REPRODUCTION

REVIEW

# The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective

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## Abstract

**The introduction of nuclear transfer (NT) and other technologies that involve embryo reconstruction require us to reinvestigate patterns of mitochondrial DNA (mtDNA) transmission, transcription and replication. MtDNA is a 16.6 kb genome located within each mitochondrion. The number of mitochondria and mtDNA copies per organelle is specific to each cell type. MtDNA is normally transmitted through the oocyte to the offspring. However, reconstructed oocytes often transmit both recipient oocyte mtDNA and mtDNA associated with the donor nucleus. We argue that the transmission of two populations of mtDNA may have implications for offspring survival as only one allele might be actively transcribed. This could result in the offspring phenotypically exhibiting mtDNA depletion-type syndromes. A similar occurrence could arise when nucleo–cytoplasmic interactions fail to regulate mtDNA transcription and replication, especially as the initiation of mtDNA replication post-implantation is a key developmental event. Furthermore, failure of the donor somatic nucleus to be reprogrammed could result in the early initiation of replication and the loss of cellular mtDNA specificity. We suggest investigations should be conducted to enhance our understanding of nucleo–cytoplasmic interactions in order to improve NT efficiency.**

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## Introduction

Nuclear transfer (NT) involves the fusion of either an embryonic or somatic donor cell with an enucleated recipient oocyte (Campbell *et al.* 1996). The resultant reconstructed oocyte can then be activated electrically or biochemically and allowed to develop in culture. Viable embryos, from various stages of development, are then transferred into surrogates. However, this technology is fraught with low success (Wilmut *et al.* 1997). In the case of Dolly, the first cloned sheep derived from Somatic Cell NT (SCNT), the generation of 283 embryos resulted in one viable offspring. Although offspring from various species are now produced with a greater intensity, it is still evident that these offspring are liable to serious defects (Cibelli *et al.* 2002). These include phenotypes such as circulatory distress, placental oedema, umbilical hernia, hydrallantois, respiratory problems, immune dysfunction and kidney/brain/liver malformation. Furthermore, large offspring syndrome is associated with bovine and ovine offspring and involves pathological changes such as extended gestation length and increased birthweight (Lazzari *et al.* 2002). However, very few of the pathologies reported have taken mitochondrial dysfunction into consideration but closer analysis suggests that mitochondrial disorder might indeed persist. Examples include myopathies and liver disorders (see Cibelli *et al.* 2002). The aim of this review is to analyse the potential impact that aberrant mitochondrial DNA (mtDNA) transmission can have on NT outcome and how modifications to its transmission could result in potentially greater success. It also emphasises the importance of nucleo–cytoplasmic interaction and nuclear reprogramming to mtDNA differentiation.

## The role of mitochondria

Mitochondria are the key generators of cellular ATP. ATP can be produced through the Kreb's cycle, β-oxidation and oxidative phosphorylation (OXPHOS). In the vast majority of cases, the primary pathway for ATP production is OXPHOS via the electron transfer chain (ETC; see Fig. 1),


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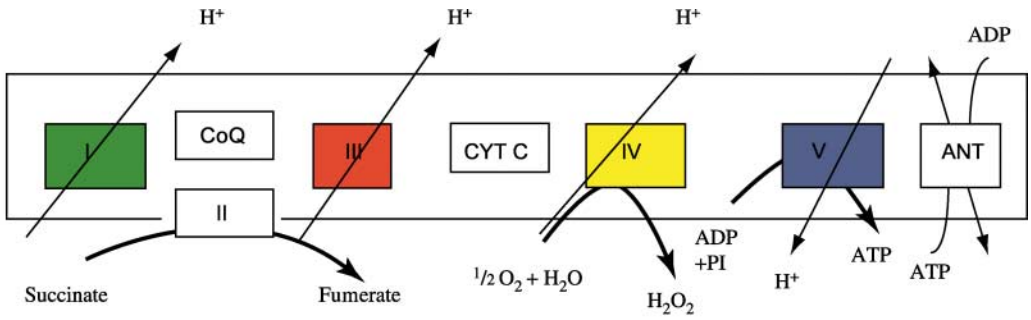


| Complex | I | II | III | IV | V |
|---|---|---|---|---|---|
| nDNA subunits | >18 | 4 | 8 | 10 | 10 |
| mtDNA subunits | 7 | 0 | 1 | 3 | 2 |

**Figure 1** The electron transfer chain (ETC). The ETC is a major component of OXPHOS, the main ATP generating pathway in the cell. Almost all protein subunits of the ETC (complex I, NADH dehydrogenase; complex III, ubiquinol:cytochrome c; complex IV, cytochrome c oxidase (COX); complex V, ATP synthase) contain mtDNA encoded subunits. There is one exception, complex II (succinate:ubiquinone oxidoreductase) which is entirely nuclear encoded.

which unlike any other cellular pathway is encoded for by two distinct genomes, the nuclear (nDNA) and mitochondrial (mtDNA) genomes. Mammalian mtDNA encodes 13 of the polypeptides that constitute part of the ETC (Anderson *et al.* 1981; see Fig. 2). It further consists of 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) thus making it a semi-autonomous molecular entity that requires interaction with the nucleus to instigate transcription and replication (see Clayton 1998).

## MtDNA homoplasmy and heteroplasmy - the clinical phenomenon

The state of homoplasmy exists when all copies of mtDNA within a cell or tissue are identical either as wild type (WT) or mutant or deleted/rearranged. A mixing of two variants or the existence of WT and rearranged molecules results in heteroplasmy. The phenotypic onset of mtDNA-type disease is often dependent on the ratio of mutant:WT. Patient studies have indicated that in Leber's hereditary optic neuropathy (LHON) for example, >60% mutant mtDNA load is required before the characteristic phenotype is observed (Chinnery *et al.* 2001). Other studies have indicated that a critical threshold level exists where over 85–90% of mutant mtDNAs must be present for a biochemical or clinical disease phenotype to be expressed (Boulet *et al.* 1992). Amongst the maternally inherited point mutations, there are: mutations in individual rRNA/tRNA complexes associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS; Goto *et al.* 1990); myoclonic epilepsy and ragged-red fibre disease (MERRF, Shoffner *et al.* 1990) and deafness (Torroni *et al.* 1999); mutations in the coding region, e.g. neurogenic weakness, ataxia, retinitis pigmentosa (NARP; Fryer *et al.* 1994); mitochondrial encephalomyopathy (Clark *et al.* 1999), and LHON (Wallace *et al.* 1988, Howell *et al.* 1991); and a combination of mitochondrial disease symptoms resulting from a point mutation at nucleotide 8993 (Holt *et al.* 1990). Mutations can also be transmitted in Mendelian fashion, e.g. chronic progressive external opthalmoplegia (CPEO; Cormier *et al.* 1991). This group arises from mutations in nuclear encoded components vital to mtDNA transcription and replication. This includes adenine nucleotide translocator (ANT) 1, an ADP/ATP translocator (Kaukonen *et al.* 2000), thymidine kinase 2 (Saada *et al.* 2001) and the mitochondrial deoxyguanosine kinase (Mandel *et al.* 2001) - both enzymes that are involved in a scavenger pathway that provides dNTPs for mtDNA replication, and polymerase gamma (PolG), the DNA polymerase specific to mtDNA (Van Goethem *et al.* 2001). In addition, mitochondrial transcription factor A (TFAM) is a vital transcription factor that binds to the D-loop, or control region, of the mitochondrial genome and regulates transcription and replication. Decreased TFAM expression has been associated with mtDNA depletion syndrome (TFAM; Poulton *et al.* 1994). Finally, large-scale deletions are harboured as sporadic single deletions or as an insertion-duplication of mtDNA, which can act as an intermediate, as for example in Kearns-Sayre syndrome (KSS; Schon *et al.* 1989), and is often characterised by the 4977 bp 'common' deletion. Multiple large-scale deletions are also observed in post-mitotic tissues such as muscle (Holt *et al.* 1988), and can be induced through ageing mechanisms such as the generation of free radicals (Hayakawa *et al.* 1995).

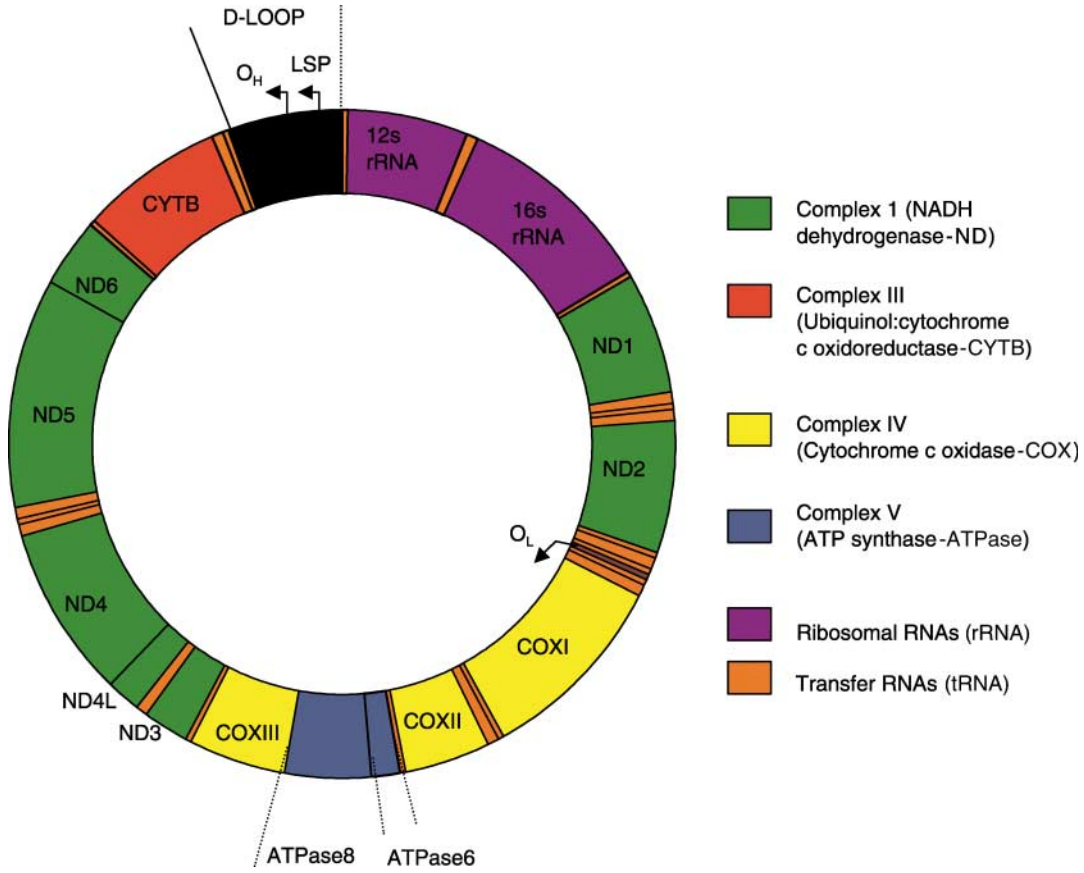


**Figure 2** Mammalian mtDNA. Mammalian mtDNA is a double stranded molecule, composed of a H (heavy) strand and a L (light) strand and is approximately 16.5 kb in size (e.g. human mtDNA is 16.569 kb; Anderson *et al.* 1981). The origin for H-strand replication ($O_H$) and the HSP and LSP transcription promoters are located in the D-loop (displacement loop). The origin for L-strand replication ($O_L$) is located two thirds away around the genome from the D-loop. MtDNA encodes thirteen proteins of the electron transport chain (ETC). Twelve genes are located on the H-strand and only one gene is located on the L-strand. mtDNA also has 22 tRNAs and 2 rRNAs involved in mtDNA transcript production and processing.

## Heteroplasmy derived from the coexistence of two distinct alleles

The coexistence of two or more mtDNA alleles, defined as sequence variants, can result from sperm transmission from interspecific crossings or from supplementation arising through cytoplasmic transfer or nuclear transfer. These techniques contravene the strict mechanism that regulates mtDNA transmission post-fertilisation. mtDNA is primarily transmitted through the oocyte and consequently is maternally inherited (Birky 1995, 2001). This is perhaps best demonstrated in intraspecific crossings of mice that appear to eliminate sperm mitochondria by the late pronuclear stage (Kaneda *et al.* 1995, Shitara *et al.* 2001), and before the 8-cell stage in cattle and the rhesus macaque (Sutovsky *et al.* 1999). This process is thought to be mediated through the labelling of mitochondria in the spermatogonia by ubiquitin resulting in their subsequent targeting by oocyte-driven ubiquitination (Sutovsky *et al.* 1999). Interspecific crosses behave differently by ignoring this ubiquitin-mediated event and allowing sperm mtDNA to be transmitted, albeit at low levels, to the resultant offspring (Gyllensten *et al.* 1991, Shitara *et al.* 1998). However, this molecule is not transmitted to subsequent generations (Shitara *et al.* 1998). Interestingly, biparental transmission has also been observed in *Drosophila* and mussels (Fisher & Skibinski 1990, Hoeh *et al.* 1991), with male mussel offspring harbouring both maternal- and paternal-type genomes and female offspring having maternal-only genomes. Until recently, it was understood that humans transmitted mtDNA in a strictly maternal mode (Giles *et al.* 1980, Shitara *et al.* 1998, Korpelainen 1999). However, paternal mtDNA can persist in a few polyploidy embryos generated through IVF (St John *et al.* 2000), and this persistence in blastocysts can account for the mtDNA myopathy observed in a male patient (Schwartz & Vissing 2002). Other evidence exists to suggest that sperm mtDNA can persist, but again in an infrequent manner, following spermatid injection (Cummins *et al.* 1998).

Cytoplasmic transfer (CT) is a relatively new assisted reproduction technique. It involves the injection of donor cytoplasm as well as a sperm in order to supplement the defective cytoplasm of those oocytes from patients with repeated embryonic development failure (Cohen *et al.* 1997). This may arise as the mitochondria present in the mature oocyte at fertilisation provide the energy needed by the embryo for the entire preimplantation phase of development, as there is no replenishment of mitochondria until the post-blastocyst stage (Piko & Taylor 1987, Larsson *et al.* 1998). It has been shown that there is a critical number of mitochondria, approximately 100 000, that must be present in the unfertilised oocyte to allow embryo development after fertilisation in the human (Van Blerkom *et al.* 1998, Reynier *et al.* 2001, Hsieh *et al.* 2002). This number may be essential because a threshold level of ATP is required for cell division (Brenner *et al.* 2000), or a certain number of mitochondria may be required to synchronise the cellular dynamics, essential for early embryonic development. CT has been reasonably successful, showing higher than expected pregnancy rates in women unable to conceive via other methods (Cohen *et al.* 1997, 1998), particularly older women with poor quality oocytes (Barritt *et al.* 2001*a*). However, this technology results in some of the offspring possessing two cytoplasmic parents (St John & Barratt 1997, Brenner *et al.* 2000, St John 2002) as well as two chromosomal parents (sperm and oocyte chromosomal DNA). It can further result in spontaneous or selective abortion arising from Turner's syndrome (46,X0) and the onset of pervasive development disorder (Barritt *et al.* 2001*b*). Mouse studies have substantiated that foreign mtDNA injected into either the oocyte or the zygote can be transmitted in varying amounts, for example 5–80% (Laipis 1996), 0–30% (Jenuth *et al.* 1996) and 16–100% (Meirelles & Smith 1997), suggesting that those molecules introduced can be transmitted at random frequency.

A state of heteroplasmy can also result following NT where transmission of mtDNA as well as nuclear DNA from the donor cell may occur (Gaertig *et al.* 1988), as there are several thousand mtDNA genomes in most somatic cells (Michaels *et al.* 1982). Patterns of mtDNA inheritance are inconsistent in offspring generated through NT as the process bypasses the normal uniparental mechanisms of mtDNA inheritance. Consequently, NT offspring will exhibit one of three patterns of mtDNA transmission namely, homoplasmy derived solely from the recipient oocytes, homoplasmy derived exclusively from the donor somatic cell, or heteroplasmy resulting from the fusion partners.

Both homoplasmy (Evans *et al.* 1999) and varying degrees of heteroplasmy (Steinborn *et al.* 1998*a*,*b*; Heindleder *et al.* 1999, Takeda *et al.* 1999, Meirelles *et al.* 2001, Steinborn *et al.* 2002) have been observed. Homoplasmy may have resulted from a failure by donor mitochondria to enter the ooplasm following electrofusion (Evans *et al.* 1999). Alternatively, donor mitochondria could be actively destroyed by a mechanism similar to the ubiquitination of sperm mitochondria, as demonstrated in intraspecific crossings following natural conception or IVF (Ankel-Simons & Cummins 1996, Sutovsky *et al.* 1999). The existence of heteroplasmy shows that in contrast to the exclusion of sperm mtDNA following sexual inheritance, cloning can result in the mixing and the co-existence of parental mtDNAs. Donor mtDNA has been found to make up between 0 and 59% of the total cell mtDNA in those tissues analysed (see Table 1). The use of embryonic cells at different stages of development may result in less donor mtDNA being introduced into the newly reconstructed oocytes (Steinborn *et al.* 1998*b*). However, there is considerable variability between disassociated blastomeres which could account for variable levels of transmission (Van Blerkom *et al.* 2000). Most interesting, however, is the recent study of mtDNA in NT calves which clearly demonstrates a replicative advantage of donor mtDNA over the recipient oocytes mtDNA (Takeda *et al.* 2003), although it is important to note the differences in mtDNA composition between tissues of the same cloned individual (see Table 1). This is in contrast to another report where no advantage was observed for donor mtDNA (Hiendleder *et al.* 2003). These variations in reported mtDNA composition could be related to differences in the NT procedure or differences in nucleo–cytoplasmic interactions. The proportion of donor mtDNAs present may be related to the quantity of donor cell cytoplasm present post reconstruction (Takeda *et al.* 2003). This is perhaps best exemplified by those protocols that actively destroy the donor cell cytoplasm prior to its injection into the recipient oocyte's cytoplasm (Wakayama & Yanagimachi 2001).

## How is mtDNA transmission regulated following non-invasive assisted reproduction?

Primordial follicles contain as few as 10 mitochondria (Jansen & de Boer 1998) and the mtDNA molecules within them are clonally amplified (Marchington *et al.* 1997) from oogenesis up until complete maturation at metaphase II (Smith & Alcivar 1993). This clonal expansion results in more than 100 000 copies being present in those oocytes deemed capable of fertilisation and maintaining embryonic development (Reynier *et al.* 2001). The restriction to a few mtDNA copies present at the primordial follicle stage results in a genetic bottleneck (Hauswirth & Laipis 1982, Poulton 1995). This genetic bottleneck event is thought to be proceeded by random genetic drift (Jenuth *et al.* 1996) and/or non-directed segregation promoting the homoplasmic transmission of mtDNA, whenever feasible. Either of these processes would explain the vast difference in mutant load observed in a series of oocytes from one ovarian source - 0 to 95% - due to unequal levels of mtDNA present in individual segregating blastomeres (Blok *et al.* 1997).

**Table 1** Representative examples of levels of heteroplasmy detected in mammals derived from nuclear transfer. Both homoplasmy and varying degrees of heteroplasmy have been detected in mammals produced by NT. Clear differences also exist in donor mtDNA levels between tissues and at different stages in development.

| **Offspring analysed** | **Donor cell** | **Degrees of heteroplamsy observed** (% donor mtDNA) | **Reference** |
|---|---|---|---|
| 10 live sheep clones | Somatic | Homoplasmic for the recipient in all tissues analysed (blood, skeletal muscle, placenta and skin) for all sheep | Evans *et al.* (1999) |
| 4 live cattle clones | Embryonic | 13 and 18% in 2 clones derived from 24-cell morulae<br>0.6 and 0.4% in 2 clones derived from 92-cell morulae | Steinborn *et al.* (1998*b*) |
| 29 individuals of 7 cattle clones | Embryonic | 21–57% in 2 of the clones 2–4% in others | Hiendleder *et al.* (1999) |
| 11 live cattle clones (subspecies crosses, *B. indicus* and *B. taurus*) | Somatic | 0–28% (blood, muscle, skin, oocytes, follicular cells)<br>Co-existence of *B. taurus* and *B. indicus* | Steinborn *et al.* (2002) |
| 80 12-day bovine fetuses | Somatic | Blood 0–5%<br>Homoplasmic for the recipient in all other tissues analysed (skin, muscle, brain, lung, heart, rumen, jejenum, liver, spleen, kidney, cotyledon) | Hiendleder *et al.* (2003) |
| 16 embryos immediately after electrofusion | Somatic | 3 of 16 embryos showed low levels of donor mtDNA (3–4%) immediately after fusion | Takeda *et al.* (2003) |
| 11 NT calves and foetuses | Somatic | 3 of 11 NT calves/fetuses exhibited heteroplasmy. (C1: 25–51%, C2: 0–15%, C3: 8–59%). Differences between tissues were observed. | Takeda *et al.* (2003) |

C1, C2 and C3 are individual clones 1, 2 and 3.

## Consequences resulting from the transmission of two genomes – competitiveness of one genome over another

Both CT and NT bypass this early genetic restriction event resulting in heteroplasmic distribution across many tissue types and to future generations (Hiendleder *et al.* 2003). However, of considerable relevance is that replication of mtDNA does not occur until the hatched blastocyst stage of development (Piko & Taylor 1987). Before this stage, each mitochondrion and its copy of mtDNA will be transferred to just one blastomere. This segregation continues as the blastomeres divide. Mouse studies have shown that foreign mtDNA injected into the karyoplast tends to be spread equally throughout the daughter blastomeres, whereas the mitochondria injected into the cytoplast segregate preferentially to one daughter cell or another (Meirelles & Smith 1998). This is probably due to the mtDNA injected into the karyoplast being close to the nuclear genetic material, which is always divided equally among daughter blastomeres. It has been suggested that mtDNA within specific regions of a cell is preferentially replicated (Davis & Clayton 1996). However, a recent study suggests that perceived preferential replication only arises due to the higher numbers of mitochondria surrounding the nucleus, and is not due to favourable locations for mtDNA replication. To this extent, it has been demonstrated that mtDNA replication takes place throughout the cell cytoplasm in various human somatic cells (Magnusson *et al.* 2003). The position of mitochondria within an oocyte certainly plays an important role in determining where they will be located within the developing embryo and offspring (Meirelles & Smith 1998). This may therefore be due to either preferential replication or simply segregation during cell division. In any case, this could be of considerable importance in deciding where to inject supplementary mitochondria during cytoplasmic transfer.

It has been suggested that some progeny produced by NT show preferential replication of nuclear donor mtDNA (Do *et al.* 2002), perhaps due to the presence of compatible nuclear factors, for example TFAM. Those studies testing the proportion of different mtDNA genotypes after NT and CT have produced varying results both within and between individuals. These range from complete homoplasmy to equal proportions of two or more genotypes. The range of heteroplasmic states reported so far may be due to a number of factors such as nucleo–cytoplasmic incompatibility. The ideal outcome for CT would be the supplementation of oocytes with mtDNA from the same genotype (St John 2002).

## Use of cybrid technology as a means of evaluating nucleo–cytoplasmic interactions

Cybrids, or cytoplasmic hybrids, have been used to study nucleo–cytoplasmic interactions in humans (King & Attardi 1988, 1989). More recently, the fate of mtDNA populations against different nuclear backgrounds have been analysed (Dunbar *et al.* 1995, Barrientos *et al.* 1998, 2000, Moraes *et al.* 1999, Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003). Typically, a homoplasmic cybrid is formed by the fusion of an enucleated cell (cytoplast) with an mtDNA-depleted cell possessing a somatic nucleus (karyoplast). Heteroplasmic cybrids can be generated through either the fusion of a heteroplasmic cytoplast, containing for example a mixture of mutant mtDNA and WT mtDNA, with a mtDNA-depleted cell (Dunbar *et al.* 1995, Inoue *et al.* 2000), or the fusion of a

**Table 2** Nucleo–cytoplasmic variation: differences in OXPHOS, mtDNA replication and transcription in homoplasmic and heteroplasmic cybrids.

| **Cybrid** (nuclear background–cytoplast) | **Homoplasmic/ heteroplasmic** | **OXPHOS** | **Respiratory complex activity** | **Oxygen consumption** | **Mitochondrial protein synthesis** | **mtDNA replication and transcription** | **Lactate** (indicates the level of ATP production) |
|---|---|---|---|---|---|---|---|
| Murine–murine (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Homoplasmic for murine mtDNA | Unaffected (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Unaffected (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Unaffected (Dey *et al.* 2000) | Unaffected (Dey *et al.* 2000, McKenzie & Trounce 2000) | The replication of murine mtDNA was unaffected (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Unaffected (McKenzie *et al.* 2003) |
| Murine–rat (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Homoplasmic for rat mtDNA | Compromised (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003) | Complex I and IV activity was 50% lower in murine–rat cybrids compared with parental cell lines and murine–murine cybrids (Dey *et al.* 2000). Complex I, III and IV activities were reduced to 46%, 37% and 78% that of the murine–murine cybrids (McKenzie & Trounce 2000) | 50% lower in murine–rat cybrids compared with parental cell lines and murine–murine cybrids (Dey *et al.* 2000) | Unaffected (Dey *et al.* 2000, McKenzie & Trounce 2000) | Rat mtDNA was replicated efficiently (McKenzie & Trounce 2000) | Increased cellular production of lactate (decreased ATP production) (McKenzie *et al.* 2003) |
| Murine–rat (Dey *et al.* 2000) | Heteroplasmic harbouring murine and rat mtDNA | Restored to levels similar to those of murine–murine cybrids (Dey *et al.* 2000) | | | | A shift in murine mtDNA levels was observed following 12 weeks of culture from 1.5 to 10% that was sufficient to restore respiration (Dey *et al.* 2000) | |
| Murine–murine harbouring mutant mtDNA (Inoue *et al.* 2000) | Homoplasmic | Compromised in cybrids with a predominance of mutant mtDNA | COX activity reduced | | Mitochondrial translation was reduced | Prolonged culture of one cybrid resulted in a shift from 30% to 83% mutant mtDNA, but then remained stable | |
| Human–non-human ape (common chimp, pigmy chimp and gorilla) cybrids (Barrientos *et al.* 1998, Moraes *et al.* 1999) | Homoplasmic for non-human mtDNA | The activities of complexes II, III, IV and V were unaltered (Barrientos *et al.* 1998) | Complex I deficiency (Barrientos *et al.* 1998) | | The level of ND1, 75 kDa and 49 kDa protein subunits of complex I were unaltered (Barrientos *et al.* 1998) | Human–non-human ape cybrids survived in culture under selection for respiratory function implying mtDNA from non-human apes can replicate and restore respiration within a human nuclear background (Moraes *et al.*. 1999) | |

**Table 2.** *Continued*

| Cybrid (nuclear background–cytoplast) | Homoplasmic/ heteroplasmic | OXPHOS | Respiratory complex activity | Oxygen consumption | Mitochondrial protein synthesis | mtDNA replication and transcription | Lactate (indicates the level of ATP production) |
|---|---|---|---|---|---|---|---|
| Human–human harbouring a mutation (Dunbar *et al.* 1995) | Heteroplasmic | Compromised | COX activity was reduced to 16% and 26% that of controls in cybrids containing 85% (lung carcinoma nuclear background) and 95% (osteosarcoma nuclear background) mutant mtDNA, respectively | | | 1/33 heteroplasmic cybrids with an osteosarcoma nuclear background had an increase in mutational load from 28-85% following prolonged culture. On the other hand, 5/25 heteroplasmic cybrids with a lung carcinoma nuclear background had an increase in WT mtDNA during culture | Increased cellular production of lactate (decreased ATP production) |

cytoplast with a cell containing mtDNA, producing a cybrid with donor and recipient mtDNA (Moraes *et al.* 1999, Dey *et al.* 2000). Cybrid technology has also been used to analyse the transmission of mtDNA to offspring by introducing mtDNA into one-cell embryos (Inoue *et al.* 2000) and also into blastocysts (Sligh *et al.* 2000). Table 2 clearly highlights the variation in mtDNA replication and transcription that can arise through nucleo–cytoplasmic variation. Of specific interest are the results arising from interspecies cybrid generation where rat mtDNA is efficiently replicated, transcribed, and translated against a murine nuclear background, although OXPHOS function is compromised, which compares unfavourably with murine–murine cybrids (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003). This is further exemplified as primate mtDNA is only replicated when the human cell's own mtDNA has been eliminated (Moraes *et al.* 1999). The anticipated problems include the variable mtDNA sequences between two populations that could result in amino acid incompatability and a dysfunctional ETC with decreased levels of ATP. Such an outcome would be similar to those human phenotypes associated with mtDNA depletion syndromes (Larsson *et al.* 1994, Poulton *et al.* 1994) arising from decreased levels of TFAM expression reducing mtDNA copy number. Indeed, mtDNA mutations, which are characteristic of mtDNA depletion, result in phenotypic changes in animals including growth and milk quality in Holstein cows (Schutz *et al.* 1994, Nagao *et al.* 1998). These outcomes certainly necessitate the importance of establishing nucleo–cytoplasmic compatibility before attempting NT and CT. This is especially so following the recent report of human embryonic stem cells (hESCs) being derived by autologous SCNT (Hwang *et al.* 2004). Consequently, human–human cybrids would provide an excellent model for studying the diversity of a distinctive somatic nucleus against that of an embryonic genome. This would also provide a valuable insight into the compatibility of the nucleus from one cell type with that of the cytoplasmic background from another individual. Such investigations would clearly determine nucleo–cytoplasmic efficiencies related to ESC derivation.

## Could upstream epigenetic errors impact on mitochondria numbers in cells and respective copies of mtDNA per mitochondrion?

Somatic cell NT utilises a differentiated cell as its source of chromosomal DNA, which requires reprogramming prior to the first cell division. The genotype of a series of cells from an individual organism is identical, but cell-specific gene expression results in an individual cell's identity related to function and morphology (Shi *et al.* 2003). Consequently, these donor nuclei should be reprogrammed to adopt the characteristics of a newly fertilised zygote that has either commenced or completed

recombination of the male and female pronuclei. The naturally or *in vitro* fertilised oocyte undergoes a series of epigenetic events (Reik & Dean 2003). These include methylation and deacetylation, regulators of imprinting, which are key to developmental events. However, evidence from studies related to IVF and intra cytoplasmic sperm injection (ICSI) has shown that correct imprinting patterns are essential to normal development. Consequently, Beckwith Wiedemann syndrome, an imprinting disorder, has been reported in more severe instances following assisted reproduction (Maher *et al.* 2003). More importantly, imprinting disorders can be phenotypically lethal (see Lee *et al.* 2002). In addition, other key markers of early differentiation, such as certain pluripotent genes, are expressed during normal embryo development although they are not necessarily present in somatic cloned embryos nor beforehand in their contributing somatic nucleus (Boiani *et al.* 2002, Bortvin *et al.* 2003). The most-studied is Oct-4 and associated family members, including Dppa1, Pramel4, Pramel5 and Pramel6 (Bortvin *et al.* 2003).

We hypothesise that failure of somatic donor nuclei to express key markers of an unprogrammed nucleus indicates that aberrant gene expression or silencing compromises early embryonic development that will deviate markedly from the expected pattern. This will perhaps best be identified in the failure of the highly regulated nucleo–cytoplasmic interaction. Consequently, the nucleus which regulates mtDNA transcription and replication through its expression of specific transcription factors, for example TFAM, may result in subsequent blastomeres adopting the phenotype of the nuclear donor cell type, resulting in the inner mass cells and those contributing to the foetus phenotypically expressing mitochondrial morphology and mtDNA copy number of that particular cell type. This would influence mitochondrial morphology, mtDNA copy number, and the number of mitochondria per cell and thus OXPHOS capacity for organs or tissues. This is particularly critical as the number of mitochondria and the mtDNA copy number are unique to each cell type and related to OXPHOS requirement, as predicted for individual post-mitotic cell types (see Moyes *et al.* 1998).

Multiple copies of the mtDNA genome are found in individual mitochondria in somatic cells although only a single copy is found in those of the oocyte (Jansen & de Boer 1998). The degree of multiple copies is hypothesised to be regulated by the ATP requirement of individual cells (Moyes *et al.* 1998). For example, in rat tissue the mean number of mtDNA copies per mitochondrion for the ventricle is 0.7, for liver 2.7, for red muscle 0.9 and for white muscle 1.9. Furthermore, the mtDNA copy numbers per cell type have been demonstrated to be significantly different between cell types. For example, in skeletal and cardiac muscle there are $3650 \pm 620$ and $6790 \pm 920$ mtDNA copies per diploid nuclear genome respectively, representing a significant difference of $P = 0.006$ (Miller *et al.* 2003). The mtDNA copy number in peripheral blood mononuclear cells is $409 \pm 148$ copies per cell and in subcutaneous fat it is $2049 \pm 391$ (Gahan *et al.* 2001), whilst cultured fibroblasts possess $823 \pm 71$ copies/cell (Zhang *et al.* 1994). Interestingly, there are $2.6 \times 10^5$ copies per bovine oocyte whilst bovine fetal heart fibroblasts possess $2.6 \times 10^3$ copies/cell (Michaels *et al.* 1982). Considerable variation has also been demonstrated in rat tissues, with ventricle tissue possessing $279 \times 10^9$ mtDNA copies per gram tissue, liver $743 \times 10^9$, red muscle $230 \times 10^9$, and white muscle $116 \times 10^9$ (Wiesner *et al.* 1992). Failure of the nucleus to be fully reprogrammed can result in failure of appropriate mtDNA segregation during embryogenesis and can affect ATP production and cellular development. A mechanism for regulating such outcomes during early embryo development would be to analyse the differences between mtDNA transcription and replication of CT- and NT-generated embryos and the distribution of the heteroplasmic genomes to individual blastomeres. Consequently, the outcomes of mtDNA supplementation are being tested under the regulation of the embryonic and somatic nuclear genomes throughout early embryogenesis.

To further support our hypothesis, analysis of the initiation of mtDNA transcription and replication post fertilisation provides key evidence. mtDNA transcription and replication is mediated by nuclear-encoded transcription factors that are imported into the mitochondria (Clayton 1998). Transcription proceeds from the 2-cell stage in the developing murine embryo although mtDNA copy number is constant until the blastocyst stage (Piko & Taylor 1987). MtDNA copy number is probably maintained by nuclear respiratory factor (NRF)-1, where homozygous null mice survive up to embryonic day (E) 6.5 (Hou & Scarpulla 2001). However, there is continual dilution of the mtDNA genome to each newly generated blastomere until replication is initiated post-implantation. One of the major regulators of this event is TFAM. In those homozygous murine TFAM knockout embryos, severe mtDNA depletion is observed with embryo survival persisting as far as E10.5. This is in contrast with heterozygous offspring that phenotypically present with cardiomyapthy, a severe debilitating mtDNA disorder (Larsson *et al.* 1998).

## Conclusion

It is evident that nucleo–cytoplasmic interaction is vital to the successful generation of live offspring. Whilst epigenetic factors related to imprinting can result in earlier onset syndromes, it is imperative that full investigations are conducted on the genetic compatibility of the nucleus and the mitochondrion to fully initiate an appropriate level of differentiation in order that the cytoplasm can effectively generate appropriate levels of ATP. The consequence of reduced ATP levels would promote mtDNA-depletion syndromes as evidenced in certain clinical conditions categorised in the human. Furthermore, a cytoplasm over-populated with mitochondria would lead

to cellular expansion that might be indicative of the reported large-offspring syndromes. This under-researched area of investigation could provide clear answers to some of the developmental abnormalities witnessed in NT offspring and aborted foetuses, whether mediated through failure of somatic cell reprogramming or independently.

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