

Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells

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Submitted on September 30, 2009; resubmitted on December 21, 2009; accepted on January 19, 2010

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BACKGROUND: Mitochondrial DNA (mtDNA) encodes key proteins associated with the process of oxidative phosphorylation. Defects to mtDNA cause severe disease phenotypes that can affect offspring survival. The aim of this review is to identify how mtDNA is replicated as it transits from the fertilized oocyte into the preimplantation embryo, the fetus and offspring. Approaches for deriving offspring and embryonic stem cells (ESCs) are analysed to determine their potential application for the prevention and treatment of mtDNA disease.

METHODS: The scientific literature was investigated to determine how mtDNA is transmitted, replicated and segregated during pluripotency, differentiation and development. It was also probed to understand how the mtDNA nucleoid is regulated in somatic cells.

RESULTS: mtDNA replication is strictly down-regulated from the fertilized oocyte through the preimplantation embryo. At the blastocyst stage, the onset of mtDNA replication is specific to the trophoctodermal cells. The inner cell mass cells restrict mtDNA replication until they receive the key signals to commit to specific cell types. However, it is necessary to determine whether somatic cells reprogrammed through somatic cell nuclear transfer, induced pluripotency or fusion to an ESC are able to regulate mtDNA replication so that they can be used for patient-specific cell therapies and to model disease.

CONCLUSIONS: Prevention of the transmission of mtDNA disease from one generation to the next is still restricted by our lack of understanding as to how to ensure that a donor karyoplast transferred to an enucleated oocyte is free of accompanying mutant mtDNA. Techniques still need to be developed if stem cells are to be used to treat mtDNA disease in those patients already suffering from the phenotype.

Key words: embryology / genetic disorders / mitochondria / nuclear transfer / stem cells

Introduction

The mitochondrion exists within the cytoplasm of nearly all eukaryotic cells. Along with mediating processes such as steroidogenesis, apoptosis, homeostasis and cell division, mitochondria are also essential generators of ATP through the process of oxidative phosphorylation (OXPHOS) which takes place within the electron transfer chain (ETC). OXPHOS, unlike any other cellular process, is highly dependent on the expression of proteins encoded by the mitochondrial genome [mitochondrial DNA (mtDNA)] and chromosomally encoded genes. Although the transmission of mtDNA is dependent on the mitochondrion as its vehicle, both the mitochondrion and mtDNA should be considered as independent entities that co-exist, just as the mitochondrion co-exists within the cytoplasm of a cell but its origins are bacterial (Cavalier-Smith, 2009). The role played by the mitochondrial genome in reproductive biology has been largely ignored. However, since the advent of more sophisticated reproductive technologies, such as nuclear transfer, its importance is becoming increasingly recognized, especially as mixed and diverse populations of mtDNA co-existing together could severely affect cellular function and thus offspring survival and quality of life.

Search methods

A computerized search was carried out using MedLine and Web of Knowledge for key areas associated with the mitochondrial genome, mtDNA replication, the factors controlling mtDNA replication, mtDNA transmission and mtDNA segregation. The search initially focused on somatic cell biology and then the relevance of those areas related to mtDNA disease. The search then concentrated on how those events were regulated following natural fertilization which included the generation of offspring and stem cells through intra- and interspecific crosses. We then investigated the transmission of donor cell mtDNA following nuclear transfer and also sperm mtDNA in diverse crosses. After including interspecies nuclear transfer, we then probed the literature to understand how various other technologies could affect the normal transmission of mtDNA in order that we could make predictions about transmission from those technologies that have yet to be

investigated. We restricted our search to mtDNA, but did discuss mitochondrial and ETC function when, specifically, the effects of heteroplasmy might affect cellular function and offspring survival. The literature was also interrogated for mtDNA-type disease and how this might be prevented through oocyte manipulation and stem cell therapy.

The mitochondrion

Mitochondria exist in various topographical forms, which reflect the type and functions of the cells they serve and thrive off. In maturing oocytes and early cleavage stage embryos, they tend to be oval and spherical while in the differentiating and mature cells, they adopt a more elongated configuration (Sathananthan *et al.*, 2002). Furthermore, the density of mitochondria per cell is often indicative of the cell's requirements for ATP production (Moyes *et al.*, 1998). Consequently, cells such as neurons and muscles tend to have higher densities, though these are considerably fewer than the metaphase II stage oocyte, which possesses between 150 000 and 300 000 mitochondrial profiles (Jansen and De Boer, 1998).

What is mtDNA?

In humans, the circular, double-stranded mtDNA genome is ~16.6 kb in size (Fig. 1) and resides within the inner membrane of the mitochondrion. It consists of a heavy (H) and a light (L) strand, which encode 13 of the polypeptides contributing to Complexes I, III, IV and V of the ETC (Fig. 2). The remaining genes and those of Complex II are derived from chromosomal DNA (Anderson *et al.*, 1981). Specifically, mtDNA codes seven subunits of NADH dehydrogenase (Complex I), one subunit of cytochrome *c* reductase (Complex III), three subunits of cytochrome *c* oxidase (Complex IV) and two subunits of the ATP synthase (Complex V; Fig. 1). mtDNA also encodes some of its own transcriptional and translational machinery, i.e. 22 tRNAs and 2 rRNAs, emphasizing the semi-autonomous nature of this genome as these processes are also dependent on chromosomally encoded genes. Furthermore, the chromosomally encoded transcription and replication factors interact with the only non-coding region of the

genome, the displacement loop (D-loop). Otherwise, the mtDNA genome contains no introns interspersed between the coding regions with some of the coding regions even overlapping each other, namely ATPase 6, ATPase 8, ND4 and ND4L (Anderson et al., 1981). Furthermore, some of the other genes do not possess

termination codons, as these are generated through post-transcriptional polyadenylation (Ojala et al., 1981).

mtDNA nucleoid

mtDNA replication is mediated by several nuclear-encoded transcription and replication factors, which along with mtDNA are packed within a space of ~ 70 nm in diameter, forming the mitochondrial nucleoid (Chen and Butow, 2005). There are ~ 30 nucleoid proteins conserved between different species (Chen and Butow, 2005). Some of these are responsible for the maintenance and packaging of mtDNA, whereas the others have, in addition to their role in mtDNA maintenance, a metabolic function (Kucej and Butow, 2007). A structural model has been suggested where the nucleoid is composed of a central core and a peripheral region where the central core proteins are involved in nucleic acid synthesis (Fig. 3; Table I), whereas translation and protein assembly occur in the peripheral region (Bogenhagen et al., 2007). The number of mitochondrial nucleoids per mitochondrion ranges from 1 to 10 (Sato and Kuroiwa, 2004). There is a dispute about mtDNA copy number as the estimated copies/mitochondrion ranged from 1 to 15 (Sato and Kuroiwa, 2004), whereas others report that there are between 2 and 8 copies of mtDNA in each nucleoid (Legros et al., 2004).

Both mitochondrial transcription factor A (TFAM; Ekstrand et al., 2004) and ATAD3 (He et al., 2007) have been proposed to provide either the key-linking protein or the backbone to the nucleoid. ATAD3 (also known as TOB3) is an AAA domain protein, consisting of two subdomains, ATAD3f1 and ATAD3f2 (Wang and Bogenhagen 2006; He et al., 2007). Although it is tightly associated with the inner mitochondrial membrane, its relationship with mtDNA is controversial. One study suggested that ATAD3 is attached to mtDNA at the

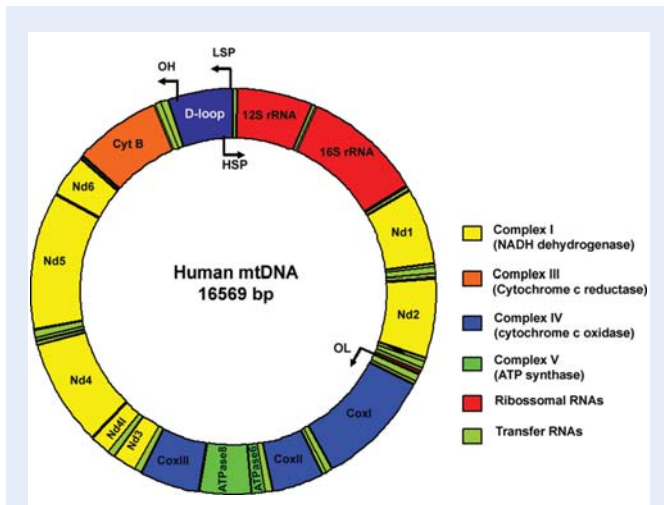


Figure 1 The human mtDNA genome encodes 13 polypeptides of the ETC.

These include seven subunits of Complex I (ND 1–6 and ND4L), one subunit of Complex III (CytB), three subunits of Complex IV (COX I to III) and two subunits of Complex V (ATPase6 and ATPase8). It also encodes two rRNAs (12S and 16S) and 22 tRNAs (Anderson et al., 1981). The main control region is the D-loop which contains the H-strand promoter region (HSP), the LSP region and the origin of H-strand replication (O_H). The second control region consists of only 30 bp and is located between ND2 and COXI and is the site of the origin of L-strand replication (O_L).

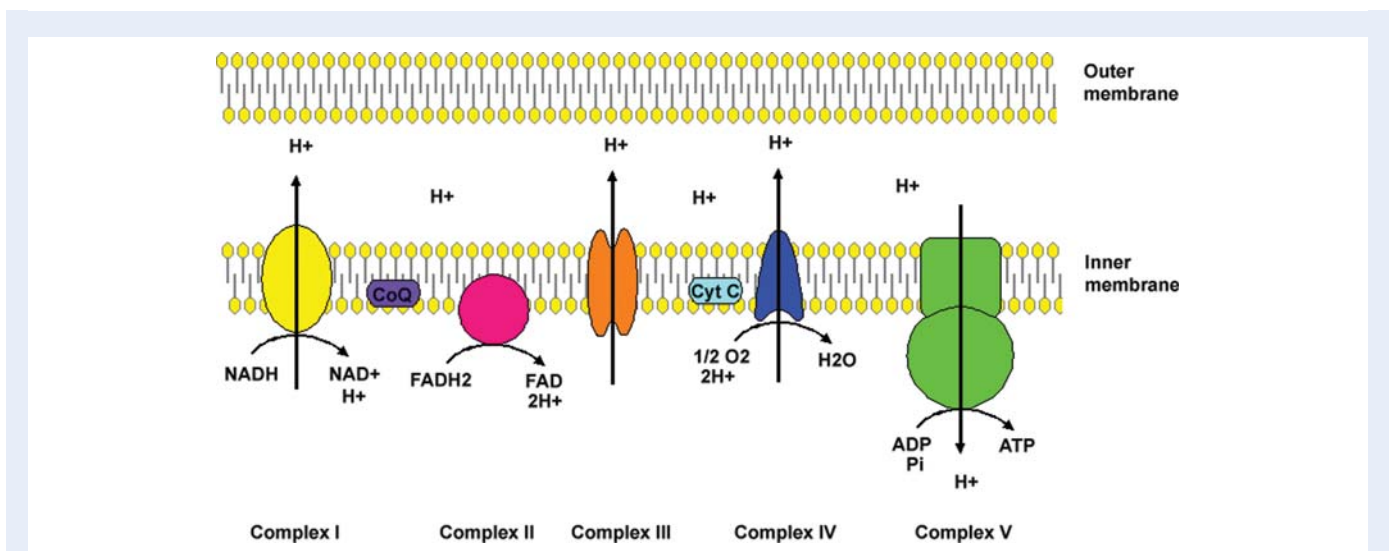
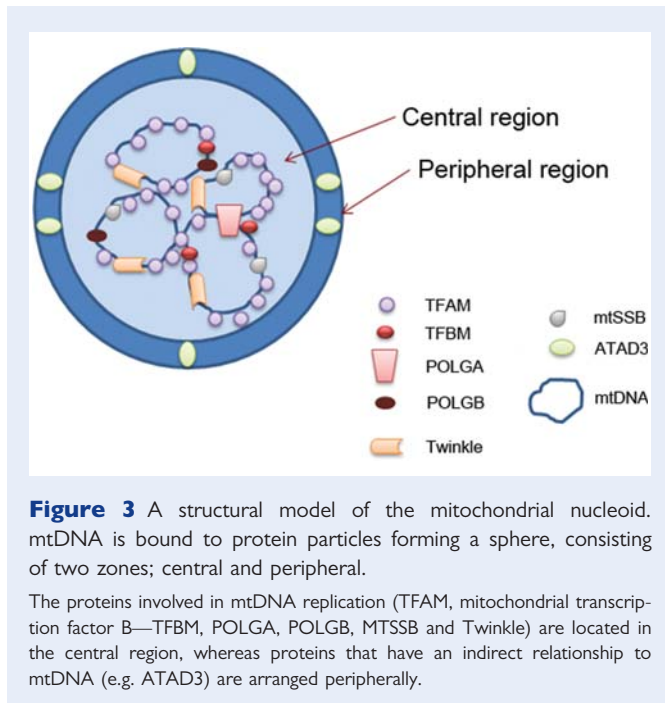


Figure 2 The electron transfer chain (ETC) is the major generator of cellular ATP through OXPHOS.

The high energy electrons derived from NADH and $FADH_2$ are transferred into Complexes I and II, respectively. The electrons then flow to the lipid-soluble carrier co-enzyme Q which in turn donates electrons to Complex III. Cytochrome c is the next electron acceptor and donates electrons to Complex IV. The energy released during the transfer of electrons is used by Complexes I, III and IV to pump protons across the mitochondrial inner membrane and to generate an electrochemical gradient. Complex V uses the mitochondrial membrane potential to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate. Complex I (ND); Co-enzyme Q (CoQ); Complex II (SD); Complex III (cytochrome c reductase); Cytochrome c (CytC); Complex IV (COX); Complex V (ATPase).



D-loop and it is involved in mitochondrial nucleoid formation and segregation (He *et al.*, 2007). However, immunofluorescence and protease susceptibility studies indicated that ATAD3 does not interact directly with mtDNA (Bogenhagen *et al.*, 2007). On the other hand, TFAM belongs to the high mobility group (HMG) box family of genes, which consists of a subset of genes characteristically associated with chromosomal DNA packaging, such as histones (Bianchi and Agresti, 2005). It has a molecular weight of ~25 kDa (Parisi and Clayton, 1991) and has three functions, namely binding, unwinding and bending of mtDNA.

The first function suggests a direct relationship between TFAM and mtDNA levels whereby over-expression of TFAM leads to an increase in mtDNA copy number (Ekstrand *et al.*, 2004; Hance *et al.*, 2005; Pohjoismaki *et al.*, 2006). Furthermore, the molar ratios of TFAM and mtDNA are ~900–1000:1 (Ekstrand *et al.*, 2004) again suggesting that mtDNA is extensively packaged with TFAM (Alam *et al.*, 2003). Indeed, the role of TFAM as an mtDNA-packaging molecule has been further supported by more recent studies demonstrating its co-localization to mtDNA through immuno-electron microscopy (Kaufman *et al.*, 2007). The latter two functions suggest an important role in mtDNA transcription further supporting its functional similarity to other HMG proteins (Fisher *et al.*, 1992; Takamatsu *et al.*, 2002; Bianchi and Agresti, 2005; Kaufman *et al.*, 2007). Furthermore, up-regulation of TFAM expression results in an increase in expression of the mitochondrial-specific polymerase gamma (Polg) (Ekstrand *et al.*, 2004; Hance *et al.*, 2005) implying a role in initiation of mtDNA replication.

POLG mediates the replication, recombination and repair of mtDNA (Grazieswicz *et al.*, 2005), thus demonstrating its essential role in mtDNA maintenance (Spelbrink *et al.*, 2000). Although it is capable of replicating mtDNA, it cannot replicate mammalian chromosomal DNA, perhaps resulting from its similarities to other members of the family A group of DNA polymerases. These include *Escherichia*

coli DNA polymerase I and the T7 DNA polymerase (Fridlender and Weissbach 1971; Fridlender *et al.*, 1972; Beese *et al.*, 1993a, b). It consists of two subunits in animal cells and one in yeast (Grazieswicz *et al.*, 2005): POLGA is the catalytic subunit that has 3' → 5' exonuclease activity and accounts for the high fidelity of this enzyme. POLGB is the accessory subunit that forms a heterotrimer in humans (in other mammalian species, it forms a heterodimer) at a ratio of 2:1 with POLGA (Carrodeguas *et al.*, 1999, 2001), thus stabilizing the enzyme to increase efficiency. In addition, a recent study has suggested that POLGB is the key factor that determines mtDNA copy number within the nucleoid as it is required for D-loop synthesis (Di Re *et al.*, 2009).

Two other factors play a key role in mtDNA replication. The helicase, Twinkle, was originally identified in association with autosomal dominant progressive ophthalmoplegia, a mitochondrial disorder characterized by mtDNA deletions (Spelbrink *et al.*, 2001). It shares a structural similarity to the C-terminal helicase part of bacteriophage T7 gene 4 protein which exhibits both helicase and primase activities (Bernstein and Richardson, 1989; Spelbrink *et al.*, 2001). Although mammalian Twinkle seems to lack primase activity (Spelbrink *et al.*, 2001), Twinkle homologues in *Plasmodium falciparum* (Seow *et al.*, 2005) and nearly all eukaryotes (Shutt and Gray, 2006) possess both helicase and primase activities. The mitochondrial single-stranded DNA-binding protein (mtSSB) mediates the unwinding of mtDNA through its physical interaction with Twinkle (Korhonen *et al.*, 2003), which co-localizes with both TFAM and mtSSB. These proteins act not only as structural components but also stabilize mtDNA during replication (Garrido *et al.*, 2002).

mtDNA replication

Both the asymmetric and the coupled leading- and lagging-strand synthesis models have been described to explain mtDNA replication. The asymmetric model has been the traditional approach to understanding how mtDNA replication is mediated (reviewed in Shadel and Clayton, 1997). It is initiated from the origin of H-strand replication (O_H), located within the D-Loop region, where TFAM binds to the enhancer of the light-strand promoter (LSP) and induces structural changes that expose the promoter region to the mitochondrial-specific RNA polymerase. This allows an RNA primer to be generated, which is used by POLGA to initiate mtDNA replication. mtDNA replication then continues two-thirds round the genome to the origin of L-strand replication (O_L). L-strand synthesis then proceeds in the opposite direction.

The coupled leading- and lagging-strand synthesis model proposes that both H- and L-strands are replicated bidirectionally from the same initiation cluster site (Yasukawa *et al.*, 2005). This mechanism is thought to occur in addition to the asymmetric model, but is also typical of cells repopulating mtDNA. The coupled leading- and lagging-strand synthesis model has now been refined to include the presence of replication intermediates which allow gaps within the replicating mtDNA on the lagging-strand to be filled to complete the replicon (Yasukawa *et al.*, 2006). It has more recently been proposed that replication is driven from several origins of replication within the vicinity of the O_H . However, to date, there has been little resolution as to which model is the most appropriate with entrenched views being expressed by the two opposing groups (Bogenhagen and Clayton, 2003; Holt and Jacobs, 2003).

Table 1 The key mtDNA nucleoid proteins associated with transcription and replication.

Nucleoid protein	Molecular weight (kDa)	Chromosomal location (in human)	Function	References
TFAM	24.4	10q21	Transcription and packaging	Parisi and Clayton (1991)
POLGA	125–140	15q25	mtDNA maintenance	Ye et al. (1996); Lecrenier et al. (1997); Wernette and Kaguni (1986)
POLGB	35–55	17q	mtDNA maintenance, putative role as a regulator of mtDNA copy number	Carrodeguas et al. (2001); Di Re et al. (2009); Wernette and Kaguni (1986)
Twinkle	77	10q24	mtDNA unwinding	Li et al. (1999)
mtSSB	17.26	7q34	Stabilizes D-loop and stimulates Twinkle activity	Takamatsu et al. (2002); Tomaska et al. (2001)
ATAD3 f1	59	1p36.33	Chaperone-like activity; backbone to the nucleoid	Bogenhagen and Clayton (2003); He et al. (2007)
ATAD3 f2	56.4	1p36.33	Chaperone-like activity; backbone to the nucleoid	Bogenhagen and Clayton (2003); He et al. (2007)
TFB1M	39.5	16q25.1–q25.3	Accessory transcription factor and translation of mitochondrial proteins	Falkenberg et al. (2002)
TFB2M	45.3	1q44	Accessory transcription factor and translation of mitochondrial proteins	Falkenberg et al. (2002)
mTERF	45.7	7q21–q22	Control rRNA and mRNA synthesis	Roberti et al. (2006)
mtRNA pol	138.6	19p13.3	Initiation of transcription at a specific mtDNA site	Gaspari et al. (2004)

It has been generally accepted that the mtDNA genome is not under direct epigenetic regulation. For example, mammalian cells characterized for mtDNA methylation are highly hypomethylated (Pollack et al., 1984). However, an important question that remains to be determined is how the expression of the key genes associated with mtDNA replication is mediated. Recent analysis of the promoter region of *Tfam* produced inconclusive data as to whether its expression was regulated by DNA methylation (Choi et al., 2004; Gemma et al., 2009). Nevertheless, more recent analysis of *PolgA* has however suggested that specific CpG islands within exon 2 of this gene are targeted (Oakes et al., 2007) and could thus account for changes in *PolgA* expression during spermatogenesis. This approach would certainly compliment the observation of an 8- to 10-fold decrease in the number of mtDNA molecules as these cells differentiate (Hecht et al., 1984). At this stage, *Tfam* expression is also decreased in the human (Larsson et al., 1997) and, in the mouse, an isoform is expressed that does not possess a mitochondrial targeting sequence (Larsson et al., 1996). This reduction in *Tfam* expression and mtDNA copy number would match other reports that indicate an association between *Tfam* and mtDNA copy number (Ekstrand et al., 2004), most likely restricting it to a packaging role in this instance. This suggests that replication could then be regulated by either a conformational or a numerical change to TFAM that would result in exposure of O_H or the broader initiation cluster coupled with a change in the DNA methylation status of *PolgA*. Indeed, DNA methylation of *PolgA* would present a truly interesting scenario, namely that the replication of a bacterial-originating genome was mediated by epigenetic regulation of a mammalian nuclear-encoded gene(s). It would also therefore be worth considering other members of the mtDNA nucleoid to determine whether their expression is also mediated in a similar manner, or by other processes such as microRNAs.

mtDNA replication in the pluripotent preimplantation embryo

Pluripotent blastomeres of mammalian preimplantation embryos and embryonic stem cells (ESCs) share a number of mitochondrial characteristics such as limited oxidative capacity and a greater dependence on anaerobic respiration (reviewed in Facucho-Oliveira and St John, 2009). This is further highlighted, following fertilization of the oocyte, by either whole embryo mtDNA copy number remaining constant during mouse preimplantation development up to the morula stage (Thundathil et al., 2005) or the reduction observed in porcine (Spikings et al., 2007) and bovine embryos (May-Panloup et al., 2005). Nevertheless, during the early cleavage stages, mammalian embryos express either very low levels of mtDNA replication factors or there is no expression (May-Panloup et al., 2005; Thundathil et al., 2005; Spikings et al., 2007). Consequently, mtDNA replication does not take place up to and including the morula stage and the number of mtDNA copies/blastomere is progressively decreased after each embryonic cell division. This results in these blastomeres progressively losing their capacity to generate ATP through OXPHOS as they become more reliant on anaerobic respiration (Leese and Barton, 1984; Leese 1995; Van Blerkom et al., 2002). Indeed, mouse (Batten et al., 1987), cattle (Stojkovic et al., 2001), pig (Spikings et al., 2007), monkey (Squirell et al., 2003) and human preimplantation embryos (Wilding et al., 2001) contain low numbers of spherical and immature mitochondria which are located in the perinuclear regions of the cytoplasm. The loss in mtDNA copy number is mirrored by mtDNA replication factor activity. For example, in pig and sheep, POLGA, POLGB and TFAM are only briefly detected between the 4- to 8-cell (Spikings et al., 2007) and 8-cell to 16-cell stages (Bowles et al., 2007b), respectively, whereas in cattle, *Tfam* is first up-regulated at the morula stage

(May-Panloup *et al.*, 2005). These brief mtDNA replication events are most likely associated with the generic turnover of a multiple sets of genes, i.e. during embryonic genome activation (EGA; Ma *et al.*, 2001), as demonstrated by the small mtDNA replication event, determined by 5-bromo-2'-deoxy-uridine staining, which takes place in pre-nuclear and 2-cell stage mouse embryos (McConnell and Petrie, 2004).

mtDNA replication and embryonic differentiation

At the blastocyst stage, there is an up-regulation in the expression of mtDNA replication factors which leads to reactivation of mtDNA replication in those mammalian species so far investigated (Piko and Taylor, 1987; May-Panloup *et al.*, 2005; Thundathil *et al.*, 2005; Spikings *et al.*, 2007). At the same time, mitochondria start to differentiate into elongated organelles containing swollen cristae (Stern *et al.*, 1971; Piko and Matsumoto, 1976; Wilding *et al.*, 2001), acquire a higher membrane potential and levels of oxygen consumption, and increased OXPHOS activity leading to ATP production (Trimarchi *et al.*, 2000; Wilding *et al.*, 2001; Houghton, 2006). However, these replication events are most likely specific to the trophoblast cells, the cells that give rise to the placenta and will mediate the process of implantation if the embryo is to continue to develop. These cells are no longer pluripotent, i.e. have the potential to differentiate in to any cell type of the body, and typically express markers such as *Cdx2* (Niwa *et al.*, 2005). The inner cell mass (ICM) cells, which are pluripotent and give rise to the fetus and ESCs, continue to express high levels of the pluripotency genes such as *Oct4*, *Nanog* and *Sox-2* (Chen and Daley, 2008). They also continue to express low levels of POLG and TFAM (Spikings *et al.*, 2007). As such, the high levels of expression of pluripotency genes appear to be associated with low levels of expression of mtDNA replication factors and anaerobic metabolism (reviewed in Facucho-Oliveira and St John, 2009).

mtDNA replication, pre-gastrulation and undifferentiated ESCs

The ICM cells continue to divide before they undergo gastrulation or give rise to ESCs and thus progressively gain an increased nucleocytoplasmic ratio. At the blastocyst stage, there are ~483 000 copies of mtDNA per single cell in the ICM (Cao *et al.*, 2007). Consistent with the undeveloped mitochondrial network in the pluripotent ICM, undifferentiated pluripotent mouse and human ESCs contain ~20 mitochondria profiles with poorly developed cristae (Rivolta and Holley, 2002; Baharvand and Matthaei, 2003; St John *et al.*, 2005a, b; Cho *et al.*, 2006; Chung *et al.*, 2007), and high levels of glycolytic enzymes (Kondoh *et al.*, 2007). As the ICM cells progress to become ESCs, they also express low levels of mitochondrial replication factors, such as *PolgA*, *PolgB* and *Tfam* (St John *et al.*, 2005a, b; Facucho-Oliveira *et al.*, 2007) and progressively reduce their mtDNA copy number so that they possess 30–45 copies/cell (Facucho-Oliveira *et al.*, 2007; Spikings 2007). Similarly, undifferentiated hESCs also contain very low levels of mRNA for *Tfam*, *Tfb1m*, *Tfb2m* and *PolgA* (St John *et al.*, 2005a, b). Consequently, any mtDNA replication observed in the ongoing pluripotent cell will most likely be associated with replenishment rather than active increases in mtDNA copy number which will occur prior to division to ensure that each newly divided mitochondrion has sufficient copies of mtDNA for transcription and transmission.

mtDNA replication and cellular differentiation

The onset of mtDNA replication and cellular differentiation can be studied in ESCs and parallels studies in the mouse embryo. Indeed, the analyses of the expression of a panel of lineage-specific markers showed a strong correlation between days of spontaneous differentiation of mESCs and stages of mouse embryo development (Leahy *et al.*, 1999). To this extent, it has been demonstrated that embryoid bodies (EBs) at Days 1 and 2 are equivalent to mouse embryos at E4.5 to E6.5; EBs at Days 3–5 are equivalent to E6.5 to E7.0 and that EBs after Day 6 are equivalent to E 7.5. Moreover, the embryos at E4.5 and E6.5 are at pre-gastrulation which correlates with the up-regulation of pluripotency genes in Days 1 and 2; R1 mESCs (Facucho-Oliveira *et al.*, 2007). At E6.5 to E7.0, embryos are in gastrulation as determined by detection of markers associated with the endoderm, mesoderm and ectoderm (Leahy *et al.*, 1999), and therefore, down-regulation of the pluripotency genes and up-regulation of lineage-specific markers would be expected (Days 3–4). At E7.5, mouse embryos are in the early stages of organogenesis (Leahy *et al.*, 1999). During Days 1 and 2 of spontaneous differentiation of ESCs, the expression of the pluripotent markers *Nanog*, *Dppa5*, *Pramel7* and *Ndp5211* were up-regulated (Facucho-Oliveira *et al.*, 2007; Facucho-Oliveira and St John, 2009). These pluripotency genes were then down-regulated and the percentage of Vimentin⁺, Nestin⁺ and β -tubulin III⁺ cells increased during Days 3 and 4. At this stage, the expression of *PolgA*, *PolgB* and *Tfam* and the number of mtDNA copies/cell were minimal. By Day 6, the expression of *PolgA*, *PolgB* (Facucho-Oliveira *et al.*, 2007; Fig. 4), and the nuclear-encoded ATPase5b and the mtDNA-encoded COXI (Fig. 5) were up-regulated in line with an increase in the number of mitochondria and mtDNA copies/cell (Facucho-Oliveira *et al.*, 2007). This indicates that the onset of organogenesis requires an increase in expression of the mtDNA replication factors and ETC subunits. This is further supported by homozygous *PolgA* knockout mice dying between days E7.5 and E8.5 (Hance *et al.*, 2005) and homozygous *Tfam* knockout mice between E8.5 and E10.5 (Larsson *et al.*, 1998).

Modification of mtDNA replication

Stem cells have the ability to respond to the environment in which they exist. For example, the stroma has been proposed as a mediator of stem cell maintenance and thus the regulator of the stem cell niche and can mediate differentiation (e.g. see McKinney-Freeman and Daley, 2007). Equally, ESCs can undergo targeted differentiation into specific lineages to generate enriched populations of single cell types. This can be mediated through addition of chemical reagents such as retinoic acid (RA; Dani *et al.*, 1997; Rohwedel *et al.*, 1999) or growth factors (Reubinoff *et al.*, 2001) to the culture media, through genetic engineering (Xiong *et al.*, 2005) or epigenetic modification to key developmental transcriptional factors (Kawamura *et al.*, 2005). For example, the treatment of mESCs with RA has been shown to enhance neuronal differentiation (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Rohwedel *et al.*, 1999) and can also induce amplified transcription of the mtDNA-encoded NADH dehydrogenase subunit 5, COXI and 16S rRNA (Li *et al.*, 1994; Gaemers *et al.*, 1998) genes. However, induction of differentiation with RA not only increased the number of neuronal-type cells expressing Nestin and

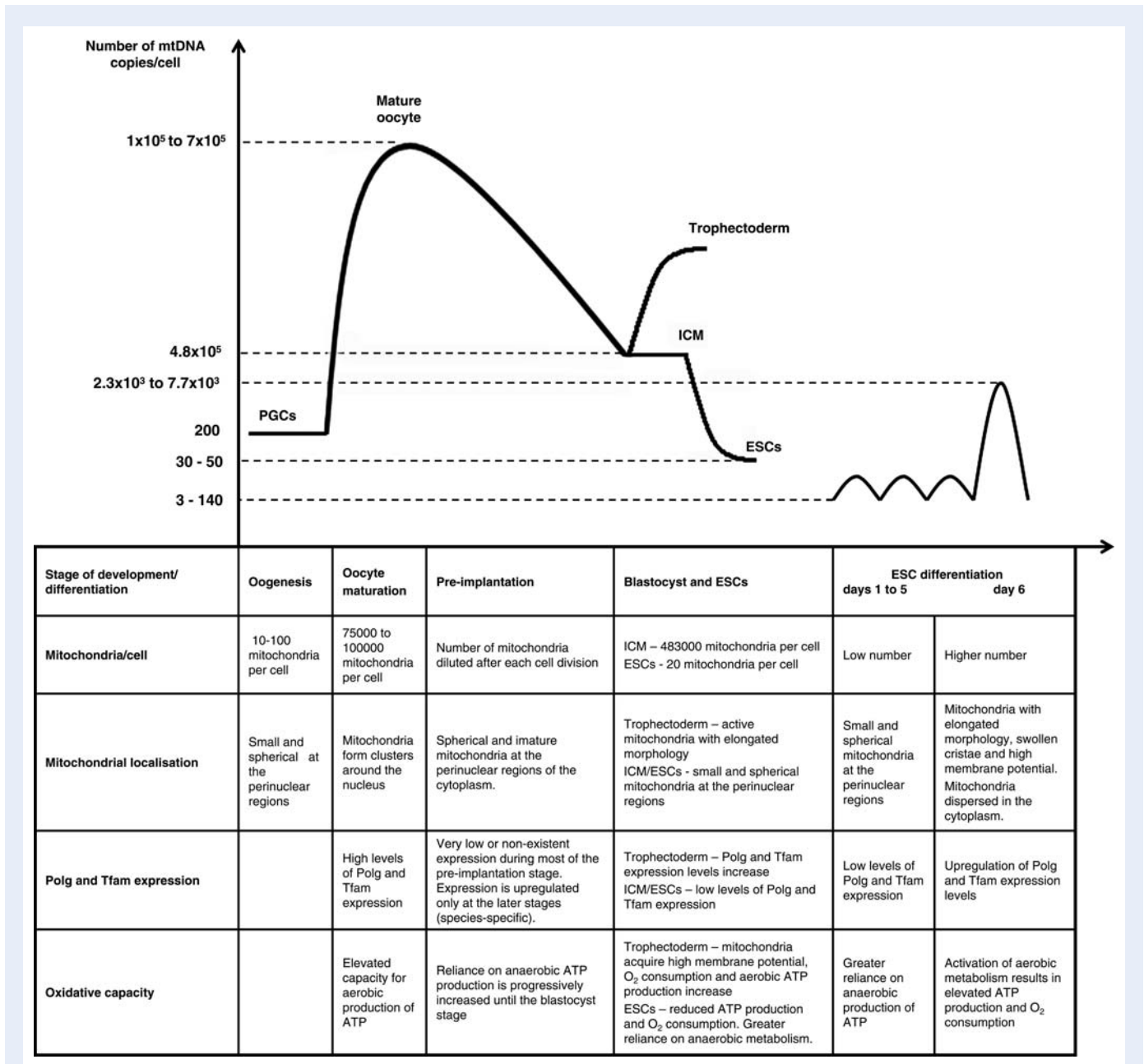


Figure 4 The main events of mitochondrial biogenesis and mtDNA proliferation.

Both mitochondrial biogenesis and mtDNA replication are tightly regulated processes that ensure sufficient numbers of mitochondria and copies of mtDNA are available for the onset of OXPHOS as the oocyte is fertilized. mtDNA replication is then significantly reduced during preimplantation development with only increases taking place in the trophoblast to aid implantation. However, the cells of the ICM continue to decrease their numbers with each cell division with undifferentiated and differentiating ESCs maintaining low copy number.

β-tubulin, when compared with spontaneous differentiating cells, but it also produced significantly different patterns of mtDNA replication (Facucho-Oliveira *et al.*, 2007) and was marked by a significant increase in expression of *Tfam*, *PolgA* and *PolgB*.

During Days 1–5, the RA-induced D3 mESCs had higher levels of *PolgA*, *PolgB* and *Tfam* expression, a higher number of mtDNA copies/cell (Facucho-Oliveira *et al.*, 2007; Fig. 4) and higher percentage of cells with increased ATPase5b and COX1 expression (Fig. 5) than the D3 mESCs differentiated in the presence of 25 mM glucose

(standard glucose concentration for ESC culture). RA exposure also enhanced neuronal differentiation in comparison to the D3 mESCs differentiated in 25 mM glucose. RA-induced D3 mESCs had higher percentages of Vimentin⁺ and β-tubulin III⁺ cells on Day 1, a lower percentage of Vimentin⁺ cells during Days 2–7 (Facucho-Oliveira *et al.*, 2007; Fig. 4) and a higher percentage of β-tubulin III⁺ cells on Day 14 (Fig. 5).

The presence of glucose also influences metabolic activity. This is demonstrated by D3 mESCs differentiated in the presence of 1 and

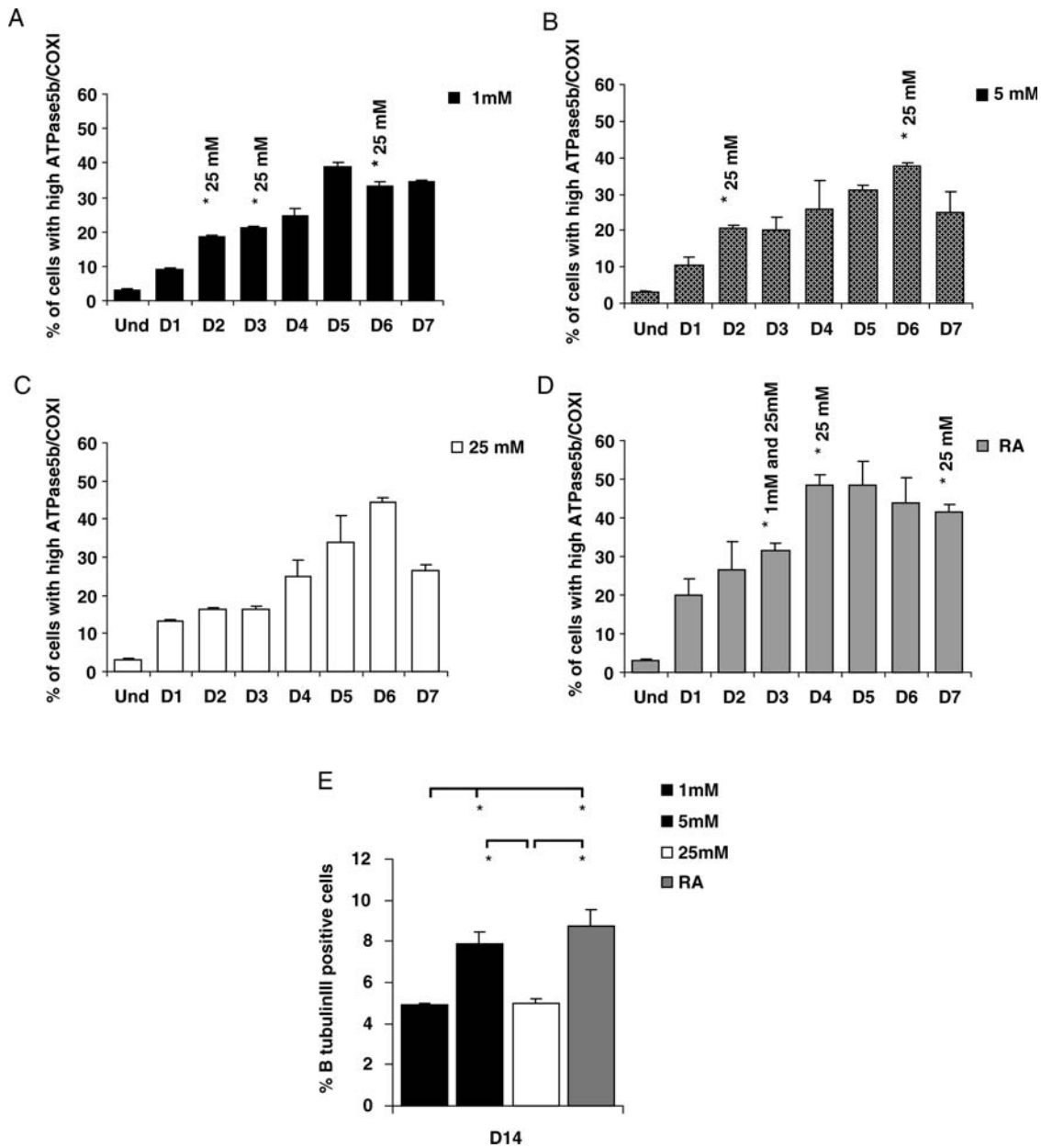


Figure 5 Percentage of cells with increased ATPase5b and COXI expression during differentiation of D3 mESCs in the presence of 1 mM (A), 5 mM (B) and 25 mM (C) of glucose and RA-induced differentiation (D). D3 mESCs differentiated in the presence of 1 and 5 mM of glucose had a higher percentage of cells with increased ATPase5b and COXI expression than D3s differentiated in the presence of 25 mM of glucose on Days 2 and 3 (A–C). However, the D3s differentiated in the presence of 25 mM of glucose had a higher percentage of cells with elevated levels of ATPase5b and COXI than the D3 differentiated in 1 and 5 mM of glucose on Day 6 (A–C). The RA-induced D3 mESCs had the highest percentage of cells with increased ATPase5b and COXI expression on Days 1–5 and 7 (D). The percentage of β -tubulin III⁺ neurons was significantly higher in the RA-induced D3s and D3s differentiated in the presence of 5 mM of glucose than in the D3s differentiated in the presence of 1 and 25 mM of glucose on Day 14 (E). Bars represent mean \pm s.e.m.; significant differences in the percentage of cells with increased ATPase5b and COXI expression are indicated (* $P < 0.05$).

5 mM glucose having a higher percentage of cells with increased ATPase5b and COXI expression than the D3 mESCs differentiated in the presence of 25 mM glucose on Days 2 and 3 (Fig. 5). Furthermore, the D3 mESCs differentiated in 5 mM glucose showed a higher percentage of β -tubulin III⁺ cells than the D3 mESCs differentiated in 25 mM glucose on Day 14 (Fig. 5).

mtDNA transmission

Mammals normally inherit their mtDNA from the population present in the oocyte just prior to fertilization (reviewed in Birky, 1995, 2001) when mtDNA replication has been completed (Spikings *et al.*, 2007). These copies tend to be identical and thus the mtDNA transmitted to

the offspring is homoplasmic (Giles *et al.*, 1980). Following natural fertilization, sperm mtDNA tends to be eliminated thus ensuring the maintenance of homoplasmy, though this is primarily restricted to intra-specific crosses. This arises as, during early spermatogenesis, spermatogonia are labelled with ubiquitin, which appears to be 'masked' as spermatozoa transit through the epididymis and is then present following ejaculation (reviewed in Sutovsky *et al.*, 2004). Having entered the oocyte, sperm mitochondria, and thus its mtDNA complement, are eliminated prior to embryonic gene activation (EGA) for that particular species. For example, in the mouse, sperm mitochondria and mtDNA persist to the 2-cell stage (Kameda *et al.*, 1995; Shitara *et al.*, 2000), whereas in larger mammals, such as sheep (Zhao *et al.*, 2004) and cattle (Sutovsky *et al.*, 1999), it is eliminated by the 8-cell stage, and in the rhesus macaque (St John and Schatten, 2004; Sutovsky *et al.*, 1999) and humans (St John *et al.*, 2000), this tends to be at the 4–8-cell stage (St John *et al.*, 2000). This suggests that the process of ubiquitination is unlikely to be associated with new genes transcribed during the initial round of transcription following EGA, but is dependent on mRNA carried over from the oocyte and subsequently translated.

Following interspecific crossing, e.g. the mating of two different strains of mouse (Gyllensten *et al.*, 1991; Shitara *et al.*, 2000) or different breeds of sheep (Zhao *et al.*, 2004) or subspecies of rhesus macaques (St John and Schatten, 2004), sperm mtDNA can be transmitted through the offspring. Although only low levels of sperm mtDNA tend to be transmitted, there is no evidence to suggest that this is transmitted to subsequent generations (Gyllensten *et al.*, 1991). Consequently, the molecules recognized by the ubiquitination process are likely to be species-specific. However, the mechanism that precludes sperm mtDNA from being transmitted within a species, such as in the human, is not absolute. Indeed, there is one reported case of sperm mtDNA recombining with oocyte mtDNA creating a new hybrid molecule that segregated to one but not other tissues giving rise to a muscle myopathy in a male patient (Schwartz and Vissing, 2002; Kraysberg *et al.*, 2004). Furthermore, abnormal human embryos can also fail to eliminate their sperm mtDNA (St John *et al.*, 2000). These outcomes assert the argument that transmission is dependent on mtDNA replication, where sperm will contribute copies to the fertilization process, and if not eliminated, will be available for both replication and transmission. Interestingly, though poor-quality sperm have significantly higher numbers of mtDNA (Diez-Sanchez *et al.*, 2003; May-Panloup *et al.*, 2003; Amaral *et al.*, 2007). However, although poorer quality sperm are then used in ICSI treatment, to date there have been no reported cases of transmission resulting from this treatment (Danan *et al.*, 1999; Marchington *et al.*, 2002).

Transmission of sperm mtDNA is evident in both invertebrates and vertebrates. For example, blue mussels transmit both maternal- and paternal-type genomes to male offspring and maternal only genomes to female offspring (Zouros *et al.*, 1992) suggesting an important outcome for mtDNA transmission in sex selection and sex determination, although this is now contested (Kenchington *et al.*, 2009). Both intra- and interspecific crosses of *Drosophila* can transmit both oocyte- and sperm-derived mtDNA to their offspring (Kondo *et al.*, 1992). However, there is no reported evidence from such models to suggest that the transmission of potentially diverse mtDNA molecules would give rise to any type of mitochondrial-related disease

or whether the resultant offspring exhibit specific advantageous or disadvantageous traits. Although increased genetic distance between the sperm and the oocyte is likely to influence fertilization outcome, the transmission of sperm mtDNA is normally at significantly low levels that it is unlikely to induce a phenotypic disadvantage, except in rare cases. Consequently, the potential for sperm mtDNA to be transmitted is most likely restricted to those crosses where the degree of genetic distance does not prevent mtDNA from restricting fertilization outcome. However, there is likely to be merit in conducting a wide-ranging inclusive study of sperm mtDNA transmission in humans. Although a few studies have indicated that sperm mtDNA is not transmitted (Taylor *et al.*, 2003), they tend to have been centred on samples from patients with mtDNA disease. They would not necessarily provide an insight into the overall levels of sperm mtDNA leakage within a population; this necessitates a large, wide-ranging study.

There are a large number of mtDNA mutations and deletions, which have been associated with a large number of clinical phenotypes (see Wallace, 1999, for review). For example, Leber's hereditary optic neuropathy (LHON; Wallace *et al.*, 1988; Howell *et al.*, 1991) and neurogenic weakness, ataxia and retinitis pigmentosa (Fryer *et al.*, 1994) are associated with single point mutations in coding genes, whereas mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS; Goto *et al.*, 1990) and deafness (Van den Ouweland *et al.*, 1992) result from single point mutations to tRNAs and rRNAs. mtDNA disease is also associated with large-scale deletions, e.g. the 4977-bp deletion resulting in Kearns–Sayre syndrome (KSS; Schon *et al.*, 1989). The onset of the phenotype for each of these diseases will be determined by the degree of mutant to wild-type molecule that persists within the affected tissue. In the case of LHON, it is predicted that over 60% mutant molecule needs to be present for the phenotypic onset of the disease to be clinically recognized (Chinnery *et al.*, 2001), whereas for MERRF, over 85% mutant needs to be present (Boulet *et al.*, 1992). However, this general rule has now been contradicted by recent findings associated with severe multisystem disorder and respiratory chain deficiency where the mutation was present at less than 25% (Sacconi *et al.*, 2008).

Do the more sophisticated reproductive approaches affect mtDNA transmission?

The introduction of more sophisticated techniques for generating embryos, ESCs and offspring, such as cytoplasmic transfer and nuclear transfer (NT) have led to a re-fashioning of the concept of heteroplasmy. Cytoplasmic transfer, the transfer of cytoplasm from a donor oocyte, was introduced into human-assisted reproduction treatment to increase embryonic developmental outcome for those women who had suffered from repeated embryonic developmental failure, most often at the post-8-cell stage (Cohen *et al.*, 1997). Here, donor oocyte cytoplasm was supplemented into such oocytes. As the oocyte cytoplasm contains not only mRNAs but also mitochondria, it was evident that two populations of mtDNA, one from the recipient oocyte and one from the donor cytoplasm, were transferred with the sperm to aid the fertilization process. Consequently, some of the resultant offspring were heteroplasmic (Brenner *et al.*, 2000). Similar outcomes have been observed following

the transfer of either mouse mitochondria into mouse oocytes (Laipis, 1996) or embryos (Nakada *et al.*, 2001) or a germinal vesicle stage or pronuclear karyoplast (Jenuith *et al.*, 1996; Meirelles and Smith, 1997, 1998; Palermo *et al.*, 2002; He *et al.*, 2003; Sato *et al.*, 2005) into a mouse oocyte. As with human cytoplasmic transfer, it was evident that variable amounts of mtDNA from either the supplementing source or the karyoplast could be identified in the resultant offspring. In three independent experiments, this has been shown to range from 5% to 80% (Laipis, 1996), 0% to 30% (Jenuith *et al.*, 1996) and 16% to 69% (Meirelles and Smith, 1997). Interestingly though, karyoplast transfer is only successful when the karyoplast is introduced into a cytoplasm from the same stage of development (Liu *et al.*, 2003).

Although cytoplasmic transfer can result in live offspring, a longitudinal study of heteroplasmic mice has demonstrated a range of physiological abnormalities when compared with control animals (Acton *et al.*, 2007). These included systemic and pulmonary hypertension, increased body mass and fat mass and abnormalities associated with electrolytes and haematological parameters. These abnormalities most likely arise from the genetic distance between the two populations of mtDNA where the genetic variability which will result in differing amino acids being encoded and could thus mimic mtDNA-type disease. This has perhaps been further exemplified through the generation of mice harbouring a large-scale deletion that induced multiple phenotypes when >85% of the deleted molecule was present (Nakada *et al.*, 2001). Although there is no direct association with human mtDNA-type disease, cytoplasmic transfer has resulted in two cases of Turner's syndrome leading to one selective and one spontaneous miscarriage, and one case of pervasive development disorder (Barritt *et al.*, 2001). Nevertheless, a recent study has demonstrated that isolated populations of mitochondria can be transferred from developmentally competent oocytes into their incompetent counterparts resulting in increased fertilization and developmental outcome. This suggests that (i) mitochondria do play a key role in early development (El Shourbagy *et al.*, 2006) and (ii) improved fertilization outcome could be achieved with mtDNA derived from the same genetic source. This could have implications for human-assisted reproductive treatment, where, for a cohort of oocytes obtained from an individual following hormonal stimulation, mitochondria could be collected from several of her oocytes and pooled. These mitochondria could then be introduced into the remaining oocytes to increase fertilization and development outcomes.

Studies involving either embryonic cell NT (ECNT) and somatic cell NT (SCNT) have demonstrated that the mtDNA accompanying the donor cell can be transmitted to the offspring at varying levels along with recipient oocyte mtDNA. This occurs as the process of NT often uses a whole donor cell (Campbell *et al.*, 1996), with donor cell mtDNA being either eliminated during preimplantation development, resulting in homoplasmic transmission of the recipient oocyte's mtDNA only, or persists resulting in heteroplasmy (i.e. both donor and recipient mtDNA). Indeed, transmission of donor cell mtDNA ranges from 0% to 63% in preimplantation embryos (Meirelles *et al.*, 2001) and 0% to 59% in live offspring (Takeda *et al.*, 2003; see Table II). This tends to be independent of whether intra- or interspecific or interspecies ECNT or SCNT is performed (St John *et al.*, 2004; Tables II and III), e.g. bovine embryos and offspring derived by both intra- and interspecific NT (Meirelles *et al.*,

2001; Do *et al.*, 2002; Takeda *et al.*, 2003); and in caprine embryos (Jiang *et al.*, 2004) and porcine (Takeda *et al.*, 2006) and *Macaca mulatta* (St John and Schatten, 2004) offspring derived by interspecific ECNT and SCNT. It is thus evident that embryonic and somatic cell mtDNA can escape the selective mechanism that targets and eliminates intra-specific sperm mitochondria in the fertilized oocyte to maintain homoplasmy (Shitara *et al.*, 2000; Sutovsky and Schatten, 2000). Indeed, in the two non-human primates derived by interspecific ECNT, both oocyte and sperm mtDNA contributing to the donor blastomere were transmitted (St John and Schatten, 2004). However, the factors that allow for the large differences in heteroplasmy between individuals remain to be determined experimentally.

In order to determine why donor cell mtDNA might be transmitted, an early study indicated that the amount of mtDNA that was transmitted was proportional to the amount of mtDNA accompanying the embryonic donor cell at the time ECNT was performed. The donor cells were from varying stages of preimplantation development thus containing decreasing levels of mtDNA following division during preimplantation development (Steinborn *et al.*, 1998). However, closer examination of these data suggests that this is perhaps not as decisive an outcome as first thought. A further study set out to understand why donor cell mtDNA persists at levels inconsistent with its original contribution. Intra- and interspecific SCNT-ovine embryos, using donor cells depleted to 0.01% (residual – mtDNA^R) and 0.1% of their original mtDNA content, were generated and compared with time-matched non-depleted (mtDNA⁺) cells (Lloyd *et al.*, 2006). Surprisingly, donor cell mtDNA persisted to the hatched blastocyst stage for all combinations. This outcome has been attributed to the persistent expression of mtDNA-specific replication factors, such as POLGA, POLGB and TFAM, during preimplantation development, which contrasts with their *in vitro*-derived counterparts (Bowles *et al.*, 2007b).

In interspecies SCNT embryos (iSCNT; see Table III), donor cell mtDNA has been detected at the 16-cell stage in human-bovine crosses (Chang *et al.*, 2003), the blastocyst stage in macaque–rabbit (Yang *et al.*, 2003) and in a small minority of caprine-ovine embryos (4 of 40; range = 2–20+ cell embryos; Bowles *et al.*, 2007b). In this small number of heteroplasmic embryos, significant increases in donor cell mtDNA were observed again suggesting that occasionally selective replication does take place. Although the generation of live offspring is highly unlikely through iSCNT, it remains to be determined whether residual levels of mtDNA would persist and proliferate or be eliminated in differentiating ESCs derived through iSCNT.

It is worth, however, stressing that considerable confusion has been created by the current zoological nomenclature that assigns species status to populations that are often only subspecies. This is very common in domestic strains of animals and their distant ancestors such as *Bos taurus* and *Bos indicus* (cattle), *Capra hirus* and *Capra ibex* (goat) and *Sus scrofa* and *Sus vittatus* (pig). Here, we have collated mtDNA analyses on iSCNT embryos and offspring, based on clear distinctions between species and not as subspecies and interspecific crosses (cf. Tables II and III). However, it should be stressed that performing mtDNA analysis with different detection methods will often result in different levels of mtDNA heteroplasmy being observed, thus we have listed the relative methods used. Several conclusions can be drawn: (i) different tissues will have different mtDNA

Table II mtDNA content in cloned embryos, fetuses and offspring generated by intra- and interspecific nuclear transfer.

Species	Sample analysed	Degrees of heteroplasmy	Detection methods	References
Bovine	Sampled blastomeres, blastocysts, tissues of a newborn clone	NT-blastomere zygotes and blastocysts had similar levels of heteroplasmy (donor cell—11.0% and 14.0%, respectively), 5.8% at the 9- to 16-cell stage. Heteroplasmic levels in NT-fibroblast zygotes—4.7%, 9- to 16-cell—12.9%, blastocysts 2.2%. No donor cell mtDNA detected in gametes or tissues of newborn calf	Allele-specific PCR with fluorochrome-specific primers	Ferreira et al. (2007)
Sheep fetal fibroblast	Seven fetuses and five lambs cloned from fetal fibroblasts	Most of the heteroplasmic clones exhibited low levels of heteroplasmy (0.1–0.9%, $n = 6$). High levels of heteroplasmy (6.8–46.5%) in 1 clone	Quantitative amplification refractory mutation system (ARMS) PCR	Burgstaller et al. (2007)
Pig (<i>S. vittatus</i> and <i>S. scrofa</i>)	4 SCNT pigs derived from <i>S. vittatus</i> nuclear donor cells and <i>S. scrofa</i> recipient oocytes	0.1–1% heteroplasmy in blood and hair roots. 0–44% in tissues of one animal	SSCP and agarose gel PCR-RFLP with image analysis and SNP-PCR	Takeda et al. (2006)
Ovine fetal fibroblast with different levels of mtDNA	2-cell to hatched blastocyst	Donor cell mtDNA persisted in some blastocysts (0.01–0.9%)	Allele-specific real-time PCR	Lloyd et al. (2006)
Pig (<i>S. scrofa</i>)	5 pigs derived from 2 nuclear donor cell types (double nuclear transfer)	Donor cell mtDNA eliminated but transmission of first recipient oocyte and zygote mtDNA—bimaternal mtDNA transmission	Sequence analysis, RFLP, AS-PCR and primer extension polymorphism analysis	St John et al. (2005a, b)
Cattle (<i>B. taurus</i>)	3 calves	Heteroplasmy detected in skin and blood (1–3.6%)	Agarose gel electrophoresis and image analysis	Theoret et al. (2006)
Cattle (<i>B. taurus</i>)	5 calves	Heteroplasmy in skin samples	AS-PCR on agarose gel	Han et al. (2004)
Goat (<i>C. ibex</i> and <i>C. hircus</i>)	1-, 2-, 4-cell, and morula stage	Heteroplasmy only at 1- and 2-cell stage	AS-PCR on agarose gel and DNA sequencing	Jiang et al. (2004)
Mouse (<i>Mus musculus domesticus</i> , <i>Mus m. molossinus</i>)	25 adult mice derived from 3 nuclear donor cell types	Heteroplasmy in brain, kidney, liver and tail of all but one animal. Amount of donor cell mtDNA depended on donor cell type and tissue with significant differences between sertoli (0.78%) and fibroblast (2.4%) derived mice, and between brain (0.6%) and liver (1.7%) tissues, heteroplasmy detected in all 20 placentae analysed	Allele specific real-time PCR	Inoue et al. (2004)
Cattle	12 day fetus derived from one donor cell type; 3 recipient oocyte cytoplasms: <i>B. taurus</i> mtDNA type B, type C, and <i>B. indicus</i> mtDNA	Mainly homoplasmic with occasional heteroplasmic at 0.5–0.7%	PCR-RFLP on agarose gel Cloned PCR fragment	Hiendleder et al. (2003)
Cattle (<i>B. taurus</i>)	16 embryos from 3 nuclear donor cell types Cumulus cells (holstein), ear skin cells (Japanese black) and fetal fibroblasts (Japanese black)	3 of 9 NT calves were heteroplasmic with donor cell mtDNA ranging from 0% to 59%	PCR-mediated single-strand conformation polymorphism analysis and southern blot hybridization	Takeda et al. (2003)
Cattle (<i>B. taurus</i>)	1-, 2-, 4-, 8- and 16-cell, morula and blastocysts	Heteroplasmy at all stages	Allele specific PCR on agarose gel and direct sequencing of PCR product	Do et al. (2002)
Cattle (<i>B. taurus</i> , <i>B. indicus</i>)	<i>B. Taurus</i> nucleus and <i>B. indicus</i> mtDNA ($n = 11$)	0.6–2.8% heteroplasmy in 4 out of 11	Allele specific real-time PCR	Steinborn et al. (2002)
Cattle (<i>B. indicus</i>)	Reconstructed oocytes, blastomeres and whole morula, blastocysts and fetal and offspring tissues	0–63% in preimplantation embryos; reduction or complete elimination of donor cell mtDNA during development	PCR restriction fragment length polymorphism	Meirelles et al. (2001)
Cattle (<i>B. taurus</i>)	6 fetuses and 4 calves derived from 3 nuclear donor cell types	0.1–4% heteroplasmy in all but one sample in blood, cerebellum, hear, kidney, liver, lung, muscle and skin	Allele-specific real-time PCR	Steinborn et al. (2000)

Continued

Table II *Continued*

Species	Sample analysed	Degrees of heteroplasmy	Detection methods	References
Sheep (<i>O. aries</i>)	10 sheep derived from 3 nuclear donor cell types	No heteroplasmy in blood, milk muscle and placenta	Southern blot hybridization with phosphorimager,	Evans <i>et al.</i> (1999)
Cattle blastomeres from Japanese back cow morula	21 cloned calves produced from recipient oocytes of undefined mtDNA genotypes, and 6 cloned calves from defined mtDNA genotype	20 of 21 calves homoplasmic; 1 calf heteroplasmic	PCR-mediated single-strand conformation polymorphism	Takeda <i>et al.</i> (1999)

content at specific developmental stages and in the adult; (ii) preimplantation embryos will carry different mtDNA patterns for cloned offspring when compared with natural fertilization and (iii) the mtDNA content in blastomeres either undergoes replication or escapes degradation during cleavage and can be degraded after the blastocyst stage or lost during somatic cell development, as revealed by the lack of donor cell mtDNA at birth in some offspring.

What are the consequences of introducing somatic mitochondria and donor cell mtDNA into oocytes?

Most studies of mitochondria in mammalian oocytes and preimplantation stage embryos have focused primarily on their metabolic capacity to generate ATP. However, mitochondria are also involved in other processes such as establishing developmental competence, calcium homeostasis and apoptosis (van Blerkom, 2004). The inner mitochondrial membrane potential, morphological changes and translocation of mitochondria also play key roles in embryo development. In pig, the translocation of mitochondria from the oocyte cortex to the perinuclear area indicates positive developmental potential that was reduced in porcine SCNT embryos compared with their *in vitro* counterparts (Zhong *et al.*, 2008). Through transmission electron microscopy, these authors also demonstrated that unequal donor cell mitochondrial distribution and complex mitochondrial morphologies may influence mitochondrial translocation and affect development. Another report in goat–goat and goat–cattle embryos demonstrated that inter- and intra-species reconstructed embryos have a different pattern of developmental change to that of *in vivo*-produced embryos for mitochondria (Tao *et al.*, 2008). These ultrastructural differences might contribute to the compromised developmental potential of reconstructed embryos. In cloned mice, again, there appears to be no difference observed in the number of mitochondria, but their mitochondria displayed ultrastructural alterations (Han *et al.*, 2008). Interestingly, the authors revealed that the glucose present in the culture medium compensated for the potential effects of ATP-dependent processes being severely compromised during the first 2-cell cycles.

Somatic cell mitochondria do appear to adversely effect embryonic development. For example, it has been demonstrated that development rates to blastocyst of parthenogenetically activated oocytes supplemented with somatic mitochondria decreased in comparison to non-supplemented controls and those supplemented with oocyte cytoplasm (Takeda *et al.*, 2005). Furthermore, SCNT blastocysts

generated with mtDNA^R donor cells (SFF1 line) had significantly more embryonic cells at blastocyst compared with those generated with non-mtDNA depleted cells (Lloyd *et al.*, 2006). In addition, significantly more caprine-ovine iSCNT embryos generated with mtDNA^R donor cells reached the 20-cell stage of preimplantation development than those generated with mtDNA⁺ cells (Bowles *et al.*, 2007a). To the contrary, transferring mitochondria from granulosa cells seems to boost preimplantation development, especially for those oocytes characterized by poor mtDNA content and cytoplasmic appearance (Hua *et al.*, 2007). The difference between granulosa cells and other somatic cells may therefore be dependent on their differing maturation statuses and requirements for energy.

Although there appears to be a tolerance for interspecific crosses, the mtDNA genomes for different breeds of cattle and pigs have sequence variations in their coding genes. This is reflected in interspecific bovine (Steinborn *et al.*, 2002) and porcine (St John *et al.*, 2005a, b) offspring. It is thus likely that the subtle changes in the amino acid sequences for these two mtDNA genomes would generate an incompatibility that would affect the interactions with closely located nuclear-encoded polypeptides within the same complex of the ETC. Indeed, during the embryo-fetal period, expression of a mutated gene in specific tissues can attribute to the failure of the respiratory chain complex assembly and function (Minai *et al.*, 2008). It has been suggested that mtDNA heteroplasmy also may induce incompatibility between the nucleus and cytoplasm, which will again inhibit the development of cloned embryos (Chen *et al.*, 2002; Bowles *et al.*, 2007a, b, 2008), especially at the time of genomic activation (Sansinena *et al.*, 2005). A recent report in cat-bovine cloned embryos demonstrated that the developmental block, which normally occurs just after EGA, was associated with the presence of foreign mtDNA (Thongphakdee *et al.*, 2008). In a study of minke whale iSCNT, the granulosa–cumulus cells had the ability to dedifferentiate in bovine and porcine oocytes up to the 4-cell stage, which is again indicative of developmental block at EGA and mitochondrial incompatibility between the recipient oocytes and donor cells (Ikumi *et al.*, 2004). When using iSCNT, it is thus critical to choose compatible donor and recipient cells. Furthermore, the regulation of mtDNA copy number in the preimplantation embryo may affect outcome. For example, in some species, such as mouse, the number of mtDNA copies/embryo is stable during cleavage, with subsequent blastomeres having fewer copies of mtDNA until replication begins post-implantation (Thundathil *et al.*, 2005), whereas domestic species, such as cows and pigs, significantly reduce their mtDNA content during preimplantation

Table III mtDNA content in cloned embryos, fetuses and offspring generated by interspecies nuclear transfer.

Donor cell	Recipient oocyte	Sample analysed	Degree of heteroplasmy	Detection methods	Reference
Mixed-breed domestic cat fibroblasts	Bovine	1-, 2-, 4- and 8-cell stage embryos	Cat mtDNA stable from 1- to 8-cell. Bovine mtDNA significantly lower at the 8-cell stage than 4-cell stage	Quantitative PCR	Thongphakdee et al. (2008)
Goat fetal fibroblasts	Sheep	1-, 2- and 4-cell embryos, morula and blastocyst	Pre-morula stage, goat mtDNA 2.0; 0.012 at blastocyst stage.	Real-time PCR	Ma et al. (2008a, b)
Ovine fetal fibroblast	Bovine	Individual blastocysts and blastomeres	Ovine mtDNA constant at 1% from 1- to 8-cell stage, 0.6% at 16-cell stage and 0.1% at blastocyst stage.	Real-time PCR	Hua et al. (2008)
Postmortem wolf somatic cells (<i>Canis lupus</i>)	Dog	Six cloned wolf pups	Homoplasmic for recipient oocyte mtDNA	Species specific PCR on agarose gel electrophoresis and DNA sequencing	Oh et al. (2008)
Cattle (<i>B. taurus</i>)	Rabbit (<i>Oryctolagus cuniculus</i>)	1-, 2-, 4-/8-cell, morula and blastocyst embryos from: (i) heteroplasmic SCNT animal donor; and (ii) a homoplasmic nuclear donor	Heteroplasmy detected at all stages of preimplantation. Rabbit mtDNA remained constant to the morula stage and increased sharply in both types of blastocyst. Bovine mtDNA remained constant in SCNT donor cell group during preimplantation development but decreased sharply in the homoplasmic donor group post-morula	Species specific real-time PCR	Jiang et al. (2006)
Ibex (<i>C. ibex</i>)	Rabbit (<i>O. cuniculus</i>)	2-, 4-, 8- and 16-cell morula and blastocyst embryos	Heteroplasmy detected at all stages; rabbit mtDNA increased from the 16-cell stage. Ibex mtDNA decreased after 16-cell stage	Species specific PCR on agarose gel electrophoresis and DNA sequencing	Jiang et al. (2005)
Human granulosa and fibroblast cells	Bovine	Preimplantation stages embryos	Most embryos heteroplasmic to blastocyst stage	Species specific PCR and DNA sequencing	Illmensee et al. (2006)
Takin (<i>Budorcas taxicolor</i>)	Yak (<i>Bos grunniens</i>)	2 blastocysts	Heteroplasmy in both blastocysts	Species specific PCR on agarose gel electrophoresis and DNA sequencing	Li et al. (2006)
Macaque (<i>Macaca multatta</i>)	Rabbit (<i>O. cuniculus</i>)	1-, 2-, 4-, 8- and 16-cell, morula, blastocyst	Macaque mtDNA: 2.0% (1-cell), 1.3% (2-cell), 1.7% (4-cell), 0.6% (8-cell), 0.8% (16-cell), 1.3% (morula) and 0.011% blastocyst	Species-specific real-time PCR	Yang et al. (2004)
Red common carp	Goldfish	Blastula, gastrula, somite, muscular reaction, blood circulation, larval and adult stages	Blastulae all heteroplasmic. In late stage embryos post-vascularization only recipient oocyte mtDNA detected	Species specific PCR on agarose gel electrophoresis with image analysis	Sun et al. (2005)
Human (<i>Homo sapiens</i>)	Bovine (<i>B. taurus</i>)	2-, 4-, 8- and 16-cell, morula and blastocysts	Heteroplasmic to 16-cell stage. Homoplasmic post-morula stage	Species specific PCR on agarose gel with image analysis, DNA sequencing	Chang et al. (2003)
Human (<i>H. sapiens</i>)	Rabbit (<i>O. cuniculus</i>)	Blastocysts	Heteroplasmic	<i>In situ</i> hybridization mtDNA interspecies PCR	Chen et al. (2003)
Macaque (<i>M. multatta</i>)	Rabbit (<i>O. cuniculus</i>)	1-, 2-, 4-, 8- and 16-cell, morula and blastocyst	Heteroplasmy detected at all stages.	Species specific PCR on agarose gel, DNA sequencing	Yang et al. (2003)
Giant panda (<i>Ailuropoda melanoleuca</i>)	Rabbit (<i>O. cuniculus</i>)	Blastocysts and 2 fetuses	Heteroplasmy detected in blastocysts. Both fetuses homoplasmic for donor cell	Species specific PCR on agarose gel, sequencing of PCR product	Chen et al. (2002)
Gaur (<i>Bos gaurus</i>)	Cattle (<i>B. taurus</i>)	3 fetuses	No heteroplasmy in brain eye, gonad, hear, intestine, kidney, liver, lung, muscle skin and tongue	PCR-PFLP and phosphorimager	Lanza et al. (2000)

development with mtDNA replication first taking place as the expanded blastocyst forms (May-Panloup *et al.*, 2005; Spikings *et al.*, 2007). Indeed, this outcome may merely reflect a difference between domestic and non-domestic mammals.

Is there an association between the genetic distance of donor cell mtDNA and recipient oocyte mtDNA?

A small-scale study comparing the effects of haplotype between two breeds of cattle demonstrated that one mtDNA lineage produced a greater number of blastocysts following SCNT (Bruggerhoff *et al.*, 2002) and the other showed improved developmental competence following IVF (Tamassia *et al.*, 2004). In SCNT-bovine embryos, divergence up to 0.0787% is tolerable for the generation of live offspring (Bowles *et al.*, 2008). In SCNT-ovine embryos, divergence up to 0.0391% appears to be tolerable for development to blastocyst, with the propagation of *Ovis aries* using *Ovis musimon* oocytes providing the limits for the generation of live offspring (Loi *et al.*, 1998), being ~2 million years apart. However, a 4-fold increase (mean = 0.4114%) in genetic distance, i.e. by generating caprine-ovine interspecific embryos, resulted in the failure of development to blastocyst (Bowles *et al.*, 2007b).

A model approach using handmade cloning and phylogenetic analysis has gone some way to clarifying the relationship between donor cell and recipient oocyte mtDNA (Bowles *et al.*, 2008). Handmade clones can be generated by fusing a donor cell to one or more enucleated oocytes of varying genetic distance to determine whether the donor cell will preferentially replicate a population of mtDNA that is closer or genetically more divergent. The outcomes of these studies demonstrated that, within limits of genetic distance, the donor cell favours a genetically more diverse mtDNA haplotype to that of its own with the most divergent genetic distance being at 0.0787%. Fusion with very close genetic partners appears to hinder development.

What can be learnt from other cellular models?

Cytoplasmic hybrids, commonly known as cybrids, have been used to study nuclear–cytoplasmic interactions in a variety of species. This technology requires either the karyoplast to be completely or partially depleted of its mtDNA and it is then fused to an enucleated cytoplasm (King and Attardi, 1989). The resultant fusions can then be used to understand either the proliferation of mtDNA mutations for specific cells in the disease state or the interactions between a nucleus from one species with a population of mtDNA from another. The latter approach provides a significant amount of knowledge for those wishing to generate iSCNT-derived embryos and ESCs. For example, the generation of mouse–rat cybrids, where rat mtDNA was efficiently replicated, transcribed and translated by the mouse nuclear transcription and replication factors, had decreased ATP output and oxygen consumption, and increased levels of cellular lactate and reduced activity in complexes I, III and IV of the ETC compared with the parental cell lines and mouse–mouse cybrids (Barrientos *et al.*, 1998; Dey *et al.*, 2000; McKenzie and Trounce, 2000). Similarly, in murine–murine cybrids where the mtDNA mutation was homoplasmic, there was compromised OXPHOS and COX activity with also decreased levels of mitochondrial translation (McKenzie and Trounce, 2000). Prolonged culture of

one cybrid resulted in a shift from 30% to 80% mutant, which then remained stable throughout culture, perhaps similar to the transmission of mtDNA following interspecies NT, where the accumulation of one population of mtDNA may outnumber the other dependent perhaps on the nuclear background of the cell. Similar outcomes have been observed as primate mtDNA can only be replicated by a human nucleus when the human mtDNA has been eliminated (Barrientos *et al.*, 1998). In this instance, the activities of complexes II–V were unaltered, but there was a deficiency in complex I activity.

Mitochondrial DNA replication and transmission following induced pluripotency

Induced pluripotent cells (iPSCs) are somatic cells that have been induced to become ESC-like due to the introduction of defined factors (Takahashi and Yamanaka, 2006). They then express key pluripotent markers such as *Oct4*, *Nanog* and *Sox-2* and have the ability to differentiate into multiple cell types. It however remains to be determined whether such cells have the ability to regulate their mtDNA copy number in their undifferentiated state as ESCs do. It further needs to be demonstrated whether these cells can effectively regulate their patterns of mtDNA replication as the process of differentiation takes place in ESCs (Facucho-Oliveira *et al.*, 2007). It is unlikely that those cells generated through NT would be able to do so, due to the up-regulation of the mtDNA replication factors during early embryonic development (Bowles *et al.*, 2007b). However, the iPSCs should, in line with pluripotent cells (Facucho-Oliveira *et al.*, 2007; Facucho-Oliveira and St John, 2009), be able to demonstrate low levels of mtDNA replication to maintain existing populations. Attempts at reprogramming cells using cell fusion (Sumer *et al.*, 2009), where an adult cell is fused to an ESC, may cause several problems in terms of the regulation of mtDNA. These may include heteroplasmy, because of two different sources of cells being used to generate the newly fused reprogrammed cell, and the failure to adopt appropriate numbers of mtDNA copies. As reprogrammed cells have been proposed as models that can be used for the study of specific diseases and to screen drugs for their efficacy (Bowles *et al.*, 2007a), they need to be carefully investigated to determine their appropriateness for such analyses. This is because such experimental protocols may contain two experimental variables, namely increased numbers of mtDNA copy and/or heteroplasmy, which may result in irregular ATP production and the genetic rearrangement associated with the particular disease. Consequently, it would not be clear from the experimental outcome whether the cellular dysfunction arose through the genetic rearrangement or the cell's inability to generate sufficient levels of ATP through OXPHOS. Equally so, the inability of the drug to induce, e.g. appropriate calcium oscillations in neuronal or cardiac cells, might also be due to impaired OXPHOS function. However, as such cells offer considerable hope as clinical entities, then it is worth investigating whether it is feasible to simply regulate the mtDNA content of these cells so that they are suitable for therapeutic and experimental use.

mtDNA segregation

The segregation of mtDNA molecules tends to follow a pattern of random genetic drift (reviewed in Shoubridge and Wai, 2007).

Consequently, it is not possible to predict which cell types or tissues mutant molecules will migrate to during early development. This is the case for both somatic and germ cells and specifically relevant to female primordial germ cells (PGCs), which, when harbouring mutant molecules, would thus ensure their continued transmission to subsequent generations. PGCs originate from the extra-embryonic endoderm and migrate to the germinal ridge (Surani et al., 2007). At this stage, they contain ~200 copies of mtDNA and maintain relatively fewer numbers during the early stages of oogenesis (Smith and Alcivar, 1993; Cao et al., 2007; Cree et al., 2008; Fig. 4). As PGCs differentiate and progress from meiosis I to II, mtDNA is clonally expanded (Smith and Alcivar, 1993). Consequently, those mature mouse oocytes that are competent for the process of fertilization or oocyte reconstruction will have between 180 000 and 200 000 mtDNA copies (Cao et al., 2007; Cree et al., 2008; Wai et al., 2008) with 1–2 copies/mitochondrion (Piko and Matsumoto, 1976; Piko and Taylor, 1987), whereas bovine (May-Panloup et al., 2005), porcine (Spikings et al., 2007) and human (Steuerwald et al., 2000; Reynier et al., 2001; Almeida-Santos et al., 2006) oocytes have between 100 000 and 700 000 copies (Fig. 4). Consequently, the combination of random genetic drift and clonal expansion of mtDNA would explain the large variation in mutant molecules found within the cohorts of oocytes from those women who are potential carriers of mtDNA mutations. For example, mtDNA mutation:wild type for a specific mitochondrial disorder, MELAS, ranged from 0% to 97% (Blok et al., 1997).

Following fertilization of the oocyte, mtDNA copy number remains constant during mouse preimplantation development up to the morula stage but is reduced in porcine (Spikings et al., 2007) and bovine (May-Panloup et al., 2005) embryos. mtDNA replication is then initiated at the blastocyst stage in those mammalian species so far investigated (May-Panloup et al., 2005; Thundathil et al., 2005; Spikings et al., 2007). However, this replication is specific to the trophectodermal cells, the cells that give rise to the placenta, whereas those cells giving rise to the embryo or ESCs, the ICM cells, have very few mitochondria present and thus copies of the mtDNA genome (Spikings et al., 2007). The ICM cells are likely to continue to reduce their mtDNA copy number as they continue to divide before they undergo gastrulation, as evidenced by human and mouse ESCs having as few as 10–20 mitochondria (St John et al., 2005a, b) containing between 30 and 45 copies of the mitochondrial genome (Facucho-Oliveira et al., 2007). This selective reduction in mtDNA copies in those cells giving rise to the fetus ensures that very few copies are available for clonal amplification in gametes and other tissues and that the maternal homoplasmic transmission of a single type of molecule is transmitted to the next generation. This process, otherwise known as the mitochondrial bottleneck (Hauswirth and Laipis, 1982), also ensures that mutated molecules are either eliminated or fixed in the female germline according to the severity of the mutation within a generation so that either quality mtDNA is transmitted or the mutation is lethal and the oocytes do not mature (Fan et al., 2008; Stewart et al., 2008). However, as the female PGCs, which constitute the vehicle for the recycling of mtDNA from one generation to the next, possess ~200 copies of mtDNA, this represents a potential small increase in mtDNA copy and suggests that the bottleneck may have occurred prior to the establishment of the PGCs.

The variable transmission of mtDNA has questioned the feasibility of using preimplantation genetic diagnosis (PGD) on embryos generated following either *in vitro* fertilization or intra-cytoplasmic sperm injection protocols to determine whether individual preimplantation blastomeres contain equal loading for mutant molecules. Interestingly, although mutant load can be different in various mature cell types (reviewed in McFarland et al., 2007), there appears to be uniformity in individual blastomeres (Dean et al., 2003) suggesting that segregation is a post-gastrulation event. Although this is a reassuring outcome, it suggests that PGD is only useful as a predictor that mature cells might have high mutant mtDNA loading when a critical threshold of mutant loading is reached in preimplantation embryos. For those preimplantation embryos with low levels of mutation, it can inform patients that their offspring would most unlikely be affected (Poulten et al., 2009). However, the overriding question is: how sure can we be?

As mtDNA replication in fetal cells is initiated post-gastrulation and from a few molecules present in uncommitted cells, it allows individual cell types to be populated with the appropriate numbers of mtDNA copies to match their requirements for OXPHOS-derived ATP (Moyes et al., 1998). Such examples would be neuronal, muscle and cardiac-type cells where neuronal cells would have between 8 and 10 copies/mitochondrion (Sato and Kuroiwa, 1991). It is likely that there is a refinement of mtDNA copy at various stages of development and these are likely to be linked to the expression of certain genes during the various different stages of development (Facucho-Oliveira et al., 2007; Facucho-Oliveira and St John, 2009). Early phases of replication are likely to be associated with replenishment of a genome and also to determine whether the replication machinery is capable of sustaining phasic changes, thus ensuring that, at various stages of differentiation, the cell is capable of adaptation. Consequently, PGD may not reflect later stage outcomes. This is further likely as it appears that mtDNA rearrangements and wild-type molecules are able to intermix so that they do not remain as separate entities within distinct organelles or cells (Nakada et al., 2001).

Does heteroplasmy following reproductive technologies have implications for embryo, fetal and offspring survival and wellbeing?

Currently, there are no effective intervention strategies available to prevent the transmission of mtDNA disease from mother to offspring. Experimental approaches concentrate on determining whether germinal vesicle or pro-NT might be appropriate approaches. In this case, the germinal vesicle or pronuclei from the affected oocyte would be transferred into an enucleated egg that was not carrying the mutant population (Meirelles and Smith, 1998). The degree of donor cell transmission that arises or transfer of donor mitochondria following cytoplasmic transfer when at its higher levels could mimic conditions associated with mitochondrial disease. Studies, for example, using mouse strains with variants in the coding sequences between the two strains would be similar to clinical-onset heteroplasmy and would produce similar outcomes to those observed following SCNT when different breeds of cattle and pig contributed by a donor cell and a recipient oocyte. As previously demonstrated, some replication of such mtDNA can take place during preimplantation development (Meirelles and Smith, 1998) and persist at high levels in a few offspring

(Sato *et al.*, 2005). Consequently, it is worth questioning whether the degree of mtDNA heteroplasmy in cloned offspring would indeed account for some of the phenotypic diseases observed from NT. Although there is no direct evidence currently to support this, there is substantive evidence that is associated with those malformations in tissues from fetuses and offspring following SCNT (Cibelli *et al.*, 2002). A recent study has also demonstrated that donor ooplasm transfer can affect growth in early life with interspecific germinal vesicle transfer causing growth abnormalities post-natally (Cheng *et al.*, 2009). Such tissues that might be affected are lung, heart and liver (Bowles *et al.*, 2008), which are high ATP users and would therefore be more likely to be indicative of failure to produce sufficient compatible genomes for the ETC. This would be further exaggerated following iSCNT where the nuclear and mtDNA-encoded subunits of the ETC would show varying degrees of incompatibility.

As the transmission of donor cell mtDNA in cloned offspring could also be explained by random genetic drift, as these molecules tend to segregate in a non-uniform manner, the up-regulation of mtDNA replication factors during preimplantation development (Bowles *et al.*, 2007a, b) and their preferential selection for one molecule over another (Bowles *et al.*, 2008) will influence the number of molecules present as segregation takes place. Consequently, any assisted reproductive technology that uses an oocyte cytoplasm to reprogramme the donor cell and allows mixing of mtDNA populations will affect any PGD outcomes as the population present in the preimplantation embryo will continue to be replicated unlike its fertilized counterpart and would be only predictive of that stage of development. This outcome may be equally valid for the transfer of a germinal vesicle or a pronuclei which will bring some accompanying mtDNA as part of the transfer. Consequently, entire elimination of donor cell mtDNA would be perhaps an appropriate safeguard for such approaches (Bowles *et al.*, 2007a, b). A recent attempt in *M. mulatta* has suggested that this might be achieved (Tachibana *et al.*, 2009) but these results must be viewed with considerable caution as it still needs to be verified whether the transferred spindle has accompanying mtDNA and whether this was transmitted to various tissues of the offspring.

Conclusions

mtDNA transmission and replication from the gamete through the preimplantation embryo into the fetus and into the offspring is a highly regulated process where mtDNA replication is initiated at precise stages throughout development. The task for scientists at the moment is to elicit when these key stages are and how many of these key stages exist. Once we have a clearer understanding of these, it may be possible to regulate mtDNA transmission in order to prevent the onset of severe mitochondrial diseases which affects the quality of life of ~1:1 000 in northeast England (Schaefer *et al.*, 2008) and 1:6000 for A3243G mutation in Finland (Majamma *et al.*, 1998; McFarland *et al.*, 2007). We have approaches that may be able to regulate mtDNA transmission in the new offspring through assisted reproductive technologies. The task for scientists now is to develop approaches using stem cells that will allow dysfunctional tissues affected by mtDNA to be replaced with non-heteroplasmic populations which will alleviate the severe phenotype on the offspring.

Funding

We acknowledge the Medical Research Council and the British Heart Foundation for their generous support.

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