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Role of mitochondrial DNA replication during differentiation of reprogrammed stem cells

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ABSTRACT Mitochondrial DNA (mtDNA) is a 16.6 kb genome that encodes for 13 of the 100+ subunits of the electron transfer chain (ETC), whilst the other subunits are encoded by chromosomal DNA. The ETC is responsible for the generation of the majority of cellular ATP through the process of oxidative phosphorylation (OXPHOS). mtDNA is normally inherited from the population present in the mature oocyte just prior to fertilisation. However, following somatic cell nuclear transfer (SCNT), mtDNA can be transmitted from both the donor cell and the recipient oocyte. This heteroplasmic transmission of mtDNA is a random event and does not appear to be related to the amount of mtDNA contributed by the donor cell. The distribution of mtDNA is randomly segregated between blastomeres and differentiating tissues, and therefore the mtDNA complement transmitted to offspring tissue cannot be predicted. mtDNA divergence between the cytoplast and the donor cell in intra- and inter-specific crosses favours a slightly more diverse mtDNA haplotype. However, this is limited as interspecies SCNT (iSCNT) genetic divergence contributes to developmental failure. SCNT embryos demonstrate a plethora of aberrantly reprogrammed characteristics including the uncoordinated regulation of the mtDNA replication factors. This results in increased mtDNA copy number during preimplantation development and propagates the replication of donor cell mtDNA. These failures are likely to be a consequence of incompatible nuclear- and mtDNA -encoded proteins interacting within the ETC thus reducing ATP production. The outcomes would be similar to the severely debilitating or even fatal mtDNA diseases associated with genetic rearrangements to mtDNA or mtDNA depletion type syndromes and have serious implications for any form of karyoplast transfer approach. The only method to overcome the problems of heteroplasmy in SCNT embryos is to completely deplete the donor cell of its mtDNA prior to SCNT.

KEY WORDS: *mitochondria, mtDNA, somatic cell nuclear transfer, oxidative phosphorylation*

Introduction

Somatic cell nuclear transfer (SCNT) offers a technological approach to studying the biochemical and molecular dynamics of development as well as offering the propagation of desirable genetic traits (Bowles *et al.* 2007a), preservation of endangered species (Loi *et al.* 2001) and the derivation of patient specific stem cells through intra- and interspecies SCNT (iSCNT; (St John and Lovell-Badge 2007; St John *et al.* 2008). The development rates for these SCNT embryos is extremely poor and many factors have been attributed to developmental failures, most notably nuclear reprogramming. Furthermore, the amalgamation of genetically divergent mitochondria from different breeds or species may have deleterious implications and deserves extensive experimentation considering the dynamic functions of mitochondria during development as well as the implicated technological and therapeutic applications of SCNT. To this extent, defective interactions between the donor chromosomally-encoded mitochondrial DNA (mtDNA) transcriptional and replication factors with recipient oocyte mtDNA would result in compromised expression of mitochondrial proteins and propagation of mitochondrial genes. This,

Abbreviations used in this paper: ETC, electron transfer chain; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; PGC, primordial germ cell; POLGA, polymerase gamma; SCNT, somatic cell nuclear transfer.

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in turn, would compromise the given cellular demand for ATP generated through oxidative phosphorylation (OXPHOS) thus potentially triggering embryonic arrest or subsequent disease phenotypes (Wallace 2005; Wallace *et al.* 2010). Ineffective interactions of the chromosomally- and mtDNA-encoded complexes of the electron transport chain (ETC) could also result in deleterious phenotypes pre- and post-term. Indeed, SCNT embryos (Alexopoulos *et al.* 2008), foetuses (Hiendleder *et al.* 2003; Burgstaller *et al.* 2007) and offspring (Steinborn *et al.* 1998; Hiendleder *et al.* 1999; Meirelles *et al.* 2001; Takeda *et al.* 2003) can exhibit different mitochondrial profiles, which has been suggested to be a common cause of premature death resulting from reproductive cloning (St John 2002). The aim of this review is to describe and discuss these outcomes.

Mitochondria

Along with energy production, mitochondria are involved in the regulation of steroidogenesis (Bose *et al.* 2002), reactive oxygen species (ROS; (Nemoto *et al.* 2000), nitric oxide (Dedkova *et al.* 2004), calcium signalling (Brini 2003; Dumollard *et al.* 2006); and apoptosis (Joza *et al.* 2001). The eukaryotic mitochondrion most likely originated from α-protobacterium, an event that took place approximately 1 to 2 billion years ago. Over hundreds of millions of years, an endo-symbiotic relationship evolved, whereby key proto-mitochondrial genes, located on a circular genome within the mitochondria, were transferred to the eukaryotic chromosomal genome (Gray *et al.* 1999). As a result, the mammalian chromosomal genome contains many of the genes encoding for each of the complexes of the electron transfer chain (ETC) along with nearly all the necessary proteins for the transcription and replication of mtDNA, along with numerous factors involved in protein translation of the mitochondrial genes (Lang *et al.* 1999; Andersson *et al.* 2003).

Mitochondrial DNA

The double stranded mammalian mtDNA is between 16.2 and 16.7kb in size (see Fig. 1) and encodes 13 of the 100+ subunits of the electron transfer chain (ETC; See Fig. 2; Anderson *et al.* 1981; Bibb *et al.* 1981; Anderson *et al.* 1982; Ursing and Arnason 1998) 2 rRNAs and 22 tRNAs that flank most of the coding genes. The ETC is an intra-mitochondrial apparatus that generates the vast majority of cellular ATP through the process of OXPHOS (Wallace *et al.* 2010). OXPHOS, an aerobic mechanism, is by far the most efficient process for generating ATP as it produces 32 molecules to every 2 generated through glycolysis, an anaerobic form of metabolism (Brown 1992; Pfeiffer *et al.* 2001). Consequently, OXPHOS is essential for aerobic cells, such as neurons, cardiomyocytes and oxidative skeletal muscle, which have high energy demands, to mediate their complex cellular functions, such as maintaining heartbeat through pacemaker cells or initiating and driving action potentials (Erecinska and Silver 1989; Wong-Riley 1989; Heineman and Balaban 1990). The importance of these genes in maintaining vital cellular functions is demonstrated by mutations and deletions, which can result in severe cellular impairment or can be lethal (Wallace 1999).

mtDNA replication

Whilst the mitochondrial genome is not interspersed with introns and exons as for the chromosomal genome, it has one non-coding region, the displacement or D-Loop (see Fig. 1). This is the site of interaction of several nuclear-encoded mtDNA-specific transcription and replication factors that mediate mtDNA replication (Anderson *et al.* 1981; Anderson *et al.* 1982; Shadel and Clayton 1996; Shadel and Clayton 1997). Mitochondrial transcription factor A (TFAM) binds to the enhancer of the Light Strand Promoter within the D-Loop to induce structural changes that expose the promoter region to the mitochondrial-specific RNA polymerase (Falkenberg *et al.* 2007). This allows an RNA-DNA hybrid primer to be generated, which is then used by the mitochondrial specific DNA Polymerase Gamma (POLGA) to initiate replication (Fisher and Clayton 1985; Xu and Clayton 1992). POLGA is supported by its accessory subunit, POLGB, the mitochondrial single-stranded DNA-binding protein (mtSSB) and the helicase, Twinkle (Korhonen *et al.* 2003). Decreased expression and mutation to the mtDNA replication factors result in fewer genomes being available for transcription and mediate the onset of mtDNA depletion syndromes (Copeland 2008).

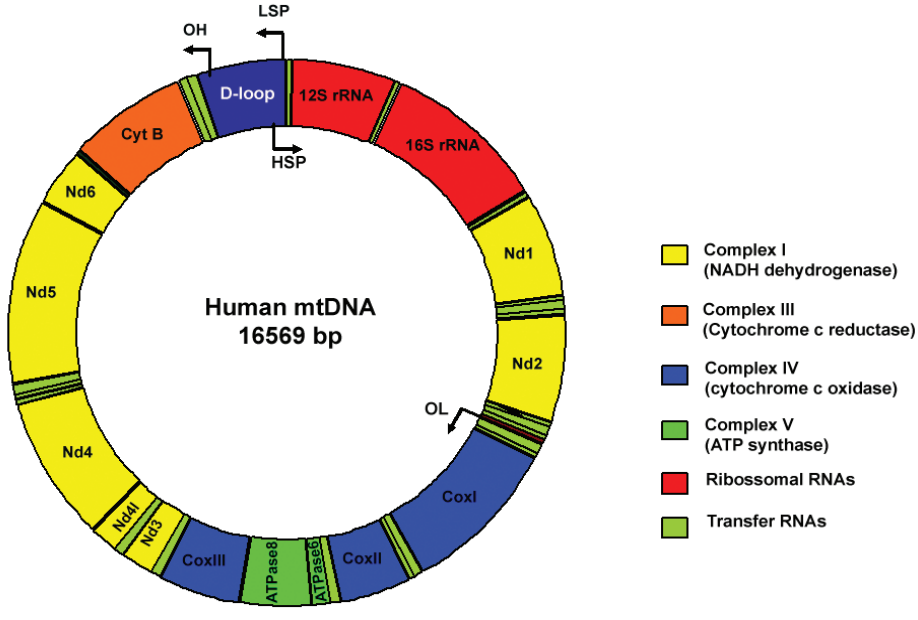

Fig. 1. The mammalian mtDNA genome encodes 13 of the subunits of the electron transfer chain (ETC). *For Complex I, these consists of ND 1, 2, 3, 4, 4L, 5 and 6); Complex III (CytB); Complex IV (COX I, II and III); and Complex V (ATPase6 and ATPase8). mtDNA also encodes two rRNAs (12S and 16S rRNAs) and 22 tRNAs. The D-loop is the main control region, which houses the H-strand promoter region (HSP), the L-strand promoter region (LSP), and the origin of H-strand replication (O_H). A second control region, consisting of only 30 bp, is located between ND2 and COXI and is the site of the origin of L-strand replication (O_L).*

Two methods have been described to explain mtDNA replication. The assymetric model proposes that replication is initiated from the origin of H-strand replication, located within the D-Loop region, and continues two-thirds round the genome to the origin of L-strand replication (Shadel and Clayton 1997). L-strand synthesis then proceeds in the opposite direction. The coupled leading- and lagging-strand synthesis model suggests that both H and L strands are replicated bidirectionally from the same initiation cluster site (Yasukawa *et al.* 2005; Yasukawa *et al.* 2006). This mechanism is proposed to occur in addition to the asymmetric model, but is typical of cells repopulating mtDNA.

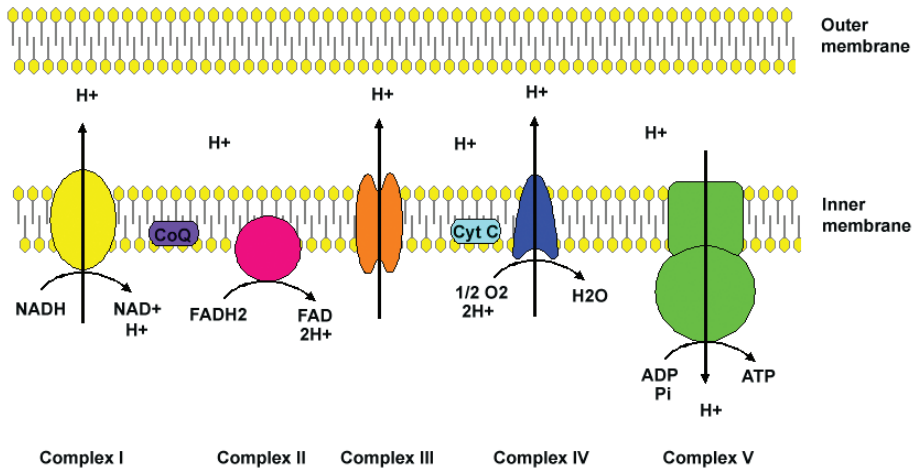

Fig. 2. The electron transfer chain (ETC). *The ETC is the major generator of cellular ATP through OXPHOS. Electrons are derived from NADH and $FADH_2$ and transferred into Complexes I and II, respectively. Following transfer into coenzyme Q (Co Q), electrons are transferred to Complex III. In turn, Cytochrome c (Cyt C) accepts electrons and then donates them to Complex IV. The energy released as electrons is used by Complexes I, III and IV to generate an electrochemical gradient by pumping protons across the mitochondrial inner membrane. Complex V utilises this membrane potential to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate.*

Maternal inheritance of mtDNA

Mitochondrial DNA (mtDNA) is normally inherited unimaternally with all copies being identical (homoplasmy; Birky, 1995; Birky, 2001). This population originates from approximately 200 copies present in each primordial germ cell (PGC), which are laid just after gastrulation (Shoubridge 2000; Shoubridge and Wai 2007). Although this tends to be true for intra-specific crosses, sperm mtDNA can be transmitted through inter-specific crosses generated with gametes from different breeds or strains (Lansman *et al.* 1983; Gyllensten *et al.* 1991). In intra-specific crosses, sperm mitochondria are selectively ubiquitinated within the fertilized oocyte just prior to the onset of embryonic genome activation (EGA) demonstrating that this is an oocyte rather than embryo mediated event. This prevents the potential transmission of sperm mtDNA during subsequent development (Sutovsky *et al.* 1999; Sutovsky *et al.* 2000). This ubiquitinated-targetted process does not regulate sperm mtDNA transmission in interspecific crosses resulting in variable transmission to offspring. However, sperm mtDNA does not appear to be fixed in the germ line, as subsequent generations do not inherit this paternal source of mtDNA.

mtDNA and developmental competence

The mitochondria of the mature metaphase II (MII) oocyte contain only a single mtDNA copy (Satoh and Kuroiwa 1991), but have significantly more mitochondria present meaning a total mtDNA content that is at least a 10-fold higher than that of somatic cells. Thus, mtDNA potentially contributes to ~50% of the total DNA present in the oocyte at fertilization (Reynier *et al.* 2001). The number of mtDNA copies present in mature mammalian oocytes is on average greater than $1x10^5$ copies although mtDNA copy number varies dramatically between oocytes and between mammalian species. The mechanisms of these variations and the purpose of high copy numbers at fertilization have been matters of great debate recently.

It has been demonstrated that oocytes with higher copy numbers of mtDNA are thought to be associated with higher fertilisation rates. Glucose-6-phosphate dehydrogenase (G6PD) is a metabolic enzyme involved in the pentose phosphate pathway, and is essential for cellular growth (Tian *et al.* 1998). G6PD breaks down Brilliant cresyl blue (BCB) dye, which has been applied in the determination of oocyte developmental competence (Rodriguez-Gonzalez *et al.* 2002; El Shourbagy *et al.* 2006). Fully matured oocytes have decreased G6PD activity and stain blue (BCB^+), suggesting developmental competence. Increased G6PD activity reduces BCB to a colourless compound (BCB^-) and has been associated with reduced fertilisation rates (Rodriguez-Gonzalez *et al.* 2002). In the pig, competent BCB^+ oocytes contain more copies of mtDNA, and are more likely to fertilize than incompetent BCB^- oocytes (El Shourbagy *et al.* 2006). In addition, supplementation of BCB^- oocytes with purified populations of mitochondria from BCB^+ oocytes improved subsequent developmental competence post-fertilization (El Shourbagy *et al.* 2006). Indeed, human (Reynier *et al.* 2001; Santos *et al.* 2006) and bovine (May-Panloup *et al.* 2005a; Hua *et al.* 2007) oocytes with higher copy numbers are associated with increased *in vitro* developmental rates. Previously, a threshold of 100,000 mtDNA copies was hypothesised for developmental competence (Piko and Taylor 1987; Reynier *et al.* 2001). However, a recent report by Wai *et al.* (2010) demonstrated through heterozygous *Tfam* knockout mice that oocytes with as few as 4,000 mtDNA copies could be fertilized and develop to the blastocyst stage, although a minimum of 50,000 copies in mature oocytes was a critical threshold for subsequent post-implantation development (Wai *et al.* 2010). Nevertheless, *Tfam* heterozygous offspring display respiratory chain deficiency in the heart resulting in a cardiomyopathy phenotype (Larsson *et al.* 1998; Wredenberg *et al.* 2002). Their homozygous counterparts die prior to embryonic day (E)10.5, which clearly suggests that high numbers of mtDNA copy in the MII oocyte represent an investment in the long term survival of the subsequent offspring (Larsson *et al.* 1998). Furthermore, the considerable increase in mtDNA copy number as oocytes mature to MII indicates that an increase in mitochondria is required to provide sufficient energy

resource to support the multiple intracellular events that take place as fertilisation is initiated and ensues.

MtDNA present in MII occytes is believed to support embryo development to cavitation, after which glycolysis becomes increasingly important in many species (Leese and Lenton 1990; Brison and Leese 1991; Leese *et al.* 1993; Brison *et al.* 1993; Houghton *et al.* 1996; Thompson *et al.* 1996; Sturmey and Leese 2003; Houghton 2006). Furthermore, the 100,000 minimum copy number may be essential as it has been hypothesised that a threshold level of ATP is required which will synchronise mitochondrial events that support cell division (van Blerkom, 2000; van Blerkom *et al.* 2000). To this extent, following fertilisation, the totipotent zygote undergoes numerous cell divisions to form the pluripotent blastocyst, randomly segregating the maternal inherited genome between blastomeres with a progressive reduction in mitochondrial content per blastomere. This pattern is observed in all mammalian species so far studied until mtDNA replication is initiated at the blastocyst stage (Shoubridge 2000; Thundathil *et al.* 2005; May-Panloup *et al.* 2005b; Spikings *et al.* 2007). This replication event is most probably restricted to the trophectoderm cells whilst the inner cell mass (ICM) cells have very few mtDNA copies per cell (Cao *et al.* 2007) and exhibit little expression for POLGA (Spikings *et al.* 2007; Bowles *et al.* 2007b). Indeed, during the early cleavage stages, mammalian embryos express very low levels of or no mtDNA replication factor activity (Thundathil *et al.* 2005; May-Panloup *et al.* 2005b; Spikings *et al.* 2007; Bowles *et al.* 2007b). A small replication event has been described between the pronuclear and the 2-cell stage in mouse embryos, indicating replenishment rather than an increase in mtDNA copies as there was no significant change in mtDNA copy number (McConnell and Petrie 2004). Similar outcomes have been identified in porcine (Spikings *et al.* 2007) and ovine (Bowles *et al.* 2007b) embryos. Interestingly, mouse preimplantation development up to the morula stage is characterised by consistent levels of mtDNA copy number (Thundathil *et al.* 2005). However, porcine (Spikings *et al.* 2007) and bovine embryos (May-Panloup *et al.* 2005b) demonstrate a reduction in total embryo mtDNA copy number, suggesting a programmed elimination of mtDNA and/or mitochondria.

Some mtDNA and mitochondria during development will be lost through the occurrence of apoptosis in individual blastomeres. Apoptosis in embryonic cells is a potential indicator of a cellular response to suboptimal conditions, although chromosomal abnormalities, imbalance of growth factors, reactive oxygen species (ROS) and other damaging factors cause cell death (reviewed in Fabian *et al.* 2005). Apoptosis is normally first observed post-compaction in human embryos (Hardy 1999), at the blastocyst stage in mouse (Kamjoo *et al.* 2002) and porcine embryos (Hao *et al.* 2003; Hao *et al.* 2004), whilst in bovine embryos the first occurrence is at the 8-16 cell stage (Matwee *et al.* 2000; Gjorret *et al.* 2007). The apoptotic process can be triggered by staurosporine treatment in murine (Weil *et al.* 1996) and bovine (Matwee *et al.* 2000) embryos during early cleavage stages prior to EGA. In SCNT and iSCNT embryos, the incidence of apoptosis is increased compared to their *in vitro* fertilised and *in vivo* counterparts (Maddox-Hyttell *et al.* 2003), which could be a result of the heightened sensitivity of SCNT embryos to suboptimal culture conditions (reviewed in Campbell *et al.* 2007) along with the mixing of somatic and oocyte mitochondria within the oocyte's cytoplasm. Therefore, programmed cell death (PCD) events during development have the potential to eliminate blastomeres at varying stages, which could potentially result in reduced total embryo mtDNA copy number. However, this is unlikely to be the active mechanism for reducing mtDNA copy number in bovine and porcine embryos as the apoptotic events occur post-16 cell stage at which point copy number has already significantly decreased. As discussed earlier, the embryo does have the capacity to target mitochondria for destruction as evidenced by sperm-mitochondrial specific elimination. The programmed elimination of mtDNA during preimplantation, either recipient or donor in origin, is a mechanism which has yet to be described.

Mixing of somatic and oocyte mitochondria: implications for development

A mitochondrial morphological-functional relationship in preimplantation embryos has yet to be fully explored although an elongated phenotype and developed transverse cristae are considered to be characteristic of fully functional mature mitochondria capable of producing ATP through the oxidation of various substrates (Mannella 2006). Ultrastructural analysis of mammalian oocytes and embryos has identified numerous mitochondrial morphologies with some differences observed between species. However, mammalian oocytes generally contain spherical immature mitochondria, which are characterised by electron dense matrices with few or no cristae (Hillman and Tasca 1969; Hillman and Hillman 1975; King *et al.* 1996). As development progresses mitochondrial differentiation is observed during progressive cleavages to an elongated shape, with reduced electron density and transverse cristae observed at the blastocyst stage (Van *et al.* 1990; Plante and King 1994; King *et al.* 1996; Crosier *et al.* 2000; Crosier *et al.* 2001; Tao *et al.* 2008). These structural changes are associated with increased metabolic activity, oxygen consumption and CO_2 production observed in the blastocyst (Thompson *et al.* 1996; Barnett and Bavister 1996).

Nuclear transfer embryos display numerous ultrastructural changes compared to *in vitro* produced (IVP) and *in vivo* embryos (King *et al.* 1996). Nuclear transfer embryos produced using embryonic (King *et al.* 1996), foetal (Zhong *et al.* 2007; Zhong *et al.* 2008) or adult (Tao *et al.* 2008; Han *et al.* 2008) donor cells demonstrate heterogenous morphologies not seen in IVP or *in vivo* embryos that persist for several cleavages. The dynamic changes of mitochondrial structure, typical of preimplantation development, is maintained in nuclear transfer embryos. In human (Motta *et al.* 2000), pig (Katayama *et al.* 2006), primate (Squirrell *et al.* 2003) and hamster IVF embryos (Bavister and Squirrell 2000), perinuclear aggregation of mitochondrial is observed during early cleavage stages and this may be associated with developmental competence (Au *et al.* 2005). This cellular ability to translocate to perinuclear regions is reduced in porcine SCNT embryos due to the absence of factors derived from the sperm and oocyte spindles (Katayama *et al.* 2006). Mixing of mature somatic and immature oocyte mitochondria at reconstruction may be deleterious to successful development, although more sophisticated analysis is required. The additional dysfunctional translocation of mitochondria during early cleavage stages could also contribute to the subsequent failures that are characteristic of SCNT.

Although oxygen consumption remains relatively constant until an increase at the blastocyst stage, in general embryos throughout mammalian preimplantation development are reliant on OXPHOS for the generation of ATP, most probably to fuel cellular proliferation (Houghton *et al.* 1996; Thompson *et al.* 1996; Sturmey and Leese 2003). Mouse embryos depend entirely on OXPHOS until the blastocyst stage at which embryos start to metabolise glucose (Houghton *et al.* 1996). Glucose is however metabolised in human (Gott *et al.* 1990), ovine (Thompson *et al.* 1991; Thompson *et al.* 1992; Thompson *et al.* 1993), porcine (Sturmey and Leese 2003) and bovine (Thompson *et al.* 2000) embryos pre-compaction suggesting that they are not entirely reliant on OXPHOS. Furthermore, there is a metabolic switch to increase ATP production from glycolysis during compaction and blastulation (reviewed in Barnett and Bavister 1996). Cloned mouse embryos display irregular substrate requirements compared to IVF and *in vivo* embryos (Heindryckx *et al.* 2001; Chung *et al.* 2002; Gao *et al.* 2003; Han *et al.* 2008). Cumulus cell clones (Chung *et al.* 2002; Han *et al.* 2008) have a preference for glucose containing media as early as the 1-cell stage, which enhances development to the blastocyst stage. In addition, SCNT embryos produced using myoblast donor cells fail to thrive in standard embryo culture media, but flourish in somatic media favoured by the original donor cells (Gao *et al.* 2003). These cloned embryos continued to express the somatic glucose transporter Glut-4, indicating inadequate reprogramming of the somatic genome and that the somatic genome significantly modifies the embryos metabolic phenotype. These findings highlight the essential requirement for nuclear-cytoplasmic compatibility during development, which in some cases seems to be absent in cloned embryos. However, any links between developmental competence, perturbed metabolic output and disrupted phenotypes characteristic of SCNT embryos have yet to be elucidated.

Transmission of mtDNA following SCNT: homoplasmy or heteroplasmy

Somatic cells contain from a few hundred up to several thousand mitochondria each containing between two and ten copies of mtDNA (Michaels *et al.* 1982) reflecting the cells requirement for ATP (Moyes *et al.* 1998; Moyes 2003). Indeed, fully differentiated cells, such as skeletal and cardiac muscle cells possess 3,650 $\pm$ 620 and 6,790 $\pm$ 920 mtDNA copies/cell, respectively (Miller *et al.* 2003), whilst sheep fetal fibroblasts (SFF; (Bowles *et al.* 2007b) and mouse embryonic fibroblasts (MEFs; Kelly *et al.* Manuscript in preparation) contain 4241 $\pm$ 411 and 807 $\pm$ 21.8 copies per cell, respectively. Oocyte mitochondria contain very few cristae, which are the inner membrane invaginations that harbour the five multimeric protein complexes of the ETC. Cristae increase the surface area of the inner mitochondrial membrane and are therefore abundant in mitochondria from highly aerobic and energy-demanding somatic tissues. Ultrastructural analysis of MII oocyte mitochondria shows an immature spherical structure of arched cristae and a dense matrix, suggesting a restricted capacity for ATP production via OXPHOS (Wassarman and Josefowicz 1978; Au *et al.* 2005; Ramalho-Santos *et al.* 2009). Somatic mitochondria are highly metabolically active and proactively undergo mitochondrial biogenesis (Moyes 2003; Nisoli *et al.* 2004). Thus, the introduction of mature somatic mitochondria into a mitochondrial quiescent environment has the potential to influence cellular metabolic function and subsequent developmental competence. Following fusion and activation, the reconstructed embryo is heteroplasmic for two mitochondria haplotypes, although the somatic-oocyte ratio at reconstruction is less than 0.01%. Paternal mitochondria are normally degraded during preimplantation development, although one exception has been documented in a male patient suffering from a mitochondrial myopathy, originating from a 2bp deletion in his father's sperm. In this instance, at fertilisation, heteroplasmy of less 0.005% contributed to a muscle disease phenotype with 90% mutant mitochondrial loading in adult tissue (Schwartz and Vissing 2002). Indeed, a similar outcome can take place when karyoplasts are transferred to mammalian oocytes, whether using somatic cells, pronuclei or MII spindles, with the potential transfer of mitochondria above threshold levels for the elimination of subsequent disease phenotypes in cloned foetuses and offspring (St John and Campbell 2010).

Following reconstruction during NT, the resultant embryo is potentially heteroplasmic (St John *et al.* 2005) for donor (somatic or embryonic) and recipient (oocyte) mtDNA. The genetic profiles of such clones results in either the elimination of donor mtDNA during gestation leading to offspring being homoplasmic (Evans *et al.* 1999) or the persistence of donor mtDNA and then being heteroplasmic for the transmission of both donor and recipient oocyte mtDNA (Hiendleder *et al.* 1999; Takeda *et al.* 1999; Meirelles *et al.* 2001; Steinborn *et al.* 2002; Takeda *et al.* 2003; St John *et al.* 2005). Thus, the offspring are authentic nuclear clones but genetic hybrids. The generation of heteroplasmic offspring may also be true for assisted reproductive techniques such as cytoplasmic transfer (St John and Barratt 1997; Brenner *et al.* 2000; St John 2002; St 2002), embryonic cell NT (ECNT; (Steinborn *et al.* 1998; St John and Schatten 2004), MII spindle transfer (St John and Campbell 2010) and pronuclear transfer (Craven *et al.* 2010). Application of these techniques for improved reproductive success or for the prevention of disease transmission to offspring requires vigilant analysis of the transferred genetic material, since heteroplasmy of 0.01% at the pronuclear stage could result in (an) adult disease phenotype(s) (Schwartz and Vissing 2002).

Numerous studies have analysed the heteroplasmic nature of cloned embryos during preimplantation development. Persistence of donor mtDNA is observed in SCNT (Do *et al.* 2002; Bowles *et al.* 2008) and interspecies SCNT (iSCNT; (Yang *et al.* 2003; Hua *et al.* 2008; Ma *et al.* 2008) and ranges from 0% to 69% during preimplantation development. Transmission of mtDNA to cloned offspring is extremely important for the production of interspecies SCNT embryos and the derivation of patient specific embryonic stem cells (ESCs; (St. John and Lovell-Badge 2007). The contributions of donor and recipient DNA detected in individual offspring derived by nuclear transfer varies considerably with reported contributions of donor mtDNA ranging from 0% in ovine (Evans *et al.* 1999) to 59% in cattle (Steinborn *et al.* 1998; Hiendleder *et al.* 1999; Meirelles *et al.* 2001; Takeda *et al.* 2003).

Genetic diversity

Differences in mtDNA sequence between donor cell and recipient oocyte are likely to give rise to proteins with slight differences in their respective amino acid sequences (St John *et al.* 2005). This is likely to result in inadequate interaction between the

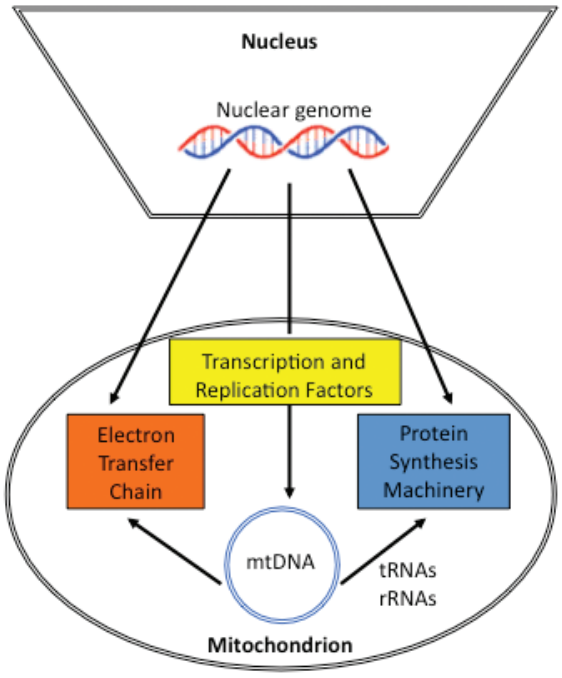

Fig. 3. Nucleo-mitochondrial interactions. *The mitochondrial genome is dependent on nuclear-encoded transcription, replication and translation factors for its propagation and to generate proteins, which contribute to the ETC. The contribution of proteins to the ETC provides cellular energy to ensure that nuclear DNA can be replicated and transcribed and mediates its epigenetic regulation, thus ensuring lineage specific gene expression.*

individual ETC subunits, which might lead to reduced oxidative capacity and consequently embryonic or foetal death due to the failure to maintain the symbiotic relationship between the nucleus and the mitochondria (see Fig. 3). Studies have demonstrated the effects of mtDNA haplotypes between different breeds of cattle where they have the potential to produce greater numbers of blastocysts following SCNT (Bruggerhoff *et al.* 2002) and improve developmental competence of IVF embryos (Tamassia *et al.* 2004), possibly via epigenetic mechanisms (Yan *et al.* 2010). The degree of genetic divergence between recipient and donor cell mtDNA permitted for ovine blastocyst development is 0.0391% (Loi *et al.* 1998) whilst for the production of cloned cattle up to 0.0787% is tolerable (Bowles *et al.* 2008). However, a 4-fold increase (mean = 0.4114%) in genetic divergence, by generating caprine-ovine clones, has demonstrated the failure of such embryos to develop to the blastocyst stage (Bowles *et al.* 2007b). Interestingly, 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 between very close genetic partners appears to hinder development. Fig. 4 demonstrates the potential relationship between the mtDNA genetic donor cells and recipient oocytes.

Lessons from genetic diversity in somatic-cell fusions

The effects of diverse nuclear-mitochondrial communication are demonstrated by interspecies cybrid fusion models of human disease. Fusion of Orang-utan (Pongo pygmaeus) with human mtDNA-less cells (Kenyon and Moraes, 1997), Chimpanzee (Pan troglodytes) with gorilla (Gorilla-gorilla) cells (Barrientos *et al.* 1998) or rat (Rattus rattus) with mtDNA-less mouse (Mus musculus) cells results in compromised respiratory capacity due to inadequate interaction of the ETC subunits. Although the direct role of mitochondrial-nuclear dysfunction in SCNT embryos remains to be elucidated, there is some evidence to demonstrate that somatic cell mitochondria adversely affect embryonic development. For example, development rates to blastocyst of parthenogenetically activated oocytes supplemented with somatic mitochondria are lower compared to non-supplemented controls (Takeda *et al.* 2005). Heteroplasmic mice produced by cytoplasmic transfer, where two distant populations of mtDNA are present, demonstrate numerous physiological abnormalities including systemic and pulmonary hypertension, increased body mass and abnormalities associated with electrolytes and haematological parameters (Acton *et al.* 2007). The increased genetic distance between the two populations of mtDNA would result in different amino acids being encoded thus mimicking mtDNA type disease.

Lessons from mitochondrial haplotypes and disease

Many mitochondrial diseases are caused by genetic defects to the mitochondrial genome, which can be either spontaneous or maternally inherited. On the other hand, mutations to chromosomally-encoded ETC genes are inherited in Mendelian fashion. In both cases, these result in the disruption of protein complexes involved in mitochondrial transcription, replication and energetics (DiMauro and Schon 2001; Copeland 2008; DiMauro and Schon 2008). Mitochondrial disorders preferentially affect tissues with high energy demands and thus have been implicated in forms of blindness, deafness, movement disorders, dementias, cardiomyography, myopathy, renal dysfunction and aging (Wallace and Murdock 1999). The epidemiological frequency of disease phenotypes is believed to be approximately 1 in 5000 although mutations have been detected at 1 in 200 in newborn umbilical cords (Schaefer *et al.* 2004; Schaefer *et al.* 2008). The conse-

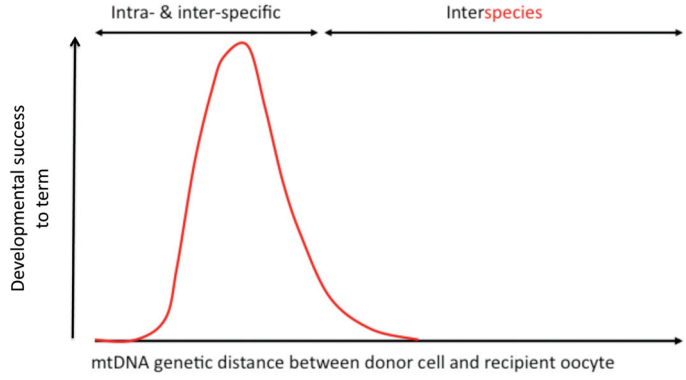

Fig. 4. The role of mitochondrial genetic distance in SCNT outcome. *The degree of genetic distance between the donor cell and recipient oocyte is a key determiner for successful preimplantation development and to term. Increasing the genetic distance between two genetic sources enhances developmental outcome. Whilst the optimal genetic distance is within the same species, once the species barrier is crossed, there is significantly reduced developmental outcome. Red represents same species.*

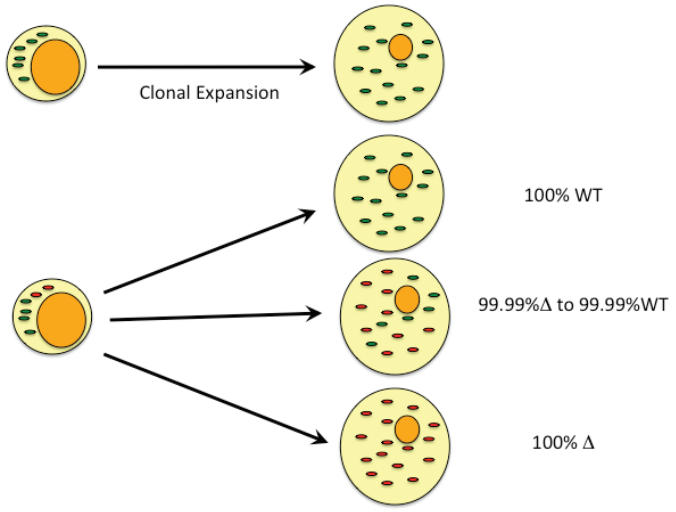

Fig. 5. mtDNA segregation during development. *The mitochondrial genome is randomly segregated during early development. Consequently, the degree of heteroplasmy, where cells will possess mutant and WT mtDNA (natural fertilisation) or possess donor cell and recipient oocyte mtDNA (all forms of nuclear transfer), or homoplasmy can not be predicted in any particular tissue. Additionally, during development, one molecule may be preferentially selected for replication over another, potentially increasing nucleo-cytoplsmic dysfunction.*

quence of these defects uncouples the ETC, altering energy production, production of ROS, modulation of calcium ion uptake and tendency to apoptosis. MtDNA is more susceptible to mutation than genomic DNA most likely due to the lack of histone packaging and the proximity to ROS (Nachman *et al.* 1996; Schriner *et al.* 2000; Wiesner *et al.* 2006). This is, however, contentious as TFAM is presumed to have a DNA packaging role also (Kaufman *et al.* 2007). Upon mutation of mtDNA, cells become heteroplasmic containing two types of mtDNA which are randomly segregated at mitosis, resulting in genetic drift towards mutated or wild type molecules. A threshold for cellular mutant loading exists which, once crossed, reduces energy output, disease phenotype prevails and the onset of necrosis or apoptosis is more likely. Interestingly, due to the random segregation to daughter cells, different phenotypes are observed for the same mutation (See Fig. 5; Wallace and Fan 2010). Variations of mitochondrial proteins can uncouple ATP production and influence the amount of calories used by the respiring mitochondria.

In humans, mitochondrial haplotypes are generally associated with specific populations and geographic regions, and permitted our ancestors to adapt to a range of new environments. Specific haplogroups predispose individuals to a wide range of metabolic and degenerative diseases as well as to cancer and longevity (Wallace *et al.* 2010; Wallace and Fan 2010). Cattle haplotypes can influence the ATP content of *in vitro* produced embryos (Bruggerhoff *et al.* 2002; Tamassia *et al.* 2004) and the compatibility of mtDNA haplotypes between donor cells and recipient cells in SCNT can influence developmental outcome and the epigenetic status of such embryos. Indeed, it has been hypothesised that the energetic state of a particular cell is communicated to the nucleus by modifications of nuclear chromatin via phosphorylation and acetylation mechanisms (Wallace *et al.* 2010; Wallace and Fan 2010).

mtDNA copy number set point

At fertilisation, the mitochondria present in mature oocytes are thought to be derived from a finite pool within PGCs (reviewed in Jansen and de Boer, 1998; Shoubridge and Wai, 2007). Random segregation within post-mitotic cells excludes predicting mitochondrial allele frequency in heteroplasmic individuals. The restoration of homoplasmy over a few generations in bovine (Hauswirth and Laipis 1982; Laipis *et al.* 1988; Ashley *et al.* 1989), human (Blok *et al.* 1997) and mouse (Meirelles and Smith 1997) progeny imply that during oogenesis a mtDNA bottleneck minimises heteroplasmic inheritance, maintaining homoplasmy perhaps due to a female germ line selection mechanism (Fan *et al.* 2008). PGCs are thought to contain approximately 10-200 copies of mtDNA and maintain relatively low numbers during the early stages of oogenesis (Smith and Alcivar 1993). During oogenesis, the expansion of the differentiating PGCs is accompanied by the clonal expansion of mtDNA (Chen *et al.*, 1995; Poulton *et al.*, 1998; Smith and Alcivar 1993). However, recent concordant studies have proposed alternative mechanisms for mtDNA segregation during PGC differentiation into a mature oocyte. It has been proposed that the mitochondrial bottleneck occurs without reduction of mtDNA content in mouse female germ cells (Cao *et al.* 2007; Cao *et al.* 2009) whilst others demonstrated mtDNA reduction during embryogenesis at 7.5 dpc in PGCs (Cree *et al.* 2008). In addition, Wai *et al.* (2008) concluded that the bottleneck is a result of replication of a subpopulation, not during embryogenesis but during postnatal oocyte maturation (Wai *et al.* 2008). Moreover, large levels of mtDNA replication have been shown to take place during the final stages of oocyte maturation (Jansen and de Boer, 1998; Spikings *et al.*, 2007). These studies suggest further examination is required into the precise mechanism behind the bottleneck, although they highlight that the expansion of mtDNA copy number during oogenesis is an essential genetic mechanism.

Increased oxygen consumption, CO_2 production and ATP output at the blastocyst stage occur in the differentiating trophectodermal cells (Houghton 2006; Spikings *et al.* 2007). Pluripotent embryonic stem cells derived from the inner cell mass (ICM) express numerous markers of pluripotency, such as Oct4 Sox2 and Nanog. Mouse ESCs (Facucho-Oliveira *et al.* 2007) and human ESCs (Cho *et al.* 2006; Prigione *et al.* 2010) contain low mtDNA copy numbers and structurally immature mitochondria (Kelly *et al.* submitted) compared to somatic cells. In addition, induced pluripotent stem cells (iPSC) display similar copy numbers and morphological characteristics to ESCs (Kelly *et al.* submitted). Therefore, it appears that the expression of pluripotent genes influences the levels of mtDNA copy number and mitochondrial function. Indeed, this association permits the developing embryo to establish the mtDNA 'set point' prior to gastrulation or cellular differentiation so that once a pathway of differentiation is initiated the progenitor cell may proliferate the mitochondrial complement in accordance with the precise cellular demands of its mature, adult form (See Fig. 6).

Generating homoplasmic offspring and iSCNT embryonic stem cells

Prior to reconstruction, donor cells and/or recipient oocytes can be depleted of their mtDNA using various chemical reagents.

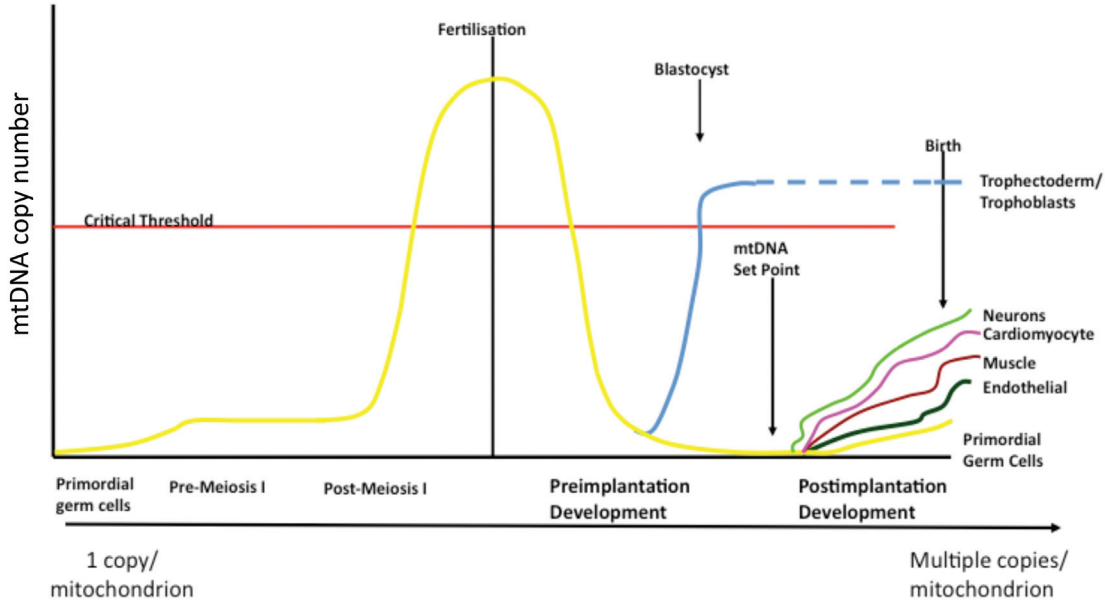

Fig. 6. mtDNA replication during development. *MtDNA replication is strictly regulated during oogenesis, preimplantation development and pre-gastrulation. The mature oocyte possesses sufficient copies of mtDNA to meet its need for OXPHOS-derived ATP during fertilisation. MtDNA replication is then significantly reduced during preimplantation development with increases most likely taking place in the trophectoderm to aid implantation. The ICM cells continue to reduce copy number after each cell division thus establishing the 'mtDNA set point'. Undifferentiated and differentiating ESCs maintain very low copy number until they commit to specific lineages.*

Donor cells containing all their mtDNA complement (mtDNA^{+}) and cells depleted to residual levels of mtDNA (mtDNA^{R}) both transmit their mtDNA to the blastocyst stage (Lloyd *et al.* 2006; Bowles *et al.* 2008). The advantage of depleting cells is, that following fusion of the somatic cell with the enucleated oocyte, the mtDNA replication factors, which are up-regulated, would be unable to replicate any persisting donor cell mtDNA thus ensuring transmission of mtDNA in a manner similar to natural fertilisation. Nevertheless, these depleted cells can support development to term and produce live lambs in which no donor somatic mtDNA is transmitted with the resultant offspring being homoplasmic for recipient oocyte mtDNA-only whilst mtDNA^{+} fetuses possessed donor cell mtDNA (Lee *et al.* 2010). Whilst SCNT protocols have concentrated on reprogramming and the effects of aberrant epigenetic regulation, it is important to note that homoplasmy, rather than heteroplasmy is a tightly regulated developmental process that ensures, in nearly all cases, the healthy status of the offspring is maintained. Not only is heteroplasmy with mutant mtDNA disadvantageous to the offspring but mixing of mtDNA haplotypes has similar outcomes (Barritt *et al.* 2001; Acton *et al.* 2007).

In iSCNT embryos donor cell mtDNA has been detected at the 16-cell stage in human-bovine crosses (Chang *et al.* 2003), the blastocysts stage in macaque-rabbit (Yang *et al.* 2003) and in a small majority of caprine-ovine embryos (Bowles *et al.* 2007b). In the small number of heteroplasmic embryos, significant increases in donor cell mtDNA were observed again indicating that donor mtDNA was preferentially replicated. The production of live interspecies offspring is extremely unlikely, however, for the generation of embryonic stem cells, nuclear cytoplasmic regulation is paramount for any clinical applications. Thus, the generation of iSCNT embryos and stem cells requires the elimination of recipient oocyte mtDNA as opposed to normal SCNT so that respiratory function, subsequent mitochondrial proliferation and differentiation are appropriate for cell function.

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