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

Justin C St John, Rhiannon E I Lloyd, Emma J Bowles, Emma C Thomas and Shahinaz El Shourbagy

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

Correspondence should be addressed to Justin C St John, Room EF20, The East Wing, The Medical School, The University of Birmingham, Birmingham B15 2TJ, UK; Email: j.stjohn@bham.ac.uk

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.

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),
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 homoplasy and heteroplasy - the clinical phenomenon

The state of homoplasy 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/rrNA 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 (Torrioni 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 (Kauponen 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 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.
Heteroplasmia 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. 2001a). 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. 2001b). 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 (Michaela 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, homoplasy derived solely from the recipient oocytes, homoplasy derived exclusively from the donor somatic cell, or heteroplasm resulting from the fusion partners.

Both homoplasy (Evans et al. 1999) and varying degrees of heteroplasmy (Steinborn et al. 1998a,b; Heindleder et al. 1999, Takeda et al. 1999, Meirelles et al. 2001, Steinborn et al. 2002) have been observed. Homoplasy 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. 1998b). However, there is considerable variability between disassociated blastomeres which could account for variable levels of transmission (Van Blerkem 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 nuclear–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 (BlokJ 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.

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

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 blastocomes divide. Mouse studies have shown that foreign mtDNA injected into the karyoplast tends to be spread equally throughout the daughter blastocomes, 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 blastocomes. 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 nuclear–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.

<table>
<thead>
<tr>
<th>Cybrid (nuclear background–cytoplast)</th>
<th>Homoplasmic/heteroplasmic mtDNA</th>
<th>Respiratory complex activity</th>
<th>Oxygen consumption</th>
<th>Mitochondrial protein synthesis</th>
<th>mtDNA replication and transcription</th>
<th>Lactate (indicates the level of ATP production)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine–rat (Dey et al. 2000)</td>
<td>Heteroplasmic for murine and rat mtDNA</td>
<td>Restored to levels similar to those of murine–murine cybrids (Dey et al. 2000)</td>
<td>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)</td>
<td>Mitochondrial translation was reduced</td>
<td>Prolonged culture of one cybrid resulted in a shift from 30% to 83% mutant mtDNA, but then remained stable</td>
<td>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)</td>
</tr>
<tr>
<td>Murine–murine harbouring mutant mtDNA (Inoue et al. 2000)</td>
<td>Homoplasmic</td>
<td>Compromised in cybrids with a predominance of mutant mtDNA</td>
<td>COX activity reduced</td>
<td>Mitochondrial translation was reduced</td>
<td>Prolonged culture of one cybrid resulted in a shift from 30% to 83% mutant mtDNA, but then remained stable</td>
<td>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)</td>
</tr>
<tr>
<td>Human–non-human ape (common chimp, pigmy chimp and gorilla) cybrids (Barrientos et al. 1998, Moraes et al. 1999)</td>
<td>Homoplasmic for non-human mtDNA</td>
<td>The activities of complexes II, III, IV and V were unaltered (Barrientos et al. 1998)</td>
<td>Complex I deficiency (Barrientos et al. 1998)</td>
<td>The level of ND1, 75 kDa and 49 kDa protein subunits of complex I were unaltered (Barrientos et al. 1998)</td>
<td>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)</td>
<td>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)</td>
</tr>
</tbody>
</table>
cytoplasm 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 incompatibility 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, Prame4, Prame5 and Prame6 (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 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 ± 620 and 6790 ± 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 ± 148 copies per cell and in subcutaneous fat it is 2049 ± 391 (Gahan et al. 2001), whilst cultured fibroblasts possess 823 ± 71 copies/cell (Zhang et al. 1994). Interestingly, there are $2.6 \times 10^3$ copies per bovine oocyte whilst bovine fetal heart fibroblasts possess $2.6 \times 10^4$ 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 cardiomyopathy, 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 syndrome. 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.

References


Barrientos A, Muller S, Dey R, Wienberg J & Moraes CT 2000 Cytochrome c oxidase assembly in primates is sensitive to small evolutionary variation in amino acid sequence. Molecular Biology and Evolution 17 1508–1519.


King MP & Attardi G 1988 Infection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell 52 811–819.


Korpelainen H 1999 Genetic maternal effects on human life span through the inheritance of mitochondrial DNA. Human Heredity 49 183–185.


Mckenzie M, Chiotis M, Pinkert CA & Trounce I 2003 Functional respiratory chain analyses in murid xenochondamonal hybrids expose coevolutionary constraints of cytochrome b and nuclear subunits of complex III. Molecular Biology and Evolution 20 1117–1124.


Schon EA, Rizzuto R, Moraes CT, Nakase H, Zeviani M & DiMauro S 1989 A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. Science 244 346–349.


Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW & Wallace DC 1990 Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA (Lys) mutation. Cell 61 931–937.


