Introduction

Mitochondria are small endosymbiotic organelles that exist in almost all cell types and require nuclear-encoded genes for activity (Attardi and Schatz, 1988). Originally thought to simply produce energy, they are now known to influence vital processes in many different organs of the body (Wallace, 1999; Schon, 2000; Amuthan et al., 2001; Trifunovic et al., 2004). Each mitochondrion contains many copies of its own circular genome, which is continuously turned over, being synthesized by a mitochondrial specific DNA polymerase $\gamma$ (Lestienne, 1987). The exact molecular mechanism controlling the amount of mtDNA per cell is still uncertain (Moraes, 2001; Bogenhagen and Clayton, 2003). Concentrations of mtDNA are very sensitive to environmental stress, and a sub-optimal environment can cause a reduction in both the efficiency and fidelity of mtDNA synthesis with an associated reduction in the quantity of mtDNA and increase in random mutations within it (Graziewicz et al., 2002). It is also known that mtDNA is stabilized by a binding factor, Tfam, the expression of which has recently been shown to be modulated by DNA methylation of the promoter region of another nuclear-encoded gene NRF 1 (Choi et al., 2004). If the amount of Tfam within a cell type is reduced, there is a corresponding decline in the content of mtDNA (Larsson et al., 1998). Recently, clinical studies have reported correlations between altered mtDNA content and several common diseases of the developed world, including cardiovascular disease and type-2 diabetes (Berdanier, 2001; Song et al., 2001; Ballinger et al., 2002; Lamson and Plaza, 2002; Marin-Garcia and Goldenthal, 2002).

MtDNA mutations can be induced as a result of both extrinsic and intrinsic stress and tolerated for many years before reaching a damaging threshold level (Chinnery et al., 2002).
There has been particular interest in the possibility that abnormal mtDNA may be introduced during oocyte growth, when there is a massive amplification of mtDNA prior to its segregation between all founder cells of the embryo (Marchington et al., 1998; Barratt et al., 1999; Cummins, 2002; Poulton and Marchington, 2002). This focus of research has arisen because of the unusual nature of replication during the earliest stages of development (Cummins, 2002). MtDNA accumulates to high concentrations in the mature oocyte, being expanded from 10^1 copies per primordial germ cell to 10^9 copies in each mature oocyte. It was believed that no further replication of mtDNA occurs between fertilization and early post-implantation stages, since quantity of mtDNA per mouse embryo is unchanged throughout this period (Piko and Taylor, 1987). Thus, a constant amount of mtDNA is divided between increasing numbers of cells, and each founder cell of the developing embryo at embryonic day 6 will consequently contain very few copies of mtDNA. This restriction, and any environmental factors that change mtDNA prior to this stage, will be pivotal in determining the mtDNA template for all cells of the developing fetus, including the primordial germ cells, which will in turn contribute to the next generation. Because of the belief that no replication occurs between fertilization and implantation, it was supposed that these stages were relatively immune to environmental effects on mtDNA synthesis, and most studies concerned with the very early introduction and subsequent transmission of abnormal mtDNA have focused on detrimental environmental effects during oocyte maturation (Cummins, 2002).

However, several recent studies in very diverse experimental systems indicate that environmental interactions at the earliest phases of development immediately after fertilization appear to alter the adult phenotype of the offspring. Thus, stress arising from sub-optimal culture conditions during the preimplantation stages of mammalian development can lead to altered physiology in adult offspring (Tamashiro et al., 2002; Ecker et al., 2004; Fernandez-Gonzalez et al., 2004). The molecular vector of such long-term programming remains obscure, but the latency of errors in mtDNA makes the mitochondrial genome a plausible vehicle for transmission (Chinnery et al., 2002). Further evidence implicating early environmental stress to mitochondria in the permanent resetting of adult phenotype comes from detailed experimental manipulations in a simple animal model system the nematode Caenorhabditis elegans (Dillin et al., 2002). However, changes in mtDNA immediately after fertilization have not been seriously considered as a viable vector for long-term programming, partly because it was believed that there was no replication of mtDNA at these early stages (Attardi and Schatz, 1988; Cummins, 2002).

Yet, on closer examination of the literature, there are anomalies which are inconsistent with the accepted belief: certain studies involving unexpected rates of transmission of heteroplasmic DNA could be consistent with a period of mtDNA synthesis during preimplantation stages of development (Smith et al., 2002). Additionally, a detailed examination of the literature that was the source of the claim that there is no replication of mtDNA in the very early embryo can be traced back to only two studies. One used steady-state measurements to demonstrate that the concentration of mtDNA was constant throughout early development (Piko and Taylor, 1987), and the second measured incorporation of radiolabelled nucleotides and found no incorporation into mtDNA on or after the 8-cell stage (Piko, 1970). Without radiolabelled incorporation studies at all stages of preimplantation development, these studies do not preclude a narrow window of turnover prior to the 8-cell stage, where the rate of synthesis of mtDNA may equal the rate of degradation.

A series of experiments was therefore designed to discover if mtDNA replication occurred during preimplantation development and to investigate whether concentrations of mtDNA could be experimentally manipulated during this phase.

Materials and methods

Embryo collection

All animal procedures were performed, under license, in accordance with the Home Office Animal Act (1986). Virgin MF1 mice (Harlan UK Ltd, Bicester, Oxon, UK) at 5–6 weeks were used throughout. Animals were maintained at 22°C and on a 12-h light cycle. Mice were super-ovulated by sequential interperitoneal injection of 10 IU of pregnant mares’ serum (PMS) and 10 IU of human chorionic gonadotrophin 48 h apart. Both hormones were supplied by Intervet UK (Milton Keynes, UK) as a lyophilised powder and reconstituted in sterile phosphate-buffered saline (PBS) at a concentration of 100 IU/ml and stored in frozen aliquots before use. Prior to embryo collection, super-ovulated or pregnant mice were killed by cervical dislocation. Fully grown mouse oocytes (GV) were isolated from ovaries 12 h post-PMS, and unfertilized eggs (UF) were collected 12 h after HCG injection. One-cell zygote, 2-, 4-, 8-cell and blastocysts stage embryos were collected after successful mating with males of the same strain at 24, 36, 48, 60 and 72 h post-HCG. All manipulations were performed on heated stages to maintain a temperature of 37°C.

Embryo culture

Freshly recovered embryos were isolated into pre-warmed M2 medium (Sigma-Aldrich Company Ltd, Poole, Dorset, UK; cat. no. M7167) and transferred to pre-warmed M16 medium (Sigma-Aldrich; cat. no. M7292). All embryo incubations were at 37°C under 5% CO2. All inhibitors were purchased from Sigma-Aldrich: 3’-azido-3’-deoxythymidine (AZT; cat. no. A2169), dl-homocysteine (hcy; cat. no. H4628), oligomycin (Olg; cat. no. O4876) and aphidicolin (APC; cat. no. A0781). On delivery, each reagent was thawed and diluted 1/1000 in M16 media to give the following working concentrations: AZT 25 µmol/l, hcy 50 µmol/l, Olg 20 µmol/l and APC 20 µmol/l. Prior to collection for measurement of mtDNA copy number, embryos were incubated in the appropriate reagent for 4 h.
Measurement of mtDNA copy number in preimplantation embryos

Prior to this study, the reproducibility of the assay was established by first showing that replica blots gave identical results, and secondly by measuring the intersample variability by collecting 10 groups of eight embryos each. From these data, it was calculated that to detect a difference of 20% between treatment and control at a power of 80%, it was necessary to collect eight replica samples. For this reason, for each condition and each stage at least eight embryos were collected and counted into the bottom of a 0.5 ml Eppendorf under a dissecting microscope. After collection, each batch of embryos was snap frozen in liquid nitrogen and stored at ~80°C prior to analyses. Total embryo DNA was prepared using a Promega Wizard Genomic DNA preparation kit (Promega UK, Southampton, UK; cat. no. A1120). Samples were thawed briefly before adding 10 µl of lysis buffer, each sample was incubated (95°C for 10 min), pulse centrifuged at 9000 g and then 3 µl of a 1/10 dilution of the RNase solution provided with the kit was added. The samples were then incubated for 30 min (37°C) before cooling to room temperature and pulse centrifuging again at 9000 g. The entire sample was added to alternate wells of a 384-well plate. Samples were allowed to evaporate on the bench overnight covered only by filter paper. The samples were reconstituted the following day in 5 µl of water. Each sample was spotted onto a positively charged nylon membrane using a Biorobotics Microgrid II TAS microarrrayer. One spot per sample was printed and six strikes per spot were used. Two membranes were printed and treated as replicas. DNA on membranes was denatured and filters were prehybridized for at least 2 h using ULTRAhybulsensitive hybridization solution from Ambion Biotechnology Ltd (Huntingdon, Cambridgeshire, UK; cat. no. 8670). Mouse specific probes to mitochondrial specific 16S and nuclear specific 18S genes were produced using polymerase chain reaction (PCR) amplification for 16S gene, forward primer 5′-GTGGCCAAATATGTGAAAGTT-3′ and reverse primer 5′-CAGCCGGGTTTCTGTGT-3′ were used and for 18S gene, forward primer was 5′-TAAATCAGTTATGGTTCCTT-3′ and reverse primer 5′-TTGGCAATGCTTTCGCTCT-3′. PCR cycling conditions were 95°C for 2 min, (95°C 30s/65°C 30s/68°C 1 min) x35 and finally 68°C for 7 min. Probes were labelled to similar specific activities and approximately 1 x 10^6 counts/min per ml of hybridization solution was added to the pre-hybridized membrane. Filters were incubated overnight at 50°C in a rotating hybridization oven. Filters were washed twice in 1x SSC and 0.1% SDS for 30 min, rinsed in 1x SSC, and bound radioactivity was then measured on a Packard Instant-Imager (Packard, Pangbourne, Berks, UK). Data were collected and transferred to an Excel spreadsheet for analyses.

Visualization of mtDNA replication

MtDNA replication was detected using a commercially available kit [FLUOS® in-situ cell proliferation kit; Roche Applied Science (cat. no. 1 810 740) Lewes, UK] with minor modifications to the manufacturer’s instructions. For each stage, embryos were first incubated in APC (20 µmol/l) for 1 h to prevent nuclear replication and to facilitate visualization of the less intense mtDNA replication (Davis and Clayton, 1996) and then incubated in medium containing APC and bromo-deoxyuridine (BrdU) for 4 h (as in the manufacturer’s instructions). All staged embryos were finally incubated in Mitotracker RedCM-H2XRos (Molecular Probes, Inchninnan Business Park, Scotland, UK; cat. no. M-7513) at 400 nm for 10 min prior to fixation. After fixation with 5% formaldehyde in PBS and 0.1% Tween 20, DNA was denatured using 4 mol/l HCl. Embryos were then neutralized blocked in 5% BSA in PBS and incubated with fluorescent-labelled anti-BrdU for 30 min, as in the manufacturer’s instructions. Embryos were then washed in PBS plus 5% BSA and mounted in Citifluor under coverslips. Green and red fluorescence were monitored using Bio-Rad Radiance confocal microscope (Biorad House, Hertfordshire, UK) all samples were measured at identical settings. At least 20 embryos were collected for each stage. One-cell embryos were cultured in aphidicolin for 5 h and mtDNA copy number was measured in control and treated groups. No differences in mtDNA copy number were observed, confirming that a 5 h inhibition of nuclear replication did not affect mtDNA concentrations.

Statistical analyses

For mtDNA copy number analyses, backgrounds were subtracted from individual spots and the data were then analysed by analysis of variance (ANOVA). Differences between means were then assessed by the t-statistic calculated from the residual mean sum of squares and the residual degree of freedom.

Results

Staged preimplantation embryos from germinal vesicle (GV) stage to blastocysts (B) were freshly isolated and cultured in RPMI 1640 media alone. There was a constant amount of mtDNA throughout preimplantation development [GV = 1.13 ± 0.04 x 10^3 cpm, unfertilized eggs (UF) = 1.20 ± 0.04 x 10^3 cpm, fertilized eggs (FE) = 1.22 ± 0.10 x 10^3 cpm, 2 cells = 1.06 ± 0.06 x 10^3 cpm, 4 cells = 1.18 ± 0.05 x 10^3 cpm, 8 cells = 1.14 ± 0.11 x 10^3 cpm, B = 1.14 ± 0.10 x 10^3 cpm] (see relevant histograms; Figure 1, a–g). However, when staged embryos were cultured in the presence of AZT, which is known to specifically inhibit the γ-DNA polymerase of mitochondria (Toltzis et al., 1993), there was a significant decrease in concentrations of mtDNA but only at FE (P = 0.03) and 2-cell stages (P = 0.02) [GV = 1.20 ± 0.09 x 10^3 cpm, UF = 1.24 ± 0.06 x 10^3 cpm, FE = 0.96 ± 0.07 x 10^3 cpm, 2 cells = 0.77 ± 0.06 x 10^3 cpm, 4 cells = 1.14 ± 0.09 x 10^3 cpm, 8 cells = 1.15 ± 0.08 x 10^3 cpm, blastocysts = 1.21 ± 0.10 x 10^3 cpm; compare relevant histograms illustrated below Figure 1, c and d with all other panels). In order to directly visualize synthesis of mtDNA and to confirm that AZT was ablating this synthesis, cells were incubated in BrdU alone or in BrdU plus AZT. Location and respiratory activity of mitochondria and mtDNA replication were monitored simultaneously and visualized by confocal microscopy. The results of Mitotracker fluorescence are shown in Figure 1, h–k and the corresponding BrdU incorporation profiles in Figure 1, l–p. BrdU incorporation can be visualized at the same stages where concentrations of mtDNA can be experimentally manipulated (Figure 1, m and n) but at no other stages (Figure 1, l and r). Inclusion of AZT in the labelling procedure ablates incorporation (Figure 1, r) and the cytoplasmic incorporation
Figure 1. MtDNA turnover during mouse preimplantation development is sensitive to environmental factors. Full grown oocytes (GV) were isolated from ovaries, unfertilized eggs (UF) were collected 12 h after HCG injection, and fertilized eggs (FE), 2-, 4-, 8-cell and blastocyst stage embryos were collected after successful mating at 24, 36, 48, 60 and 72 h post-HCG (a–g). MtDNA copy number was measured in groups of embryos at each stage after cultured alone, with AZT, oligomycin or homocysteine. The relative concentrations of mtDNA present in embryos at each stage after different culture conditions is represented by coded grouped histograms beneath a light field image of the relevant stage. UF, FE, 2-cell embryos and 4-cell embryos were pre-incubated with aphidicolin to prevent nuclear replication and then cultured in aphidicolin plus BrdU 10 µg/ml for 4 h. All staged embryos were finally incubated in Mitotracker RedCM-H2Xros (Molecular Probes) 400 nm for 10 min prior to fixation to pinpoint mitochondria. All samples were subsequently processed so as to visualize BrdU incorporation. Red fluorescence (Mitotracker) (h–q) and green fluorescence (BrdU) (l–r) were monitored using confocal microscopy and all samples were measured at identical settings. (q) and (r) show that when FE are incubated in aphidicolin, BrdU and AZT, all incorporation of BrdU into mtDNA is abolished. *,** denote significant difference from controls, $P \leq 0.05$ and $P \leq 0.01$ respectively. Bar = 20 µm.
of BrdU coincides with Mitotracker labelling of mitochondria (compare Figure 1, i and j with m and n).

Next, the effects of agents that may plausibly be involved in long-term programming of adult phenotype were examined. From the study in C. elegans, it has been shown that inhibition of each of the mitochondrial respiratory complexes by specific inhibitors during early development irreversibly programmes size and activity of the mature nematode (Dillin et al., 2002). The effects of culturing staged mouse embryos in oligomycin (Olg), a specific inhibitor of mitochondrial ATPase activity, were therefore investigated. This inhibitor of mitochondrial activity caused a significant decrease in mtDNA copy number from the 1-cell zygote (P = 0.05) and 2-cell stage (P = 0.03), but at no other stages (GV = 1.25 ± 0.07 × 10^3 cpm, UF = 1.25 ± 0.14 × 10^3 cpm, FE = 0.97 ± 0.11 × 10^3 cpm, 2 cells = 0.80 ± 0.07 × 10^3 cpm, 4 cells = 1.24 ± 0.10 × 10^3 cpm, 8 cells = 1.14 ± 0.07 × 10^3 cpm, B = 1.15 ± 0.11 × 10^3 cpm).

It has recently been reported that homocysteine (hcy), a toxic non-protein amino acid, is elevated during the initial phases of pregnancy in an accepted model of developmental programming (Petrie et al., 2002). Culture in the presence of hcy caused a significant increase in mtDNA content of embryos in FE (P = 0.009) but at no other stages (GV = 1.11 ± 0.10 × 10^3 cpm, UF = 1.25 ± 0.08 × 10^3 cpm, FE = 1.76 ± 0.18 × 10^3 cpm, 2 cells = 1.35 ± 0.05 × 10^3 cpm, 4 cells = 1.23 ± 0.07 × 10^3 cpm, 8 cells = 1.15 ± 0.02 × 10^3 cpm, blastocysts = 1.25 ± 0.07 × 10^3 cpm). See relevant histograms below Figure 1, a–g.

**Discussion**

The primary aim of this study was to determine if a window of mtDNA turnover occurred during preimplantation development. The data unequivocally show that there is indeed a very short period from the 1- to 2-cell stage at which the concentration of mtDNA can be altered by culturing embryos in a specific inhibitor of γ-DNA polymerase and where mtDNA replication can be clearly visualized. Previous work that relied on steady state measurements would not have detected this period of turnover where rates of synthesis must be equivalent to rates of destruction (Piko and Taylor, 1987).

Having defined this novel period of DNA synthesis, the second aim was to discover if agents that have been associated with long-term programming of adult phenotype could alter concentrations of mtDNA immediately after fertilization. Both Olg and hcy affect the absolute amounts of mtDNA present, but in opposite directions. The effect of Olg appears to be similar to that of AZT and causes a lowering of amounts of mtDNA while hcy causes an increase. In an undisturbed embryo, the concentrations of mtDNA remain constant, but in opposite directions. The effect of Olg appears to be equivalent to rates of destruction (Piko and Taylor, 1987).

Other studies have investigated the effects of hcy on cultured cell lines, and in all cases, an increase in mtDNA occurs (McConnell et al., 2003a). It is known that Tfam, a mtDNA stabilizing factor, may be regulated by levels of nuclear gene methylation (Choi et al., 2004) and that hcy disrupts normal methyl transfer reactions, causing global hypomethylation and a concomitant increase in expression of genes controlled by methylation (Petrie et al., 2002). Although hypomethylation may lead to an up-regulation of genes normally suppressed by methylation in cell lines, this mechanism is extremely unlikely in early embryos, since such a process would be global and cause premature expression of a large number of genes at a time when there is normally a tight temporal spatial regulation of gene expression (Hamatani et al., 2004). Such deregulated expression would lead to gross developmental abnormalities and arrest. Furthermore, in cell lines, inhibition of de novo transcription by alpha amanatin does not alter an hcy-induced increase in mtDNA. It seems more likely that hcy is interfering with some post-transcriptional event that is involved in regulation of mtDNA turnover.

This study has uncovered an unexpected finding. Elevated concentrations of plasma hcy have been observed in patients with low mtDNA copy number who subsequently develop type 2 diabetes, and it has been shown that hcy is elevated in a model of developmental programming where reduced amounts of mtDNA are also found in preimplantation embryos (Lim et al., 2001; Petrie et al., 2002; McConnell and Petrie, 2003). In this study, it has been shown that in vitro incubation with hcy unexpectedly increases mtDNA copy number, suggesting that it is unlikely that elevated hcy is directly responsible for reducing mtDNA copy number in vivo.

Two provocative questions arise from this study. Firstly, what is the purpose of such a short window of mtDNA turnover immediately after fertilization and secondly, what are the molecular cues that define the timing of this short period of mtDNA turnover? This period of mtDNA turnover may have evolved to eliminate sperm mtDNA that would have not been advantageous for subsequent development (Cummins, 2002). The precise molecular mechanisms operating in selective degradation remain to be elucidated. However, it may be suggested that successive rounds of turnover involving selective degradation of non-maternal mtDNA may provide an additional method of paternal mtDNA elimination, which acts in concert with the already known mechanism of ubiquination-based targeted destruction (Sutovsky et al., 2000). It is noteworthy that elimination of other forms of non-maternal mtDNA in early embryos appears to be environmentally sensitive: thus, transmission of donor mtDNA after nuclear transfer can be variable and study dependent, suggesting that rates of turnover and hence efficiency of elimination of non-maternal mtDNA may be particularly sensitive to sub-optimal culture conditions (Hendler and Wolf, 2003).

With respect to the timing of turnover, previous studies in sea urchins (Rinaldi et al., 1979) showed that mtDNA synthesis in enucleated eggs can be initiated by calcium activation and appears to be terminated by a nuclear factor. Calcium release occurs at fertilization in mouse embryos (Blancho and Seyler, 1990) and, in mice, the initial major wave of zygotic transcription occurs at the end of the two stages (Flach et al., 1982) just as mtDNA turnover ceases. It is possible that a
newly synthesized zygotic gene product may silence mtDNA replication at these early stages.

From this in-vitro study and from recent in-vivo work (McConnell and Petrie, 2003), it has been shown that a reduction in mtDNA introduced immediately after fertilization cannot be corrected after this period of turnover has ceased, and abnormalities persist till the blastocyst stage. Additionally, this study and others have reported that these reductions persist into fetal and adult life, providing a correlation with altered mtDNA and disease susceptibility (McConnell and Petrie, 2003; Park KS et al., 2003; Park HK et al., 2004). The identification of a window of mtDNA replication after fertilization provides a route in addition to that provided during oocyte maturation where environmental factors may determine mitochondrial inheritance, possibly with transgenerational effects.

It should be emphasized that measurement of mtDNA copy number was selected as a surrogate measure of γ-DNA polymerase activity, but any environmental effects that alter mtDNA copy number may also disrupt the enzyme’s fidelity and lead to unwanted mtDNA mutations (Graziewicz et al., 2002; Lee et al., 2004). The most worrying implication of the present findings is that this window of turnover could be a period when environmental factors could permanently reset the mtDNA composition of all cells of the developing fetus, including the primordial germ cells which will give rise to subsequent generations. Recent elegant work involving the genetic engineering of a defective mtDNA-specific polymerase resulted in mutant transgenic mice that aged prematurely (Trifunovic et al., 2004). These studies unambiguously link altered mtDNA with altered adult offspring physiology, but this transgenic approach is unable to identify the specific importance of environmentally induced errors in mtDNA that occur immediately after fertilization.

Firstly, the offspring derived from heterozygous mothers would only experience defective polymerase activity after activation of the zygotic genome at the late 2-cell stage. Secondly, even breeding from homozygous mutant females, it would be impossible to distinguish between long-term effects of errors in mtDNA polymerase immediately after fertilization and those consequences occurring from errors in the polymerase before or after this early period. For this reason, studies are being extended into the long-term consequences of very early alterations of the mitochondrial genome by in-vitro manipulations followed by embryo transfer and by studying in-vivo effects of sub-optimal maternal nutrition during preimplantation phases of development in several animal models of developmental programming of adult disease (Ozanne and Hales, 2002; Khan et al., 2003; McConnell et al., 2003b).

There may also be important clinical implications of these observations. Even though there may be minor interspecies differences from humans, mouse models have been invaluable in discovering the molecular basis of mammalian development in general. In-vitro culture of human preimplantation embryos is required for assisted conception programmes, pre-genetic diagnosis, and for the powerful emerging technologies associated with customized human embryonic stem cell production, and regenerative cell therapy. If deleterious changes were irreversibly programmed into the mitochondrial genome of human preimplantation embryos by sub-optimal culture during these procedures, then these progressive new technologies may inadvertently be contributing to disease in future human populations.

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References


Song J, Oh JY, Sung YA et al. 2001 Peripheral blood mitochondrial DNA content is related to insulin sensitivity in offspring of type 2 diabetic patients. Diabetes Care 24, 865–869.


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