present (Monnat *et al*., 1985). Occasionally, two or more mtDNA genotypes can be present, often because of either a pathological or a non-pathological rearrangement, resulting in heteroplasmy, as in the oocytes of women harbouring a mtDNA disease (Blok *et al*., 1997). MtDNA has a much higher mutation rate than nuclear DNA (Wallace *et al*., 1987). This is a consequence of the production of mutagenic free radicals within the mitochondria, which are further confounded by limited mtDNA repair mechanisms (Yakes and Van Houten, 1997). Furthermore, mtDNA is not stabilized with histones as is the case for nuclear DNA, although TFAM is now thought to be involved in mtDNA packaging (Alam *et al*., 2003). Despite the high mutation rate, mtDNA has been highly conserved throughout evolution, and when it arises, mtDNA heteroplasmy tends to revert back to homoplasmy sometimes within one generation (Koehler *et al*., 1991).

MtDNA replication does not occur in synchrony with nuclear DNA replication, and therefore some mtDNA genomes are not replicated during one-cell cycle, whereas others are copied many times (Sazer and Sherwood, 1990). It has been reported that there is a preferential replication of those mtDNA molecules that are closer to the nucleus (Davis and Clayton, 1996), perhaps because of the faster diffusion of nuclear-encoded regulatory factors. The introduction of exogenous mtDNA into zygotes either close to the nucleus or at the periphery influences the amount of exogenous mtDNA observed at the blastocyst stage, with higher proportions arising from karyoplast injections (Meirelles and Smith, 1998). Consequently, the position of mitochondria in the early embryo may play a role in determining which mtDNA genomes are selected for amplification.

Contradictory data have recently shown that mtDNA replication is not just restricted to mitochondria in proximity to the nucleus (Magnusson *et al*., 2003). Here, it was demonstrated that all components of the replication machinery were available in sufficient quantities throughout the cell, allowing replication to take place throughout the entire cytoplasm. This included PolG and TFAM. The previously reported preferential labelling of mtDNA near cell nuclei was suggested to be because of denser aggregation of mitochondria around nuclei (Magnusson *et al*., 2003). MtDNA replication throughout the cell cytoplasm is supported by a lack of evidence for selection against pathogenic mtDNA mutations in oogenesis or early embryonic development (reviewed by Shoubridge, 2000).

When mtDNA replication resumes following implantation (Piko and Taylor, 1987), each cell type of the developing embryo contains a few copies of mtDNA, as indicated by the few mitochondria present in undifferentiated embryonic stem cells (ESCs; Sathananthan *et al*., 2002; St. John *et al*., 2005a). Consequently, the combination of segregation followed by the amplification of selected templates produces an mtDNA purification process (Hauswirth and Laipis, 1982), which is important for the maintenance of mtDNA homoplasmy. If blastomeres inherit mutated genomes or are depleted of mitochondrial genomes, then the resulting adult tissues would be deficient in OX-PHOS capacity, which could result in the onset of mitochondrial disease (Wallace, 1992).

There are many diseases caused by mutations in mtDNA and symptoms of these diseases generally appear in tissues highly dependent on OX-PHOS (Wallace, 1992). These include Leber's hereditary optic neuropathy (LHON), which can result from mutations in the gene for the NADH-Q oxidoreductase component of complexes I and III (Brown *et al*., 1992). Myoclonic epilepsy with ragged-red fibres (MERRF) is also transmitted by maternal inheritance and results in myoclonus, epilepsy and ataxia. This can result from mutations in mitochondrially encoded tRNA genes (Hanna *et al*., 1995; Nakamura *et al*., 1995; Nishigaki *et al*., 2003; Mancuso *et al*., 2004) and protein genes (Naini *et al*., 2005). As well as point mutations, some mitochondrial diseases are caused by large-scale deletions to the genome. The most well known in this group is Kearns–Sayre Syndrome (KSS), which includes symptoms of PEO, pigment retinopathy and disorders of cardiac conduction (Klopstock *et al*., 1995; Lertrit *et al*., 1999; De Block *et al*., 2004). The clinical phenotypes resulting from mtDNA mutations are dependent on the proportion of mutated mtDNAs (Wallace, 1992). In the case of LHON, >60% mutant mtDNA load is required before the disease phenotype presents (Chinnery *et al*., 2001). In some mitochondrial diseases, including a case of MERRF, over 85% mutant mtDNAs need to be present before symptoms are observed (Boulet *et al*., 1992).

Paternal mtDNA inheritance

There is increasing evidence demonstrating that maternal-only mtDNA transmission is not merely because of dilution of the paternal mitochondria beyond the level of detection. It has been suggested that paternal mtDNA is actively eliminated by ubiquitination in the oocyte cytoplasm and subsequent proteolysis during embryonic development (Sutovsky *et al*., 1999; Nishimura *et al*., 2006). The elimination hypothesis is supported by reports demonstrating reduced paternal mtDNA content as embryonic development progresses (Cummins *et al*., 1997). Paternal mtDNA was detected only as far as the pronucleus stage following intraspecific mouse crosses compared with detection in neonates

following inter-specific mouse crosses (Kaneda *et al*., 1995). This suggests that the elimination mechanism is species specific. However, the elimination mechanism is not only species specific but also tissue specific, as donor sperm mtDNA was eliminated, whereas exogenous liver mtDNA injected into the oocyte persisted at least until the neonatal period (Shitara *et al*., 2000).

The destruction of paternal mtDNA (reviewed in Sutovsky, 2003) and the presence of the genetic bottleneck should usually result in homoplasmic individuals. Recently, the accepted theory of uniparental mtDNA inheritance has been questioned. This is especially so in the case of a male patient presenting with a mitochondrial myopathy who was found to have inherited his muscle mtDNA from his father. However, the 2bp deletion giving rise to his myopathy was *de novo* (Schwartz and Vissing, 2002). This outcome is further supported by the evidence of sperm mtDNA persistence in human polyploid IVF-generated blastocysts (St. John *et al*., 2000). Paternal leakage of mtDNA has also been reported following inter-specific crosses in fruitflies (Kondo *et al*., 1990), mice (Gyllensten *et al*., 1991), honeybees (Meusel and Moritz, 1993), birds (Kvist *et al*., 2003), sheep (Zhao *et al*., 2004) and non-human primates (St. John and Schatten, 2004) and following intra-specific crosses in mussels (Zouros *et al*., 1992) and fruitflies (Kondo *et al*., 1992). The ubiquitin-related paternal mtDNA destruction process has therefore proven ineffective in these cases. It is as yet unknown how paternal mtDNA might escape the destruction process and how the paternal mtDNA might be preferentially replicated in a particular tissue to such an extent that it resulted in a mitochondrial disease (Schwartz and Vissing, 2002). In this latter instance, a considerable selective advantage must have taken place to out-compete the maternal source of mtDNA in the affected tissue.

A possible mechanism for bypassing the paternal mtDNA destruction process is the fusion of parental mitochondria followed by recombination of the two genomes. Recombination of mammalian mtDNA was originally thought not to arise, although it is known to occur in yeast. However, more recent evidence suggests that it may also occur in mammals. For example, Ono and colleagues created two respiration-deficient cell lines from patients with mitochondrial diseases caused by different mutations (Ono *et al*., 2001). Fusion of the two-cell lines resulted in morphology and respiratory enzyme activity returning to normal within 10–14 days. These authors proposed that the outcome resulted from mtDNA recombination and genome compensation. Mammalian mtDNA recombination has since been confirmed in human cells using PCR-based techniques (D'Aurelio *et al*., 2004) and recent sequence analysis of invertebrate and vertebrate species, including primates (Tsaousis *et al*., 2005). These authors suggested that recombination may have been previously missed because of the purification process during oocyte production prohibiting the generation of new haplotypes and their transmission to the next generation. Recombination of sperm and oocyte mtDNA is now thought to explain the presence of the 2bp deletion in the male myopathy patient (Kraytsberg *et al*., 2004).

Invasive oocyte reconstruction protocols

The introduction of more advanced assisted reproductive technologies (ART) and their current use in the clinical setting and in animal production further confounds the unimaternal transmission of mtDNA. These technologies include (i) ICSI which is used clinically; (ii) cytoplasmic transfer (CT) which has been used clinically but is now, for example, banned in the United Kingdom and United States and (iii) germinal vesicle transfer (GVT), pronuclear transfer (PNT) and NT, which have not yet been used to treat humans. These more invasive ART protocols present more opportunities for the violation of the normal pattern of mtDNA inheritance. If uniparental mtDNA inheritance is not maintained following these techniques, offspring may harbour various forms of mtDNA heteroplasmy. Indeed, the introduction of these new technologies results in heteroplasmy being redefined. In this instance, either a wild-type molecule can co-exist with a rearranged molecule or two wild-type molecules may persist in the reconstructed oocyte.

ICSI

Unlike IVF, the ICSI procedure varies dramatically from the natural fertilization process. For example, the intact, non-capacitated, non-acrosome-reacted sperm is injected into the oocyte. There are also delays and alterations in post-fertilization processes following ICSI, including male pronucleus formation (Luetjens *et al*., 1999) and the calcium oscillations essential for the activation process (reviewed in Ludwig *et al*., 2001). This delayed response after sperm entry means that ICSI also has the potential to delay degradation of sperm mtDNA. Furthermore, couples who have male factor infertility and require ICSI are at a higher risk of using sperm containing abnormal mtDNA as some human mtDNA haplogroups are associated with high or reduced sperm motility (Ruiz-Pesini *et al*., 2000). This is demonstrated in those men harbouring large numbers of mtDNA deletions who are more likely to exhibit poorer semen quality (Lestienne *et al*., 1997; Reynier *et al*., 1997; St. John *et al*., 2001) as are those exhibiting point mutations (Spiropoulos *et al*., 2002). Despite the many diversions from the natural fertilization processes following ICSI, to date, it appears that sperm mtDNA is eliminated in offspring generated using this technique (Danan *et al*., 1999; Marchington *et al*., 2002). Furthermore, it appears to be eliminated before the blastocyst stage in ICSI-derived polyploid human embryos (St. John *et al*., 2000).

CT

CT is an extension of ICSI and involves the injection of supplementary cytoplasm, as well as a sperm, into the oocyte (Figure 3). The rationale for using CT is that donor cytoplasm will contain supplementary, often younger, non-mutated mtDNA and will possibly contain other important cytoplasmic components to compensate for the defective cytoplasm in the oocyte (Cohen *et al*., 1997). In the human, it has resulted in higher pregnancy rates and live births (Cohen *et al*., 1997; Barritt *et al*., 2001). CT can also result in heteroplasmy as demonstrated by the analysis of various tissues of human CT offspring (Brenner *et al*., 2000). Consequently, it remains to be demonstrated whether the donor mtDNA out-competes the recipient mtDNA in the heteroplasmic offspring. We also need to ascertain whether the coupling of two distinct genomes accounted for the two cases of Turner's syndrome, one electively aborted and the other spontaneously aborted, and the one case of pervasive development disorder (Barritt *et al*., 2001).

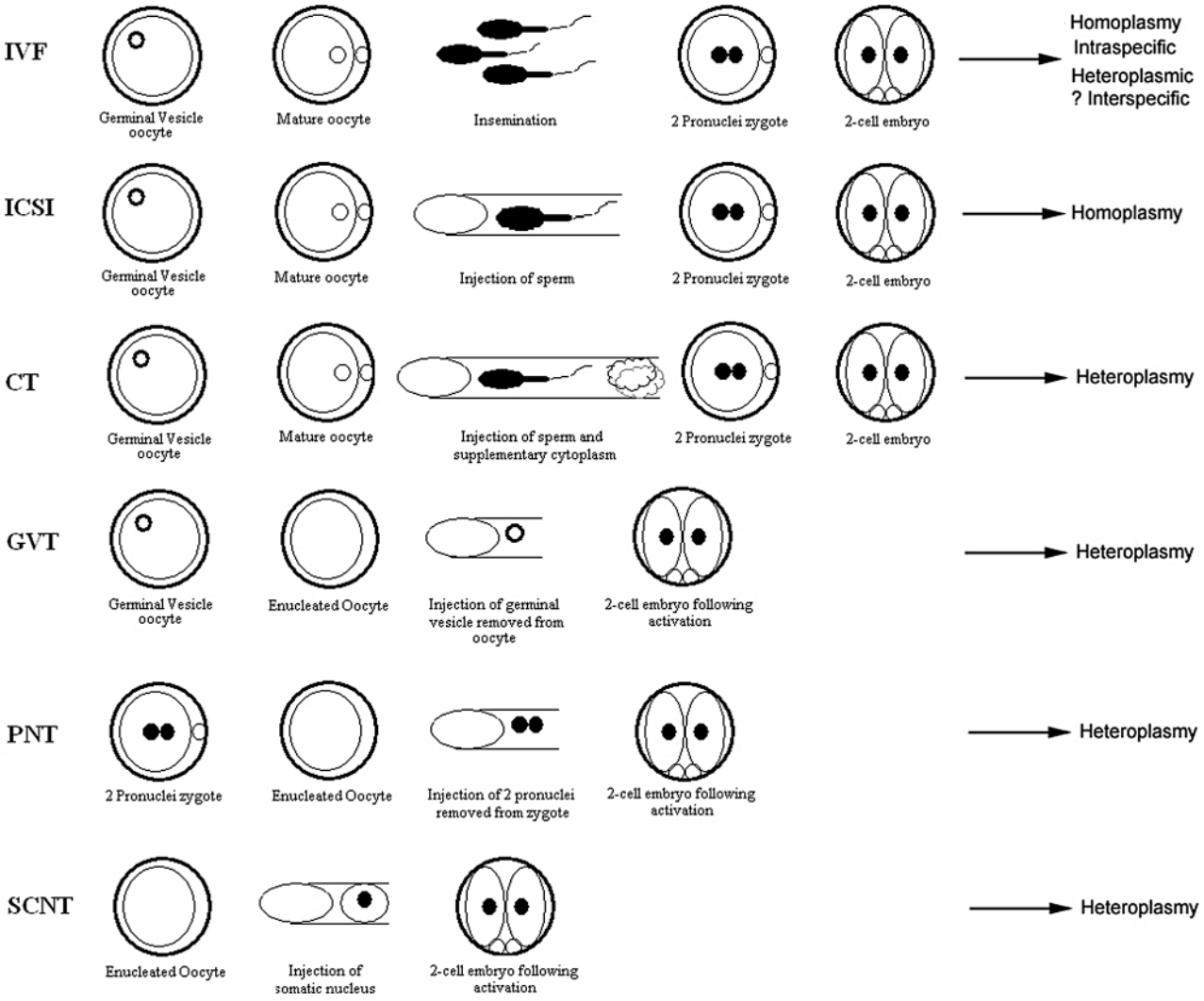


Figure 3. Assisted reproductive techniques. Predicted outcomes associated with mitochondrial DNA (mtDNA) transmission following IVF, ICSI, cytoplasmic transfer (CT), germinal vesicle transfer (GVT), pronuclei transfer (PNT) and somatic cell nuclear transfer (SCNT).

To avoid the possible harmful effects of heteroplasmy in the offspring, cytoplasm from granulosa cells of the mother rather than cytoplasm from donor oocytes has been used (Kong *et al*., 2003; 2004). These are likely to have the same mtDNA sequence as the recipient oocyte. However, one possible problem with this procedure is that mtDNA deletions in granulosa cells are more common in older women (Seifer *et al*., 2002), i.e. those that are likely to have defective oocytes and require CT. Therefore, the treatment may be unsuccessful because of continued lack of wild-type mtDNA and may even result in the transmission of mitochondrial disease to the offspring that would not have occurred without CT. Another likely problem is that granulosa cells are fully differentiated, whereas newly fertilized oocytes are pluripotent. Cells at different stages of differentiation are known to be different in terms of their mitochondrial activity and content. For example, undifferentiated human (h) ESCs (Sathananthan *et al*., 2002; St. John *et al*., 2005a) and primordial germ cells (Jansen and De Boer, 1998) have been observed to contain low numbers of mitochondria. However, differentiated cells contain higher numbers of more active mitochondria, depending on OX-PHOS requirements of specific cell types (Moyes *et al*., 1998). Mitochondria donated from granulosa cells would therefore need to be 'reprogrammed' to match those of the oocyte. We have supplemented developmentally incompetent pig oocytes with pure populations of mitochondria from developmentally competent oocytes from maternal relatives. Interestingly, we observed improved fertilization outcome and the maintenance of unimaternal inheritance (El Shourbagy *et al*., 2006).

GVT and PNT

GVT and PNT have also been proposed as techniques to overcome problems associated with aged oocytes, whereby the GV or PN is transferred into an enucleated recipient oocyte. Ideally, the enucleated recipient should be from the same stage of development as the donor nucleus (Liu *et al*., 1999). In aged oocytes, there is likely to be a larger number and higher proportion of mutant mtDNA (Keefe *et al*., 1995). It has been hypothesized that mitochondria are important not only for the production of ATP but also for correct chromosomal segregation during meiosis (Schon *et al*.,

2000). Furthermore, it has been suggested that aberrant mitochondrial function in aged oocytes may have a role in oocyte aneuploidy (Eichenlaub-Ritter *et al*., 2004), a common cause of infertility in older women (Munne *et al*., 1995; Dailey *et al*., 1996). The chances of success may therefore increase if the nuclear component of the aged oocyte was transferred into the enucleated ooplasm of a younger, healthier oocyte, rather than supplement the existing ooplasm. This transfer can be carried out at either the GV or the PN stage (Figure 3). Indeed, studies using human oocytes have shown GVT from aged oocytes to the enucleated ooplasm of young oocytes can overcome oocyte aneuploidy, with the majority of reconstructions having normal karyotypes (Zhang *et al*., 1999; Takeuchi *et al*., 2001). There may also be non-cytoplasmic components of the GV that influence chromosome segregation. This has been indicated in a series of mouse experiments mixing GVs and ooplasms of varying ages (Cui *et al*., 2005). A significantly greater number (57.1 %) of chromosomal abnormalities were found in reconstructions when the GV was transferred from older females to the enucleated ooplasm of younger females. This is in contrast to abnormalities found in reconstructions derived from the transfer of younger GVs into aged ooplasms (16.7 %).

Mitochondria are known to be involved in chromosome organization and movement. For example, the induction of mitochondrial damage in mouse oocytes prevented oocyte maturation, chromosomal segregation and spindle formation (Takeuchi *et al*., 2005). This damage was overcome by GVT of the karyoplast derived from a damaged oocyte into the ooplasm of a healthy enucleated oocyte. Cytogenetic analysis showed that 20/21 of these reconstructions had a normal number of chromosomes. Furthermore, performing ICSI on GVT reconstructions and transfer of the resulting two-cell embryos led to the generation of live mouse offspring. It has also been demonstrated that an intact mouse ooplasm could rescue mitochondrial-damaged karyoplasts induced by photoirradiation with 62% of these reconstructions maturing to metaphase II (Palermo *et al*., 2002). PNT is essentially the same procedure, except for the nuclear material being removed after fertilization. This technique has also been used in mice with considerable success as determined by the birth of live offspring (Meirelles and Smith, 1997; 1998; He *et al*., 2003; Sato *et al*., 2005). This procedure, however, involves the destruction of a zygote, which may restrict its usage because of ethical and moral considerations.

GVT and PNT have been proposed as techniques for treating mtDNA disease (Cummins 1998; Trounson, 2001). However, potential problems could arise as the transferred GV or PN is still surrounded by mitochondria, which will also be carried over into the donor ooplasm. These mitochondria remain close to the centre of the immature reconstruction at first but then disperse throughout the cytoplasm as maturation ensues (Fulka, 2004). Owing to segregation during embryogenesis, this provides an opportunity for karyoplast mitochondria to be present in all cell types of the offspring. Any disease-associated mitochondria transferred with the nuclear material may therefore continue to be transmitted.

It is further possible that, due to its initial proximity to the nucleus, karyoplast mtDNA would be preferentially replicated in the reconstructions. Indeed, fusion of enucleated somatic cells with mtDNA-depleted somatic cells can lead to the preferential expansion of one particular type of mtDNA such as the mutant molecule (Dunbar *et al*., 1995). Although, to our knowledge, there has been no analysis of mtDNA transmission following GVT, results are likely to be similar to those reported following PNT. In this instance, exogenous mtDNA accompanying the murine zygotic karyoplast is found in higher concentrations at blastocyst than mtDNA from transferred cytoplasts (Meirelles and Smith, 1998). This has been further highlighted where zygotic karyoplast transfer into zygotes resulted in varying amounts of mtDNA being transmitted to offspring (0–69%; Meirelles and Smith, 1997). Consequently, it would be difficult to predict to what extent a mutated or deleted mtDNA molecule would be selected. However, a recent report suggests that the varying levels of mtDNA deletion in mouse zygotes could be reduced to levels lower than those expected for the phenotypic onset of mtDNA disease when the zygotic nucleus is transferred into an enucleated healthy oocyte (Sato *et al*., 2005). The subsequent offspring also remained under the level for pathological onset even with the accumulative increase of the deletion with time. These authors state however that, in the human, this form of nuclear transplantation could not be applied to those mtDNA diseases where the pathogenic mutation had significant replicative advantage over wild-type mtDNA. This is most likely as in humans the prenatal period is 13 times longer than in the mouse, and this represents the window in which mutant mtDNA replication is most proliferative (Sato *et al*., 2005).

NT

NT involves the injection of a donor nucleus or a whole cell into an enucleated recipient oocyte (Figure 3). Mostly commonly, either embryonic cell (EC) NT, using a blastomere, or somatic cell NT (SCNT) are performed. Resulting embryos have then been used to generate blastocysts and offspring in a variety of species (Campbell *et al*., 2005). However, NT is renowned for its low efficiency, with low proportions of embryos developing to the blastocyst stage (White *et al*., 1999) and even fewer progressing far enough to implant, survive pregnancy and produce live offspring (Wilmut *et al*., 1997). One of the major abnormalities associated with NT offspring is the production of abnormal placentae which are thought to be the cause of many fetal deaths (Cibelli *et al*., 2002). Incorrect reprogramming of imprinted genes has also been cited as one of the major factors which leads to early fetal death. This was highlighted by non-surviving NT calves having incorrect expression of imprinted genes, namely Igf2, Igf2r and H19 in some tissues, whereas surviving adult clones had normal expression at these loci except for Igf2 in muscle (Yang *et al*., 2005). Furthermore, in mouse NT embryos and offspring irregular patterns of gene expression have been observed most likely as a result of inappropriate reprogramming (Humpherys *et al*., 2002).

Although the process of NT for human reproductive purposes is ethically unacceptable and is banned in many countries (UK – Human Fertilisation and Embryology Act, 1990; USA – H.R. 534 Office of Legislative Policy and Analysis, 2003; South Korea – see Normile and Mann, 2005), its use as a means of producing histocompatible stem cells for treatment of various diseases is likely to have a significant impact. Cell replacement therapy has great potential for the treatment of certain conditions such as myocardial infarction (Rubart and Field, 2006) and Parkinson's disease

(Taylor and Minger, 2005) and as a replacement for tissue/organ transplantation (Cortesini, 2005). To date, encouraging results have been obtained in animal studies, whereby hESC-derived neural precursors (Wernig *et al*., 2004) and cardiomyocytes (Laflamme *et al*., 2005) were successfully transplanted into animal organs. However, considerable characterization and validation of precursor cells derived from hESCs still needs to be undertaken before cell replacement can be used as an effective therapy in humans. Some success at generating NT-derived ESCs has so far been achieved in mouse models (Munsie *et al*., 2000; Wakayama *et al*., 2001), and its use as a means of delivering the patient's own chromosomal DNA with the corrected gene for a single gene disorder has been demonstrated in the mouse (Rideout *et al*., 2002).

One of the rate-limiting factors in generating autologous ESCs, i.e. stem cells possessing the patient's own chromosomal and mtDNA compositions is the regulation of the mtDNA composition. In this respect, a considerable amount can be learnt from those offspring derived from NT through either ECNT or SCNT. Here, transmission results in either homoplasmy derived from the recipient oocyte mtDNA or heteroplasmy resulting from transfer of both donor and recipient mtDNA (reviewed in St. John *et al*., 2004a,b). This is probably because of the random pattern of mtDNA segregation during embryogenesis (reviewed in Shoubridge, 2000). Consequently, it is apparent that no real mechanism is currently understood that could account for the irregular patterns of mtDNA transmission observed. One parallel that can be drawn from both ECNT and SCNT is that the range of donor mtDNA transmitted is relatively similar at 0–57% (Hiendleder *et al*., 1999) and 0–59% (Takeda *et al*., 2003), respectively. However, two explanations could exist for those studies not detecting donor mtDNA in live offspring: (i) no donor mtDNA is actually present or (ii) too few tissues have been analysed. Consequently, the true extent of heteroplasmy is difficult to determine. This is perhaps exemplified by the recent studies of pig (Takeda *et al*., 2006), cow (Takeda *et al*., 2003) and mouse (Inoue *et al*., 2004) NT-derived offspring, where varying levels of heteroplasmy has been observed between the tissues. This would indicate that if all tissues are not analysed, then these studies are not truly subjective. Furthermore, investigations should include analysis of the surrogate mother's mtDNA as leakage through the placenta into fetal blood has been reported in cattle (Hiendleder *et al*., 2004).

The initial number of donor mtDNA molecules introduced into the recipient oocyte also does not necessarily influence whether donor mtDNA is actually transmitted. Using blastomeres from more advanced embryonic stages in bovine NT has resulted in reduced levels of heteroplasmy being reported (Steinborn *et al*., 1998). However, this was not a uniform pattern as two offspring derived from 52-cell morula blastomeres contained less donor mtDNA than offspring derived from 92-cell morula blastomeres. A further issue is the stage of embryonic development of the donor cell. A recent analysis of two NT-derived monkey offspring (St. John and Schatten, 2004) demonstrated mtDNA transmission from both the recipient oocyte and the donor cell. In this instance, the early IVF blastomere (8- to 12-cell stage) still contained sperm mtDNA. Consequently, the offspring possessed three different populations of mtDNA. Furthermore, the depletion of donor cell mtDNA to residual levels (<0.02% of its original content) still resulted in the persistence of some mtDNA at the blastocyst stage in sheep NT (Lloyd *et al*., 2006).

The use of a somatic donor cell with accompanying mitochondria not only raises interesting questions with regard to the differentiated status of nucleus but also of the mitochondria. During development, from the inner cell mass to a fully differentiated cell, mitochondria increase in number to meet the metabolic requirements of that specialized cell. The proliferation of mitochondria further discriminates between the number of mtDNA copies per mitochondrion and cell type, with higher ATP users possessing greater copy numbers (Moyes *et al*., 1998). Therefore, for SCNT, the mitochondria accompanying the donor cell should also be reprogrammed to the embryonic state, as is the case for the donor nucleus. Failure to do this may result in embryos containing mitochondria with insufficient plasticity to suit a multitude of cell types that they will contribute to. The persistence of donor mitochondria could also influence the metabolic pathways used during early development and explain why improved developmental rates are observed in those embryos cultured in media that enhance anaerobic respiration (Chung *et al*., 2002). It is further evident that the injection of somatic donor mitochondria into oocytes which are then parthenogenetically activated is detrimental to embryonic development though this is not observed with donor ooplasm (Takeda *et al*., 2005).

Interaction between multiple wild-type mitochondrial genomes

It has not been clearly demonstrated whether heteroplasmy resulting from two wild-type mtDNA molecules can be detrimental to the offspring and result in mitochondrial disease. This is partly because many post-mortem results have not been published, and the cause of many deaths following NT is not documented. Consequently, we are unsure whether those offspring that survive are the exception to the rule. However, from one report, some common causes of death in sheep NT offspring are similar to symptoms of mitochondrial diseases, including brain, kidney and liver defects (McCreath *et al*., 2000) and in a series of other species (Cibelli *et al*., 2002). Sequence differences due to mtDNA from a 'foreign' source can give rise to proteins with altered amino acid sequences. This has been demonstrated in both cattle (Steinborn *et al*., 2002) and pigs (St. John *et al*., 2005b) and may result in inadequate interaction between the individual subunits of the ETC. This would result in reduced energy production capacity and symptoms common to mitochondrial disease and influence embryo and fetal survival. The question is whether handmade cloning, the fusion of one or more oocyte cytoplasts with a somatic cell (Vajta *et al*., 2001), would resolve such issues or complicate them.

The significance of nucleo–mitochondrial interaction

Intergenomic communication is vital for efficient cellular function as much interaction occurs between nuclear-encoded proteins and those encoded by the mtDNA genome (reviewed by Scarpulla, 2002). The ETC requires nuclear-encoded proteins to be transported to the mitochondria and to assemble appropriately (Stojanovski *et al*., 2003). Failure of TFAM, TFB1M and TFB2M to co-ordinate transcription would also have serious implications for a functional ETC (Falkenberg *et al*., 2002). Furthermore, failure of TFAM and PolG to co-ordinate replication would have similar outcomes for replication (Larsson *et al*., 1998; Hance *et al*.,

2005). However, NT appears to result in the continued expression of both these factors during embryo development and could explain why even residual levels of donor mtDNA persist up to the blastocyst stage (Lloyd *et al*., 2006). Failure of TFAM or PolG to interact with the mtDNA genome, post-implantation, through the diversity of the donor nucleus and the recipient oocyte's mtDNA would result in compromised transcription or replication and mimic mtDNA-depletion syndromes (Poulton *et al*., 1994).

Patient-specific ESCs derived by NT would require large numbers of good-quality human oocytes which, due to medical, ethical, legal and moral issues, are a limited resource. Added to this, the methods of recruitment of donor oocytes for the derivation of such ESCs have caused considerable outrage recently (Holden, 2005). Cross-species NT offers the opportunity to derive ESCs that would carry the chromosomal DNA from a patient propagated in a surrogate ooplasm of another species. Encouragingly, there is a single report of the generation of a hybrid ESC line following the transfer of a human nucleus into a rabbit oocyte (Chen *et al*., 2003). However, outcomes from cross-species NT further exemplify the importance of nucleo-mitochondrial compatibility for ESC derivation. Use of both human cord fibroblasts with bovine oocytes (Chang *et al*., 2003) and chicken blastodermal cells with rabbit oocytes (Liu *et al*., 2004) resulted in elimination of the donor mitochondrial genome by the morula or blastocyst stage of development. Rhesus macaque donor cells injected into rabbit oocytes resulted in heteroplasmic embryos but with only the rabbit mtDNA being replicated after the blastocyst stage (Yang *et al*., 2003), suggesting that these too could have soon become homoplasmic for the recipient oocyte mtDNA. This suggests either preferential replication of the oocyte mtDNA or degradation of the mtDNA from the nuclear donor cell.

Analysis of cross-species NT offspring provides further indications as to potential outcomes for cross-species ESCs. In two combinations of cross subspecies cow NT experiments, the donor nucleus was capable of interaction with both types of mtDNA with the production of apparently healthy heteroplasmic calves (Steinborn *et al*., 2002; Han *et al*., 2004). However, the combination of *Bos indicus* donor cells with *Bos taurus* oocytes resulted in homoplasmic embryos and one offspring (Meirelles *et al*., 2001). There has also been one report of preferential replication of mtDNA from the nuclear donor cell following cross-species NT (Chen *et al*., 2002). Here, nuclei from giant panda somatic cells were injected into rabbit oocytes, and preferential replication of the panda mtDNA was observed after analysis of the fetuses. This suggests that some combinations of cross-species NT, particularly where the species are closely related or a subspecies, may result in a functional ETC and efficient mitochondrial respiration. However, more distantly related species might preferentially replicate their own mtDNA genome because of incompatibility with that in the recipient oocyte.

Much can also be learnt from somatic cell–cell fusion studies and xenomitochondrial offspring in relation to outcomes associated with cross-species NT. For example, one study involved combinations of cells depleted of their mtDNA and exogenous mitochondria from rats and two subspecies of mouse (McKenzie and Trounce, 2000). Transfer of *Mus spretus* mtDNA into *Mus musculus* cells resulted in heteroplasmy, and no obvious OX-PHOS defects, probably due to the two types of mice, being closely related. However, when rat mtDNA was transferred into *Mus musculus* cells, its replication and transcription were unaffected, but OX-PHOS capacity was reduced. Other studies have also demonstrated increased respiratory chain defects following crosses of greater evolutionary distance both in mice (McKenzie *et al*., 2003) and in primates (Kenyon and Moraes, 1997). Effects of species differences, and also mitochondrial disease, can now be studied *in vivo* following the production of xenomitochondrial mice (McKenzie *et al*., 2004; Sokolova *et al*., 2004). Xenomitochondrial mice can be produced by (i) direct injection of foreign mitochondria into zygotes (Irwin *et al*., 1999), (ii) pronuclei fusion with enucleated oocytes (Meirelles and Smith, 1997) followed by the transfer to surrogate mothers and (iii) the production of ESC cybrids for injection into blastocysts (Levy *et al*., 1999). Again, it has been demonstrated that greater evolutionary distance between species results in offspring with increased respiratory chain defects (Trounce *et al*., 2004). It is most likely that OX-PHOS defects arose from abnormal combinations of nuclear and mitochondrially encoded ETC proteins resulting in abnormal structure of ETC complexes, a scenario very similar to outcomes that arise through cross-species NT.

There are many cases where interspecies NT embryos implant and survive pregnancy but die shortly after birth (Hammer *et al*., 2001; Han *et al*., 2004). These may be a result of inefficient ETC activity due to species differences. However, should these combinations result in apparently healthy live offspring, it is possible that phenotypic alterations may still be present. Such outcomes could affect those mtDNA haplotypes that influence, for example, milk quality in cattle (Brown *et al*., 1989), growth and physical performance in mice (Nagao *et al*., 1998), a reduction of fertility in beef cattle (Sutarno *et al*., 2002) and sperm motility in men (Ruiz-Pesini *et al*., 2000). Perhaps, the biggest note of caution is exemplified by the result of introducing a common carp nucleus into an enucleated goldfish oocyte, which resulted in the offspring developing phenotypic features of the goldfish (Sun *et al*., 2005). Consequently, the use of cross-species NT for the derivation of hESCs needs considerable investigation although it offers great potential.

Are these technologies safe?

Although some experiments have been carried out in animals and a small number of groups practise invasive ART protocols in humans, little is known about the likely outcomes of these techniques in relation to the long-term effects on offspring and future generations. It is essential that we understand the risks of these techniques before their therapeutic use becomes widespread. The use of preimplantation genetic diagnosis (PGD) has recently been proposed for early diagnosis of mitochondrial disease (Dean *et al*., 2003). The use of PGD for chromosomal genetic analysis has been implemented in many clinics. However, its effectiveness needs to be demonstrated for the detection of potential mtDNA disease transmission. Some work has indicated its usefulness in this respect. In the mouse, mtDNA genotypes in polar bodies were compared with their respective oocytes and in different blastomeres within the same early embryo (Dean *et al*., 2003). The level of heteroplasmy present in polar bodies and blastomeres was representative of that in the associated oocytes and embryos, with the authors therefore concluding that PGD was feasible for the diagnosis of mitochondrial disease. However, cloned mouse

offspring (Inoue *et al*., 2004), various farm animal species such as pigs (Takeda *et al*., 2006) and cattle (Hiendleder *et al*., 2003) and patients with mitochondrial mutations (Kirches *et al*., 2001) and diseases (Ponzetto *et al*., 1990) often demonstrate varying levels of heteroplasmy in different tissues. Furthermore, considerable variation in oocyte-derived mtDNA copy number among intra-embryo blastomeres has been demonstrated in both the pig (El Shourbagy *et al*., 2006) and the human (Lin *et al*., 2004). Therefore, even if the proportion of heteroplasmic mtDNA molecules were the same in each blastomere, the actual number of mutant molecules is likely to be different. Blastomeres containing lower numbers of mtDNA molecules will have reduced ATP-generating capacities compared with their counterparts with higher overall numbers of mtDNA. Therefore, the effects in tissues resulting from these blastomeres will be more severe than those resulting from blastomeres with a larger mtDNA complement. Furthermore, different tissues require different ATP-generating capacity. Low levels of wild-type mtDNA in some blastomeres may therefore go unnoticed if that particular blastomere develops into a low ATP-requiring tissue. However, this could result in severe mitochondrial disease should that blastomere develop into a tissue highly dependent on OX-PHOS. It is therefore unlikely that PGD would be a suitable technique to determine the risk of mitochondrial disease following oocyte reconstruction, especially following GVT, PNT and NT as all have reported preferential replication of donor mtDNA. Consequently, an alternative is required.

Conclusion

Mitochondria are vitally important during embryonic development and tissue differentiation, as demonstrated by the changes in their localization, activity and morphology in the early embryo. The propagation of mitochondria before metaphase II and the maternal transmission of mtDNA ensure that sufficient numbers of the organelle and its genome are invested into the process of early embryogenesis. Consequently, these pre-metaphase II events are under the control of the oocyte nuclear genome, which regulates transcription and replication, and ensure that transmission is not affected during a period when there is no nuclear control, i.e. before activation of the embryonic genome.

In the process of trying to treat various diseases through the generation of patient-specific stem cells and the use of GVT and PNT to prevent the transmission of mtDNA-type disease, we would be taking inappropriate risks if current strategies were introduced into the clinic. In many ways, our strategies are confounding and our ingenuity could turn into our downfall. We are attempting to provide the nucleus with a new environment in which it can prosper but, for GVT and PNT, we have forgotten that this karyoplast is still accompanied by the mutant mtDNA that we are trying to eradicate. For NT-derived patient-specific stem cells, GVT and PNT, we are asking the transferred nucleus to interact with a cytoplasm which may or may not be compatible. We also expect two or more populations of mtDNA to happily cohabit. We are however ignoring how the cytoplasm will adjust to its new nuclear companion in favour of concentrating on the nucleus and its requirement to achieve pluripotency. Cohabitation requires considerable foresight, and in this respect we need to understand considerably more about nucleo-mitochondrial harmony before we exploit such technologies. The question still remaining to be answered is: Can heteroplasmy be tolerated at a functional level even if it compromises genetic identity? Paradoxically, inheritance and therefore analysis of recipient oocyte mtDNA does, however, allow us to confirm whether oocyte reconstruction has taken place. This is a lesson we have hopefully learnt from the discredited Korean attempts at generating patient-specific ESCs.

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