Mitochondrial DNA replication during differentiation of murine embryonic stem cells

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Summary

Oxidative phosphorylation (OXPHOS), the intracellular process that generates the majority of the ATP of a cell through the electron-transfer chain, is highly dependent on proteins encoded by the mitochondrial genome (mtDNA). MtDNA replication is regulated by the nuclear-encoded mitochondrial transcription factor A (TFAM) and the mitochondrial-specific DNA polymerase gamma, which consists of a catalytic (POLG) and an accessory (POLG2) subunit. Differentiation of pluripotent embryonic stem cells (ESCs) into specific cell types requires expansion of discrete populations of mitochondria and mtDNA replication to meet the specific metabolic requirements of the cell. We determined by real-time PCR that expression of pluripotent markers is reduced before the upregulation of *Polg*, *Polg2* and *Tfam* in spontaneously differentiating R1 murine (m)ESCs, along with transient increases in mtDNA copy number. In D3 mESCs, the initial transient increase did not take place. However, precursors of neuronal and cardiomyocyte differentiation were positive for both POLG and TFAM. Similar-stage ESCs also showed active mtDNA replication, identified by 5-bromo-2′-deoxy-uridine labelling, as mtDNA copy number increased. Retinoic-acid-induced differentiation resulted in more consistent patterns of replication and upregulation of *Polg*, *Polg2* and *Tfam*, whereas siRNA knockdown demonstrated that steady-state expression of POLG is essential for maintaining pluripotency.

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Introduction

Mitochondria are organelles responsible for the production of the vast majority of ATP within eukaryotic cells. They contain their own genome, mitochondrial DNA (mtDNA), which encodes 13 of the 70+ subunits of the electron-transfer chain (ETC), 22 transfer (t)RNAs and two ribosomal (r)RNAs (Anderson et al., 1981). Mutation, deletion or depletion in one or more of these genes can result in cellular impairment, or can be lethal (Wallace, 1999).

MtDNA replication is reliant on nuclear-encoded transcription and replication factors that are translocated to the mitochondria (Clayton, 1998). Two key factors involved in mtDNA replication are mitochondrial transcription factor A (TFAM) and the mitochondrial-specific DNA polymerase gamma (Clayton, 1982; Clayton, 1998). TFAM is a high mobility group (HMG) protein that triggers mtDNA transcription in mammals (Parisi and Clayton, 1991; Garstka et al., 2003), which in turn results in generation of the RNA primer necessary for the initiation of mtDNA replication (Clayton, 1992; Xu and Clayton, 1995). Mammalian polymerase gamma is a heterotrimer enzyme composed of one catalytic subunit (POLG) and two accessory subunits (POLG2) (Carrodeguas et al., 2001). The functions of POLG2 are to recognise the primer that initiates mtDNA replication and to confer high fidelity polymerisation to POLG (Xu and Clayton, 1996; Lim et al., 1999; Carrodeguas et al., 2001).

The importance of the onset of mtDNA transcription and replication for embryo survival is demonstrated by both *Polg* and *Tfam* homozygous knockout mice: either knockout produces severe mtDNA depletion, respiratory-chain deficiency and death prior to embryonic day (E)8.5 (Hance et al., 2005) and 10.5 (Larsson et al., 1998), respectively. Because murine oocytes possess 10^5 mtDNA copies immediately prior to fertilisation and there is no increase in mtDNA copy number until post-implantation, each newly divided blastomere within the pre-implantation embryo will possess fewer copies of the genome at each stage of post-fertilisation cell division (Shoubridge and Wai, 2007). This persists until the blastocyst (Thundathil et al., 2005), the final stage of pre-implantation development. Consequently, undifferentiated human embryonic stem cells (hESCs), derived from the inner cell mass of the blastocyst, have very low numbers of mitochondria (Rivolta and Holley, 2002; St John et al., 2005) and a high nuclear-to-cytoplasmic ratio (Thomson et al., 1998). Differentiation of hESCs results in the expansion of mitochondrial number (St John et al., 2005), which is likely to be matched by increased mtDNA copy number. This expansion supports the concept that the metabolic demands of a specific cell type correlate with its oxidative capacity, and therefore with the levels of mtDNA and transcription for all the components of the ETC (Williams, 1986; Moyes et al., 1998). Consequently, successful differentiation of ESCs will require lineage-specific

transcription in the nucleus, along with effective nucleo-cytoplasmic coordination to balance mtDNA transcription and replication with nuclear transcription of ETC subunits.

Retinoic acid (RA), a vitamin A derivative, is essential for normal embryo development (Morriss-Kay and Sokolova, 1996) and induces cell-type-specific differentiation of ESCs and embryonic carcinoma cells (ECCs) in a concentration- and time-dependent manner (Dani et al., 1997; Drab et al., 1997; Rohwedel et al., 1999). Exposure of murine (m)ESCs to 10^{-7}-10^{-6} M of RA in the early days of in vitro differentiation has been shown to considerably enhance neurogenesis (Bain et al., 1995; Strübing et al., 1995). Similar to the effect of RA on nuclear genes regulating cell differentiation (Gottlieb and Huettner, 1999), RA can bind to the RA-specific receptors (RAR) present in the mitochondria (Berdanier et al., 2001), activating the RA response elements (RARE) in the regulatory region of the mtDNA (D-loop) (Wrutniak et al., 1995; Demonacos et al., 1996), which, in turn, initiates mtDNA transcription and replication during ESC differentiation.

The early stages of ESC differentiation are related to the loss of pluripotency (Pesce and Schöler, 2001). Similar to the gene encoding the pluripotency marker OCT4 (POU5F1), *Pramel* (Prame-like) and *Dppa* (developmental pluripotency associated genes) are expressed up to the epiblast stage of embryonic development and in undifferentiated mESCs, but their expression is not detected in somatic tissue (Bortvin et al., 2003). Furthermore, it has recently been demonstrated that mRNA expression for *DPPA3* and *DPPA5* is downregulated during hESC differentiation (Lagarkova et al., 2006). In vitro differentiation of mESCs gives rise to the three primary germ layers – the endoderm, mesoderm and ectoderm – that characterise early stages of embryonic development (Leahy et al., 1999). This is then followed by the expression of stage-specific markers, associated with certain lineages, such as vimentin, nestin and β-tubulin III during neuronal differentiation (Rolletschek et al., 2001; Bouhon et al., 2006) and brachyury during cardiac differentiation (Herrmann et al., 1990; Wilkinson et al., 1990). There is some initial evidence to suggest that POLG and TFAM are both expressed during early hESC differentiation, as is OCT4 (St John et al., 2005). However, it has not been demonstrated whether the initiation of mtDNA replication precedes the downregulation of genes associated with pluripotency or whether this is linked to the determination of cell commitment.

Here, we quantified the number of mtDNA copies in undifferentiated mESCs and as they undergo differentiation. We also determined whether mtDNA replication is a continuous process during differentiation and whether the onset of mtDNA replication is related to the pluripotent status of the cell. We then assessed whether these patterns are similar in another mESC line or are mESC-line specific. Finally, we established whether induction of differentiation by an agent that enriches specific populations of cells mediates mtDNA replication in a similar manner, and whether knockdown of *Polg* alters the pluripotency status of undifferentiated mESCs.

Results

Loss of pluripotency

In order to determine whether the loss of pluripotency was associated with the onset of mtDNA replication, we first analysed the levels of mRNA expression for pluripotency-associated genes in undifferentiated and spontaneously differentiating mESCs by real-time reverse transcriptase (RT)-PCR. During the initial stages of differentiation (day 2), mRNA expression was significantly upregulated for *Dppa5*, *Pramel7* and *Ndp52l1* (*Calcoco2*) by 1.25-fold ($P<0.05$), 3.23-fold ($P<0.001$) and 21-fold ($P<0.001$), respectively, when compared with undifferentiated R1 mESCs (Fig. 1). This was then followed by a considerable downregulation during days 3 and 4. *Dppa5* expression decreased to 4% and 8% of levels for undifferentiated ESCs ($P<0.001$) on days 3 and 4, respectively, whereas *Ndp52l1* was undetectable ($P<0.001$; Fig. 1). *Pramel7* expression increased to 7.7-fold compared with undifferentiated R1 mESCs on day 3 ($P>0.05$) but was downregulated to undetectable levels on day 4 ($P<0.001$). Apart from day 6, when *Dppa5* mRNA expression reached levels similar to the undifferentiated R1 mESCs, and day 5, when *Pramel7* levels were above those for undifferentiated R1 mESCs ($P<0.05$), expression of all markers remained very low or undetectable for the remainder of the culture period (Fig. 1).

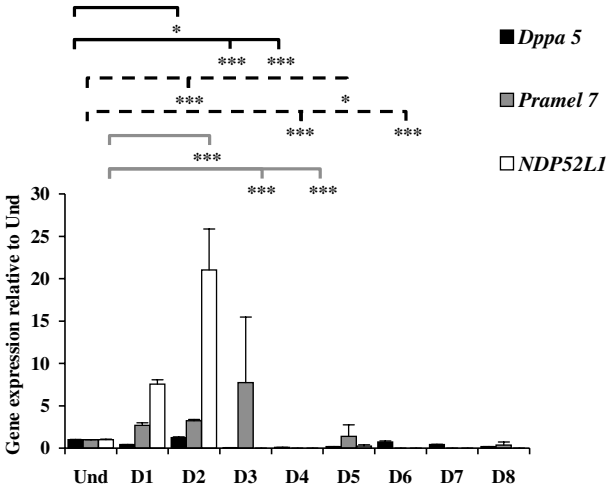

Fig. 1. Loss of pluripotency during spontaneous differentiation of R1 mESCs. Expression of the pluripotency-associated genes *Dppa5*, *Pramel7* and *Ndp52l1* was quantified on undifferentiated (Und) and spontaneously differentiated R1 mESCs (days 1-8) using RT-PCR relative quantification. Expression of individual genes was compared with the expression in undifferentiated R1 mESCs and samples were normalised to *Gapdh*. Bars represent mean ± s.e.m.; significant differences between days are indicated (*$P<0.05$, ***$P<0.001$).

The replication of mtDNA

Because the upregulation of mtDNA replication is likely to be related to the onset of differentiation, we determined whether there were specific patterns to mtDNA replication during pluripotency and the early stages of differentiation. To this extent, mRNA expression for *Polg*, *Polg2* and *Tfam*, and the number of mtDNA copies per cell, were analysed in undifferentiated R1 ESCs and during differentiation. Following spontaneous differentiation, mRNA expression of *Polg* was higher than for undifferentiated R1 mESCs on days 1 (2.7-fold, $P<0.001$), 2 (1.3-fold, $P<0.01$), 5 (2-fold, $P>0.05$), 8 (4.8-fold, $P<0.001$), 11 (1.1-fold, $P>0.05$) and 12 (4.7-fold, $P<0.001$;

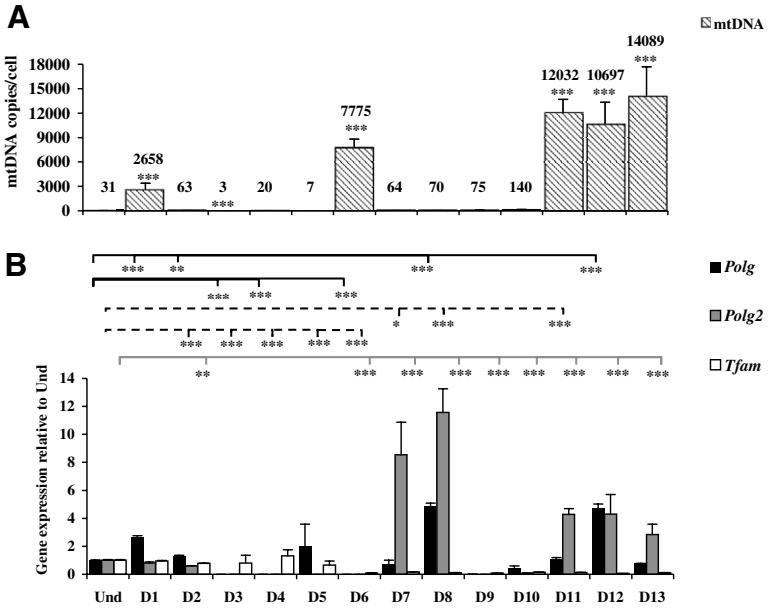

Fig. 2. MtDNA copy number/cell and expression of mtDNA replication factors during spontaneous differentiation of R1 mESCs (days 1-13). (A) MtDNA was quantified using real-time PCR and normalised to the *Gapdh* gene. The *x* axis is as in B. (B) Expression of *Polg*, *Polg2* and *Tfam* was quantified on undifferentiated (Und) and spontaneously differentiated R1 mESCs using RT-PCR relative quantification. Expression of each gene was compared with that of the undifferentiated R1 mESCs and samples were normalised to *Gapdh*. Bars represent mean ± s.e.m.; significant differences between days are indicated (**P*<0.05, ***P*<0.01, ****P*<0.001).

Fig. 2B). Similarly, *Polg2* was upregulated on days 7 (8.6-fold, $P<0.05$), 8 (11.6-fold, $P>0.001$), 11 (4.3-fold, $P<0.001$), 12 (4.3-fold, $P>0.05$) and 13 (2.9-fold, $P>0.05$; Fig. 2B). As a result, mtDNA was extensively replicated on days 1, 6 and 11-13, thus increasing the number of mtDNA copies per cell to 2658 ($P<0.001$), 7775 ($P<0.001$) and 10697-14089 ($P<0.001$), respectively (Fig. 2A).

At day 3, mtDNA copy number decreased to a minimum of three copies per cell ($P<0.001$; Fig. 2A). This coincided with undetectable levels of *Polg* and *Polg2* mRNA (Fig. 2B). Despite the significant increase in *Polg* mRNA expression on day 8 ($P<0.001$) and *Polg2* transcript levels on days 7 and 8 ($P<0.05$ and $P<0.001$, respectively; Fig. 2B), the mtDNA content of R1 mESCs derived on days 7-9 was similar to undifferentiated R1 mESCs (Fig. 2A).

Although *Tfam* mRNA expression was found to be similar to undifferentiated R1 mESCs at the early stages of differentiation (day 1 and days 3-5), it was significantly downregulated between days 6 and 13. During this period, the levels of *Tfam* expression varied between 8% (day 6, $P<0.001$) and 16% (day 7, $P<0.001$) of undifferentiated R1 mESCs (Fig. 2B). This decrease in *Tfam* expression coincided with an increase in mtDNA replication (day 6 and days 11-13, $P<0.001$; Fig. 2A).

In order to determine whether the pattern of mtDNA replication was characteristic of mESCs, we analysed another mESC line, D3. In contrast to R1 cells, spontaneously differentiating D3 cells maintained low levels of mtDNA copy number until day 6 of differentiation. A general downregulation of *Polg*, *Polg2* and *Tfam* mRNA expression between days 1 to 5 resulted in a significant decrease in mtDNA copy number when compared with undifferentiated D3 mESCs (37 copies/cell, $P<0.01$; Fig. 3A,B). Particularly low mtDNA content on days 1, 2 and 3 (3, 3 and 1 copies/cell, respectively; $P<0.001$; Fig. 3A) coincided with very significant decreases of *Polg* (days 2 and 3; $P<0.001$), *Polg2* (days 1 and 2; $P<0.001$) and *Tfam* (days 1 and 3; $P<0.001$) mRNA expression (Fig. 3B). At day 6, mtDNA copy number dramatically increased to 2730 copies per cell ($P<0.001$), which corresponded to an increase in *Polg* mRNA expression similar to those for undifferentiated D3 cells (Fig. 3A,B).

To validate the extensive mtDNA replication taking place on day 6, undifferentiated and differentiating D3 cells (days 1-7) were co-labelled with (i) 5-bromo-2′-deoxy-uridine (BrdU) and the mitochondrial-specific label MitoTracker or (ii) antibodies to the mtDNA-encoded cytochrome c oxidase I (MT-CO1) and POLG. BrdU and MitoTracker staining were co-localised and present at all time points studied, indicating that mtDNA replication was taking place during D3 mESC differentiation (Fig. 4A). Furthermore, there was increased BrdU/MitoTracker staining to match the expanded cytoplasm during differentiation. Days 1-4 had between 14.9% and 26.1% of cells with an expanded cytoplasm and positive BrdU/MitoTracker staining, whereas values on days 5, 6 and 7 were 36.5%, 34.8% and 59.3%, respectively (Fig. 4A). In addition, MT-CO1 and POLG were also colocalised tightly around the nucleus during days 1-4 (Fig. 4B), but as differentiation proceeded, both proteins were found in the expanding cytoplasm, especially at day 6 (Fig. 4B).

Cell fate commitment and mtDNA replication

Because significant mtDNA replication after the loss of pluripotency did not occur until day 6 of differentiation, we hypothesised that the onset of differentiation precedes the onset of mtDNA replication and thus analysed events within the first 7 days of differentiation. This was investigated by determining whether the onset of lineage-specific markers is coupled to mtDNA replication, using the D3 mESC line. Dual immunostaining of vimentin, nestin, β-tubulin III (neuroectoderm markers) and brachyury (mesodermal marker), with POLG and TFAM was performed on undifferentiated and spontaneously differentiating D3 mESCs (days 1-7).

Vimentin$^+$, nestin$^+$, β-tubulin III$^+$ and brachyury$^+$ D3 mESCs were also positive for POLG and TFAM (supplementary material Fig. S1A-C). Moreover, vimentin$^+$/POLG$^+$/TFAM$^+$, nestin$^+$/POLG$^+$/TFAM$^+$, β-tubulin III$^+$/POLG$^+$/TFAM$^+$ (see supplementary material Fig. S1C)

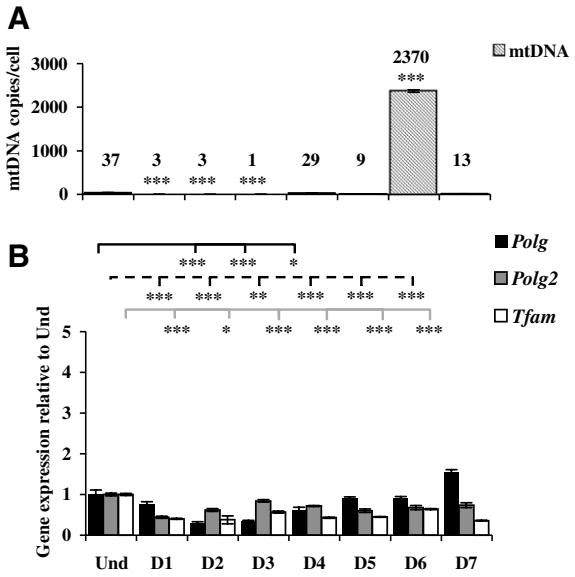

Fig. 3. The number of mtDNA copies/cell and expression of mtDNA replication factors during spontaneous differentiation of D3 mESCs (days 1-7). (A) The number of mtDNA copies/cell in undifferentiated (Und) and spontaneously differentiated D3 mESCs was determined using real-time PCR. The *x* axis is as in B. (B) The level of *Polg*, *Polg2* and *Tfam* expression was quantified using RT-PCR relative quantification. Expression of individual genes was compared with the level of expression in undifferentiated D3 mESCs. All samples were normalised to *Gapdh*. Bars represent mean ± s.e.m.; significant differences between days are indicated (**P*<0.05, ***P*<0.01, ****P*<0.001).

and brachyury$^+$/POLG$^+$/TFAM$^+$ cells were identified up to day 20 of differentiation. However, the maximum number of vimentin$^+$/POLG$^+$/TFAM$^+$, nestin$^+$/POLG$^+$/TFAM$^+$ and brachyury$^+$/POLG$^+$/TFAM$^+$ cells was found on day 3 (15.9±3.7%, *P*<0.01; 15.2±1.3%, *P*<0.001; and 11.3±6.5%, *P*>0.05, respectively), whereas the proportion of β-tubulin III$^+$/POLG$^+$/TFAM$^+$ cells increased up to day 4 (13.4±1.5%, *P*<0.001; supplementary material Fig. S1B). The vimentin$^+$/POLG$^+$/TFAM$^+$ population was then maintained at similar levels for the remainder of the differentiation period, whereas that of nestin$^+$/POLG$^+$/TFAM$^+$ decreased after day 5 (*P*<0.05; supplementary material Fig. S1B) and β-tubulin III$^+$/POLG$^+$/TFAM$^+$ cells decreased significantly on day 6 (*P*<0.05; supplementary material Fig. S1B). Indeed, because the significant increase in mtDNA replication (day 6) occurred after the downregulation of *Dppa5*, *Pramel7* and *Ndp52l1* (days 3 and 4) and after the increase in the proportion of vimentin$^+$/POLG$^+$/TFAM$^+$, nestin$^+$/POLG$^+$/TFAM$^+$, β-tubulin III$^+$/POLG$^+$/TFAM$^+$ and brachyury$^+$/POLG$^+$/TFAM$^+$ cells (days 3 and 4), this suggests that definitive lineage commitment had taken place before substantial changes in mtDNA replication were initiated.

Induction of differentiation

In order to determine whether targeted induction of differentiation would affect regulation of mtDNA replication, we stimulated neuronal differentiation by exposing undifferentiated D3 mESCs to 10^{-7} M RA for 2 days following their introduction into hanging droplets and differentiation medium. Similar to the spontaneously differentiated D3 mESCs, the vimentin$^+$, nestin$^+$, β-tubulin III$^+$ RA-induced D3 mESC were also positive for POLG and TFAM (supplementary material Fig. S2A-C). The maximum number of vimentin$^+$/POLG$^+$/TFAM$^+$ (11.7±1.5%, *P*<0.001) and nestin$^+$/POLG$^+$/TFAM$^+$ (15.3±6.5%, *P*<0.05) cells were detected on days 1 and 3, respectively (supplementary material Fig. S2B). These proportions then decreased with differentiation, resulting in only 3.6±0.8% of vimentin$^+$/POLG$^+$/TFAM$^+$ and 5.0±1.6% of nestin$^+$/POLG$^+$/TFAM$^+$ cells on day 7 (supplementary material Fig. S2B). The proportion of β-tubulin III$^+$/POLG$^+$/TFAM$^+$ RA-induced D3 mESCs significantly increased to 12.2±1.7% on day 1 (*P*<0.001) and were maintained at similar levels until day 7 (supplementary

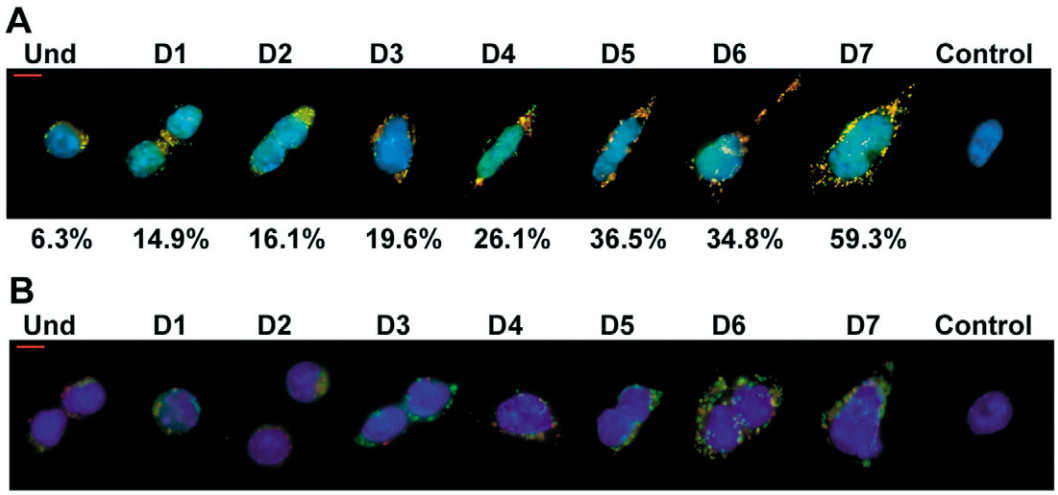

Fig. 4. Detection and localisation of mtDNA, and of the MT-CO1 and POLG proteins, during spontaneous differentiation of D3 mESCs. (A) Immunofluorescence analysis of BrdU (red) and MitoTracker (green) on undifferentiated (Und) and spontaneously differentiated D3 mESCs (days 1-7). Percentages represent the proportion of cells with an expanded cytoplasm at each time point. (B) Immunofluorescence analysis of MT-CO1 (green) and POLG (red) on undifferentiated (Und) and differentiated D3 mESCs (days 1-7). Blue, DNA. Bar, 10 μm.

material Fig. S2B). Although the percentage of nestin$^+$/POLG$^+$/TFAM$^+$ cells was not significantly different between the spontaneous differentiated and RA-induced D3 mESCs (P>0.05), RA stimulation led to a decrease in the proportion of vimentin$^+$/POLG$^+$/TFAM$^+$ cells on days 2-7 (P<0.05) and an increase in the percentage of β-tubulin III$^+$/POLG$^+$/TFAM$^+$ cells on day 1 (P<0.05).

With the earlier onset of differentiation, it was evident that RA-induced D3 mESCs had significantly higher levels of *Polg* mRNA expression on days 1-4 (P<0.001), and higher levels of *Polg2* and *Tfam* mRNA expression on days 1-7 (P<0.001), than their spontaneously differentiated counterparts (Fig. 3B, Fig. 5A,B). This is correlated with an increase in their mtDNA content between days 1 and 5 (P<0.001; Fig. 3A, Fig. 5A). In addition, the RA-induced D3 mESCs exhibited more stable levels of mtDNA copy number than the spontaneously differentiated D3 mESCs and R1 mESCs. They had 62 mtDNA copies per cell on day 3 and a minimum of 8 mtDNA copies per cell on day 7 (P<0.001; Fig. 5A), whereas the spontaneously differentiated R1 and D3 ESCs had a minimum of 3 (Fig. 2A) and 1 (Fig. 3A) mtDNA copies per cell on day 3, respectively. Most interestingly, the RA-induced D3 mESCs also showed a peak for mtDNA content on day 6 (120 mtDNA copies/cell), which was significantly different to the undifferentiated D3 mESCs (P<0.001; Fig. 5A). However, mtDNA copy number in the RA-induced D3 mESCs on day 6 was significantly lower than for R1 (P<0.001; Fig. 2A, Fig. 5A) and D3 (P<0.001; Fig. 3A, Fig. 5A) spontaneously differentiated mESCs.

POLG knockdown

Real-time RT-PCR of the spontaneously differentiated R1 and D3 mESCs and the RA-induced D3 mESCs suggested that the onset of differentiation is associated with significant variations in the levels of *Polg* expression (Fig. 2B, Fig. 3B, Fig. 5B). To evaluate whether steady-state levels of POLG expression are required to maintain pluripotency, and whether alteration of those levels are associated with the onset of mESC differentiation, we used siRNA to knockdown POLG expression in undifferentiated mESCs. To overcome any problem with contamination with feeder cells (MEFs), which could mask POLG knockdown because of the high levels of the protein, the feeder-free CEE/R mESC line was used. Densitometry analysis from western blots showed that POLG levels were decreased by 7.3-fold (Fig. 6) in the undifferentiated CCE/R mESCs transfected with the *Polg* siRNAs when compared with the undifferentiated CCE/R mESCs transfected with the negative control siRNA. Apart from the decrease in POLG, OCT4 levels were also decreased by 2.5-fold (Fig. 6) and brachyury levels increased by 1.2-fold (Fig. 6) when compared with the negative-control siRNA.

Discussion

Pluripotency and differentiation

Here we have shown that, in R1 mESCs, the pluripotency-associated genes *Dppa5*, *Pramel7* and *Ndp52l1* were upregulated during days 1 and 2 of differentiation, and downregulated during days 3 and 4 (Fig. 1). Similarly, the pluripotency marker OCT4 has been shown to increase expression by 50% during endodermal differentiation of mESCs (Niwa et al., 2000). OCT4 expression was also

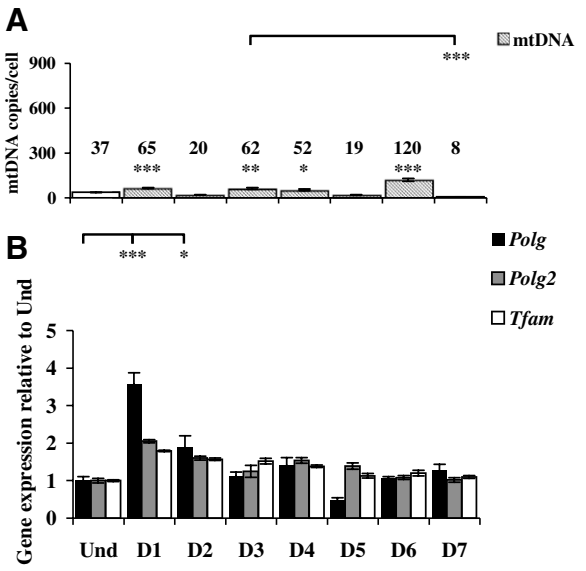

Fig. 5. Effect of RA-induced differentiation on the mtDNA copy number/cell and expression of *Polg*, *Polg2* and *Tfam* (days 1-7). (A) The number of mtDNA molecules/cell was determined using real-time PCR and normalised to the *Gapdh* gene. The *x* axis is as in B. (B) *Polg*, *Polg2* and *Tfam* expression in undifferentiated (Und) and RA-induced D3 mESCs. RT-PCR relative quantification compared the expression of individual genes to their expression in undifferentiated D3 mESCs. Values for each gene were normalised to *Gapdh* expression. Bars represent mean ± s.e.m. Significant differences between undifferentiated and days 1, 3, 4 and 6 are indicated above the respective bar representing the stage of differentiation; the significant difference between days 3 and 7 is shown next to the connecting line (*P<0.05, **P<0.01, ***P<0.001).

upregulated during migration and differentiation of the primitive endoderm into parietal endoderm during mouse-embryo development (Palmieri et al., 1994), and during RA-induced differentiation of murine F9 ECCs into visceral and parietal endodermal cells (Botquin et al., 1998). Furthermore, days 1 and 2 of mESC differentiation have been shown to be equivalent to the early post-implantation embryo at the beginning of gastrulation (E4.5-E6.5) (Leahy et al., 1999) and therefore high levels of pluripotency-associated gene expression would be expected. In addition, we have shown that the percentage of brachyury$^+$/POLG$^+$/TFAM$^+$, vimentin$^+$/POLG$^+$/TFAM$^+$, nestin$^+$/POLG$^+$/TFAM$^+$ and β-tubulin III$^+$/POLG$^+$/TFAM$^+$ D3 mESCs gradually increased to peak at days 3 and 4 (supplementary material Fig. S1B). Consistent with our results, coordinated downregulation of pluripotency-associated genes and co-expression with lineage-specific transcription factors has been reported during early stages of hESC differentiation (Laslett et al., 2007). Consequently, our data would support the view that a transient increase in the expression of these pluripotency-associated genes could initiate a cascade of events leading to the onset of ESC differentiation and commitment into a specific lineage.

mtDNA replication

Both R1 and D3 undifferentiated mESCs showed low numbers of mtDNA copies per cell (Fig. 2A, Fig. 3A), with D3 mESCs

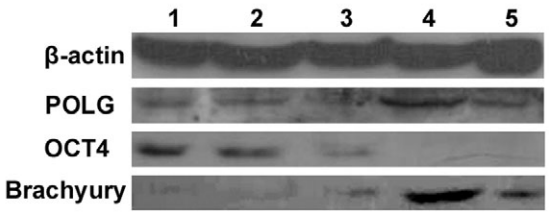

Fig. 6. The effect of siRNA-mediated knockdown of POLG in undifferentiated CCE/R mESCs. Pooled cell lysates were prepared from FACS non-transfected and siRNA-transfected CCE/R. Equal amounts of protein (100 μg) were electrophoresed on a 9% SDS-PAGE gel then semi-dry blotted onto a polyvinyl difluoride membrane. The membrane was probed for β-actin, POLG, OCT4 and brachyury with the membrane being stripped between each probing. Lane 1, non-transfected undifferentiated CCE/R mESCs; lane 2, undifferentiated CCE/R mESCs transfected with negative control siRNA (75 nM); lane 3, undifferentiated CCE/R mESCs transfected with siRNA targeting POLG (75 nM); lane 4, human embryonic kidney cells; lane 5, mouse embryonic fibroblasts.

also having low levels of MitoTracker (Fig. 4A) and MT-CO1 (Fig. 4B) staining. This low number of mtDNA copies in undifferentiated mESCs relates to the requirements of the cell for high glycolytic metabolism and low oxygen consumption, which are needed to maintain their proliferative capacity (Kondoh et al., 2007). Apart from the mtDNA replication peak on day 1 for R1 mESCs, differentiating R1 and D3 mESCs maintained low mtDNA copies per cell until day 6 of differentiation (Fig. 2A, Fig. 3A). This arises because there was a significant decrease in the transcript levels for *Polg* on days 3, 4 and 6 (R1 cells; $P<0.001$) and days 2-4 (D3 cells; $P<0.05$), for *Polg2* on days 2-6 (R1 cells; $P<0.001$) and days 1-6 (D3 cells; $P<0.01$), and for *Tfam* on day 2 (R1 cells; $P<0.01$) and days 1-6 (D3 cells; $P<0.05$). Indeed, the low levels of BrdU incorporation into the mtDNA of D3 mESCs during days 1-4 suggest that only basal levels of mtDNA replication were taking place (Fig. 4A). Moreover, MT-CO1 staining was located tightly around the nucleus and did not increase to any great extent during days 1-5 (Fig. 4B). At day 6, both R1 and D3 mESCs showed a significant increase in mtDNA content (Fig. 2A, Fig. 3A). D3 mESCs also showed an increase in BrdU incorporation into mtDNA and in the levels of MT-CO1 (Fig. 4A,B). Furthermore, BrdU incorporation and MT-CO1 were located in expanded cytoplasmic areas, suggesting an expansion of the cytoplasm and migration of the mitochondria, as previously reported during mouse and human ESC differentiation (St John et al., 2005; Cho et al., 2006; Chung et al., 2007).

Despite upregulation of *Polg* transcripts on day 8 and *Polg2* transcripts on days 7 and 8, R1 mESCs did not replicate mtDNA to any great extent (Fig. 2A,B). It is possible that this increase in expression is related to repair of the replicated mtDNA, because POLG has been shown to replace single-nucleotide gaps generated by the apurinic/apyrimidinic endonucleases and release the deoxyribose-phosphate residue to produce a substrate for the DNA ligase (Graziewicz et al., 2006). This would be a timely event because the cells from day 7 onwards would already be clearly committed to a specific lineage and would be precursors of all other lineages within that pathway. In the foetus, such an event would be crucial to ensure that a large number of rearrangements did not persist and lead to the onset of severe mtDNA-type diseases. This would also be crucial for the continued viability of ESCs, especially because these are prone to rearrangement as passage number increases (Maitra et al., 2005).

Interestingly, large levels of mtDNA replication in the R1 mESCs (day 6 and days 11-13, Fig. 2A) coincided with downregulation of *Tfam* transcript expression (days 6-13; Fig. 2B). Although increased TFAM expression is normally associated with an increase in mtDNA transcription and replication (Montoya et al., 1997; Ekstrand et al., 2004), several groups have reported that an increase in the number of TFAM molecules above the optimal mtDNA:TFAM stoichiometry has inhibitory effects on mtDNA transcription and replication (Webb and Smith, 1977; Parisi et al., 1993; Dairaghi et al., 1995; Garstka et al., 2003). Indeed, in certain circumstances the reduction of TFAM protein levels in human and *Drosophila* using RNA interference resulted in increased mtDNA transcription (Goto et al., 2001; Kanki et al., 2004). Because TFAM acts as a transcription factor but also binds mtDNA in a non-specific manner to package the mtDNA (Alam et al., 2003; Kanki et al., 2004), the decrease of mtDNA transcription and replication with high levels of TFAM has been related to extensive packaging of the mtDNA (Shen and Bogenhagen, 2001). It is likely that condensation of the mtDNA with high levels of TFAM causes restricted access of other regulatory proteins. It is therefore possible that transcription of *Tfam* during days 1-5 (Fig. 2B) resulted in accumulation of TFAM protein, whereas large mtDNA replication events such as those on days 6 and days 11-13 (Fig. 2A) required lower levels of TFAM protein.

mtDNA replication induced by RA

D3 mESCs that were differentiated in the presence of RA increased the percentage of vimentin$^+$/POLG$^+$/TFAM$^+$ and β-tubulin III$^+$/POLG$^+$/TFAM$^+$ cells more rapidly than spontaneously differentiated cells (see supplementary material Fig. S1B, Fig. S2B). Indeed, exposure of 10^{-7} M RA during the hanging-droplet stage has previously been shown to enhance neuronal differentiation of mESCs (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Rohwedel et al., 1999). Consistent with this, the RA-induced D3 mESCs showed elevated and more consistent numbers of mtDNA copy number (days 1-5; Fig. 2A, Fig. 3A, Fig. 5A) than spontaneously differentiated R1 and D3 mESCs. RA has been previously reported to upregulate transcription of the mtDNA-encoded NADH dehydrogenase subunit 5 (*mt-Nd5*), *mt-Co1* and *16S rRNA* (*mt-Rnr2*) (Li et al., 1994; Gaemers et al., 1998). Upregulation of the mtDNA transcripts is likely to result from a direct effect of RA on RARE in the mtDNA (Wrutniak et al., 1995; Demonacos et al., 1996) and RAR in the mitochondria (Berdanier et al., 2001). Given that POLG requires an RNA primer to replicate mtDNA (Chang and Clayton, 1985; Xu and Clayton, 1996), it is possible that the increase in mtDNA transcription also generated the observed increases in mtDNA replication. However, the effect of RA on the mtDNA content might also arise from stimulation of nuclear genes because higher levels of *Polg* (days 1-4), *Polg2* (days 1-7) and *Tfam* (days 1-7; Fig. 3B, Fig. 5B) mRNA expression were detected.

The relationship between POLG and pluripotency

During days 1 and 2, spontaneously differentiated R1 and RA-induced D3 mESCs increased mRNA levels for *Polg* (Fig. 2B, Fig. 5B), whereas the spontaneously differentiated D3 mESCs showed the opposite response (Fig. 3B). This suggests that defined levels of POLG might be required to maintain pluripotency and an increase or decrease in such levels could mediate differentiation of mESCs. Further investigation using siRNAs to knockdown POLG confirmed that decreased levels of POLG (7.3-fold; Fig. 6) induced differentiation of CCE/R mESCs, as demonstrated by a decrease of OCT4 levels (2.5-fold; Fig. 6) and increased levels of brachyury (1.2-fold; Fig. 6). Supporting this concept, mRNA levels for *Polg* have been shown to be dramatically increased at the blastocyst stage of pre-implantation embryonic development in a range of mammals (Thundathil et al., 2005; Bowles et al., 2007; Spikings et al., 2007). At this stage, the majority of cells are committed to the trophectoderm, in which POLG expression is likely to be upregulated, whereas the inner cell mass cells maintain persistently low levels of POLG and continue to be pluripotent. Furthermore, the alteration of POLG levels seems to be conserved beyond the mammalian species, because POLG activity has been shown to increase at the blastula stage during sea urchin-embryo development (Shioda, 1988).

The association between mtDNA copy number and differentiation

As previously mentioned, the mtDNA content of both R1 and D3 spontaneously differentiated mESCs remained minimal during days 1-5 (Fig. 2A, Fig. 3A). This period coincided with the downregulation of *Dppa5*, *Pramel7* and *Ndp52l1* expression (days 3 and 4; R1 mESCs; Fig. 1) and the upregulation in the numbers of brachyury$^+$/POLG$^+$/TFAM$^+$, vimentin$^+$/POLG$^+$/TFAM$^+$, nestin$^+$/POLG$^+$/TFAM$^+$ and β-tubulin III$^+$/POLG$^+$/TFAM$^+$ cells (days 3 and 4; D3 mESCs; supplementary material Fig. S1B). Although days 1-5 had low mtDNA content per cell, basal levels of mtDNA replication were confirmed by incorporation of BrdU into mtDNA (D3 mESCs; Fig. 4A). Low levels of mtDNA replication are most probably essential in order to replenish the mtDNA content of proliferating mESCs.

At day 6, when cells had clearly committed to a specific lineage, mtDNA was extensively replicated (Fig. 2A, Fig. 3A, Fig. 4A) and mitochondria were detected in wider areas of the cytoplasm (Fig. 4A,B). Commitment into a specific lineage, regulated by lineage-specific transcription factors (Pevny et al., 1998; Brandenberger et al., 2004), is thus likely to initiate the expansion of the number of mitochondria and mtDNA molecules per cell according to the specific metabolic demands of individual cells (Williams, 1986; Moyes et al., 1998).

The dependency of differentiation on oxidative phosphorylation

Transition from anaerobic metabolism, which characterises undifferentiated ESCs, into a more efficient mitochondrial, aerobic metabolism is crucial for successful differentiation of mouse and human ESCs (St John et al., 2005; Chung et al., 2007; Kondoh et al., 2007). Activation of the ETC and citric acid cycle (Krebs cycle) as part of this switch involves an increase in mtDNA content and expression of proteins involved in aerobic metabolism and ATP production (St John et al., 2005; Cho et al., 2006; Chung et al., 2007). Indeed, inhibition of the ETC with antimycin A or rotenone during mESCs differentiation has been shown to decrease mitochondrial volume (Spitkovsky et al., 2004; Chung et al., 2007), whereas disrupted mitochondrial networks lead to a dramatic reduction in the number of beating embryoid bodies (EBs; up to 80%) and sarcomere content of differentiating cardiomyocytes (Chung et al., 2007). Previous reports suggest that oxidative phosphorylation (OXPHOS) is activated within the first 7 days of mESC differentiation (Cho et al., 2006), and our data indicate that this occurs at day 6, perhaps as a consequence of increased transcriptional activities of the mtDNA genome, an event that precedes mtDNA replication. Most interestingly, day 6 of differentiation has been shown to mirror spatial and temporal expression of lineage-specific markers that takes place at E7.5 of mouse-embryo development (Leahy et al., 1999). If day 6 of in vitro mESC differentiation mimics the molecular events that occur during E7.5, then it is very likely that the developmental arrest of homozygous *Polg* knockout mice between E7.5 and E8.5 (Hance et al., 2005), and homozygous *Tfam* knockout mice between E 8.5 and E10.5 (Larsson et al., 1998), was caused by failure to expand the number of mtDNA molecules per cell, and resulted in aerobic-respiration deficiency. Indeed, low levels of TFAM, as argued earlier, would facilitate replication at this time, and thus explain why TFAM deficiency has a physiological impact later in development.

We demonstrated that the loss of pluripotency and the induction of cell fate commitment, upon differentiation of ESCs, occur in the presence of low levels of mtDNA replication. We suggest that a decrease in expression of pluripotency markers and increasing numbers of committed ESCs proceed in the presence of low levels of expression of POLG, POLG2 and TFAM, which ensure that sufficient mtDNA is preserved within proliferating mESCs for subsequent transmission. After mESCs have committed to a specific lineage, mtDNA is extensively replicated, leading to the enrichment of the mitochondrial content to provide the higher levels of ATP required for further differentiation. We have shown that the onset of mESC differentiation is associated with the modulation of *Polg* expression, and that steady-state levels of POLG are required to maintain pluripotency. In addition, RA stimulation led to a more rapid onset of differentiation and to increased expression of the mtDNA-specific replication factors, which emphasises that control of mtDNA copy number is crucial for successful differentiation of ESCs.

Materials and Methods

Culture of MEFs

Mouse embryonic fibroblasts (MEFs) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Gillingham, UK) with 10% foetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Invitrogen Life Technologies, Paisley, UK), 1% non-essential amino acids (NEAA; Invitrogen Life Technologies) and 1% penicillin/streptomycin solution (Sigma). Inactivation of MEFs for use as mESC feeders was carried out by γ irradiation (22.62 grays) or mitomycin C (Sigma) treatment. Confluent MEFs were treated with mitomycin C (10 μg/ml) for 2 hours at 37°C/5% CO_2, washed three times with PBS (Sigma) and cultured overnight or frozen before use as feeder cells for undifferentiated R1 or D3 mESCs.

Culture of mESCs

R1 mESCs (Nagy et al., 1993) were cultured in knockout DMEM with 15% serum replacement, 1% penicillin/streptomycin solution, 2 mM L-glutamine, 1% NEAA (all Invitrogen Life Technologies), 0.1 mM β-mercaptoethanol (Sigma) and 1000 U/ml leukaemia inhibitory factor (LIF; Chemicon, Temecula, Ca, USA). D3

(Doetschman et al., 1985) and CCE/R mESCs were cultured in high glucose DMEM (Sigma) with 15% ESC screened FBS (Hyclone, Utah, US), 1% penicillin/streptomycin solution (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies), 1% NEAA (Invitrogen Life Technologies), 0.1 mM β-mercaptoethanol (Sigma) and 1000 U/ml LIF.

Differentiation of mESCs

Spontaneous differentiation

Undifferentiated R1 and D3 mESCs were induced to differentiate using the hanging-drop method (Keller, 1995). Briefly, mESCs (day 0) were dissociated with 0.25% trypsin-EDTA (Sigma) for 2 minutes, resuspended into mESC medium (minus LIF) and plated as 20 μl droplets (approximately 450 cells per drop) on the lid of an inverted Petri dish (Sterilin, Staffordshire, UK) for 48 hours (days 1-2) to promote the formation of EBs. EBs were then placed into suspension for a further 5 (days 3-7, D3 mESCs) or 6 (days 3-8, R1 mESCs) days at 37°C/5%CO_2. EBs were then plated onto gelatin (0.1%; Sigma)-coated six-well plates (Nunc, Roskilde, Denmark) and cultured up to day 20 of differentiation.

RA-induced differentiation of mESCs (D3)

mESCs were treated as for spontaneous differentiation, except that, during the hanging-drop stage (days 1-2), RA (10^{-7} M) was added to the medium.

POLG knockdown

The following FAM-labelled siRNAs (Ambion, Huntingdon, UK) were used: a negative control siRNA complex with no homology to mouse, rat or human genomes (supplied by Ambion); and an siRNA complex targeting exons 13 and 14 of *Polg* mRNA (siRNA sequence 5′-GGACGGUAACAACUACAAUtt-3′ and 5′-AUUGUAGUUGUUACCGUCCtt-3′). Undifferentiated CCE/R mESCs underwent transfection with either of the siRNA complexes (75 nM) using Lipofectamine 2000 (Invitrogen Life Technologies), or were not transfected (control), for 24 hours in R1 mESC media (15% knockout serum). After 24 hours, fresh CCE/R media (15% Hyclone FBS) was added and the cells were maintained in culture for a further 72 hours.

Fluorescent-activated cell sorting (FACS)

All cells were sorted on a MoFlo Cell Sorter (Dakocytomation, Cambridgeshire, UK) and analysed using the CellQuest/Summit software. Undifferentiated R1 and D3 mESCs were sorted with the stage-specific embryonic antigen 1 (SSEA-1). Undifferentiated R1 and D3 mESCs were incubated with 0.25% trypsin-EDTA for 2 minutes, titrated into a single cell suspension and incubated with the SSEA-1 antibody (1:50; Developmental Studies Hybridoma Bank, University of Iowa, USA) for 30 minutes at 37°C in mESC media plus LIF. The cells were then washed three times by centrifugation, incubated with the Alexa-Fluor-488 anti-mouse (Molecular Probes, Paisley, UK) for 30 minutes at 37°C and washed three times by centrifugation. The SSEA-1-positive R1 and D3 mESCs were then sorted. The undifferentiated CCE/R cells were washed three times with PBS, trypsinised and resuspended in CCE/R mESC media 96 hours after transfection. The FAM-labelled CCE/R and non-transfected cells were sorted.

RNA extraction

Total RNA was extracted from undifferentiated, spontaneously differentiated and RA-induced (days 1-7) D3 mESCs, and from undifferentiated and spontaneously differentiated (days 1-13) R1 mESCs. Total RNA was extracted using the RNAqueous-4PCR kit (Ambion) according to the manufacturer's instructions. Samples were treated with DNase I (4 units, Ambion) for 2 hours at 37°C, after which it was inactivated with DNase inactivation reagent (Ambion). The RNA was reverse transcribed using the Reverse Transcription System (Promega, Southampton, UK). Reactions contained 800 ng/μl of RNA, 2 μl of 10× RT-Buffer, 5 mM of $MgCl_2$, 1 mM of dNTP mixture, 0.25 μg Oligo (dT) primer, 1 U/μl of RNasin Ribonuclease Inhibitor, 15 U/μg of AMV Reverse Transcriptase and ultrapure H_2O (Sigma) up to 20 μl. Reactions were incubated at 42°C for 2 hours followed by 95°C for 10 minutes to denature the AMV Reverse Transcriptase.

DNA extraction

Total DNA was extracted from undifferentiated, spontaneously differentiated and RA-induced (days 1-7) D3 mESCs, and undifferentiated and spontaneously differentiated (days 1-13) R1 mESCs. mESCs (10.0 to 7.5×10^4 cells/μl) were resuspended in ultrapure H_2O, freeze-thawed twice and vigorously pipetted to release DNA into the solution (Lloyd et al., 2006).

Real-time PCR

Each real-time PCR reaction (15 μl total volume) contained 2 μl of template (cDNA or DNA), 7.5 μl of 2× Sensi Mix (Quantace, London, UK), 0.3 μl of 50× SYBR Green I Solution (Quantace), 0.33 μM of each of the forward and reverse primers (Invitrogen Life Technologies) and ultrapure H_2O (Sigma). A 293 bp product of *Dppa5* cDNA was amplified with Dppa 5-F (5′-GCTTGATCTCGTCTTCCCTG-3′) and Dppa5-R (5′-TCCATTTAGCCCGAATCTTG-3′); 336 bp product of *Pramel7* cDNA with Pramel7-F (5′-AGAGAACCCACATGGCTTTG-3′) and Pramel7-R (5′-GGATTTGGCTTGGCATACAT-3′); 475 bp product of *Ndp52l1* cDNA with Ndp52l1-F (5′-TTGATGCTCTTGCACAGGAC-3′) and Ndp52l1-R (5′-TCACTGTTAGCACTGCCTG-3′); 188 bp of *Polg* cDNA with Polg-F (5′-GGACCTCCCTTAGAGAGGGA-3′) and Polg-R (5′-AGCATGCCAGCCAG-AGTCACT-3′); 214 bp of *Polg2* cDNA with Polg2-F (5′-ACAGTGCCTTCAG-GTTAGTC-3′) and Polg2-R (5′-ACTCCAATCTGAGCAAGACC-3′); 165 bp of *Tfam* cDNA with Tfam-F (5′-GCATACAAAGAAGCTGTGAG-3′) and Tfam-R (5′-GTTATATGCTGAACGAGGTC-3′); 366 bp of the *Gapdh* gene or cDNA with Gapdh-F (5′-GGGAAGCCCATCACCATCTTC-3′) and Gapdh-R (5′-AGAGG-GGCCATCCACAGTCT-3′); and 211 bp product of the *tRNA-Tyr* and *mt-Co1* genes of the mtDNA, amplified with tRNA-Tyr/mt-Co1-F (5′-CAGTCTAATGCT-TACTCAGC-3′) and tRNA-Tyr/mt-Co1-R (5′-GGGCAGTTACGATAACATTG-3′). Reactions were performed in a Rotorgene-3000 real-time PCR machine (Corbett Research, Cambridge, UK). Initial denaturation was performed at 95°C for 10 minutes, followed by 50 cycles of: denaturation at 95°C for 10 seconds; annealing at 53°C (mtDNA and *Tfam*), 55°C (*Polg2*) or 60°C (*Dppa5*, *Pramel7*, *Ndp52l1*, *Polg* and *Gapdh*) for 15 seconds; and extension at 72°C for 15 (*Polg*, *Tfam* and mtDNA), 20 (*Dppa5*, *Pramel7* and *Polg2*), 23 (GAPDH) or 30 (Ndp52l1) seconds. For the standards, a series of tenfold dilutions (2×10^{-2} ng/μl to 2×10^{-9} ng/μl) of the target-specific PCR product were generated.

Data were acquired in the FAM/SYBR channel during the extension phase. In order to eliminate primer dimerisation from the analysis, a fourth step with data acquisition was added. The temperature at which the specific product started to melt was determined using dissociation curves (76°C for mtDNA, 78°C for *Polg2* and *Tfam*, 81°C for *Ndp52l1* or 82°C for *Polg* and *Gapdh*) and a second acquisition phase of 15 seconds in the FAM/Sybr channel was programmed to allow measurements of fluorescence from specific product only. Melt-curve analysis was conducted by ramping from 62°C to 99°C (rising 1°C every 5 seconds) and data were acquired from the FAM/Sybr channel. Analyses were performed using the Rotor Gene software (version 7; Corbett Research). Each sample was run in triplicate in two separate reactions generating six readings per gene. From those values, only the middle four were considered in order to diminish pipetting errors (Bustin, 2000). Data were expressed as mean ± s.e.m.

The number of mtDNA copies per cell was determined using real-time PCR absolute quantification. Because samples contained an unknown number of cells, determination of the number of mtDNA copies per cell required quantification of the number of *Gapdh* gene copies and the number of mtDNA copies. The number of mtDNA copies per cell was calculated using the following formula:

$$\text{MtDNA copies/cell} = \frac{\text{(No. of copies of the } \textit{tRNA-Tyr/mt-Co1} \text{ gene)}}{\text{(No. of copies of the } \textit{Gapdh} \text{ gene/2)}}.$$

The Pfaffl method of relative expression was used to compare the expression of *Polg*, *Polg2*, *Tfam*, *Dppa5*, *Pramel7* and *Ndp52l1* in differentiating mESCs to the undifferentiated mESCs (Pfaffl, 2001). This method provides real-time RT-PCR relative quantification of samples with an unknown number of cells against a separate reaction for the housekeeping gene (*Gapdh*).

Immunocytochemistry

Undifferentiated, spontaneously differentiated and RA-induced (days 1-7 and day 20) D3 mESCs were incubated with 0.25% trypsin-EDTA for 2 minutes and titrated into a single cell suspension. D3 mESCs were then resuspended in D3 mESC differentiation media (D3 mESC media containing LIF was used for undifferentiated mESCs), plated onto gelatin-coated coverslips and incubated overnight at 37°C/5%CO_2. D3 mESCs were fixed in 2% formaldehyde (Sigma) for 1 hour, permeabilised for 30 minutes in 1% (v/v) Triton X-100 (Sigma), placed in blocking solution for at least 30 minutes and incubated with the primary antibodies. MT-CO1 (1:100; Molecular Probes) and the neuroectoderm markers vimentin (1:50; Sigma), nestin (1:200; BD Pharmingen, Oxford, UK) and β-tubulin III (1:100; Sigma) were co-labelled with the mitochondrial replication factors POLG (1:100; Abcam, Cambridge, UK) and TFAM (1:200; Santa Cruz Biotechnology, Santa Cruz, California) by incubating for 2 hours at 37°C. The mesodermal marker brachyury (1:400; R&D Systems, Abingdon, UK) was co-labelled with POLG and TFAM by incubating overnight at 4°C. The cells were washed three times with 0.1% Triton X-100 for 5 minutes at room temperature then incubated with two of 459 anti-rabbit (POLG and TFAM), 488 anti-mouse (MT-CO1, vimentin, nestin and β-tubulin III) or 488 anti-goat (brachyury) secondary antibody (Molecular Probes) for 1 hour at 37°C. The cells were further washed three times with 0.1% Triton X-100 for 5 minutes and placed onto slides using mounting medium containing DAPI (Vectashield; Vector Labs, Peterborough, England).

For BrdU and MitoTracker labelling, D3 mESCs were plated onto gelatin-coated coverslips and incubated overnight in D3 mESC media containing 10 μM of BrdU (Roche Applied Sciences, Sussex, UK) at 37°C/5%CO_2. D3 mESCs were then washed with pre-warmed PBS and incubated with 25 nM MitoTracker (Molecular Probes) in D3 mESC media for 45 minutes at 37°C/5%CO_2. Cultures were fixed for 15 minutes in 4% formaldehyde and permeabilised in 1% (v/v) Triton X-100 for 15 minutes. D3 mESCs were incubated with recommended concentrations of the BrdU antibody (1:10, Roche Applied Sciences) and the anti-mouse antibody

(1:10, Roche Applied Sciences) at 37°C for 30 minutes. A series of three washes using washing buffer (Roche Applied Sciences) was performed after the antibody incubations. D3 mESCs were placed onto slides using mounting medium containing DAPI.

Microscopy and imaging methods

Fluorescent microscopy was performed using a Nikon Eclipse E600 (Nikon, Surrey, UK). mESCs were visualised using a 40× magnification oil lens and captured on a Hamamatsu Digital Camera C4742-95. Data were collected using the IPlab 3.7 software (Nikon). For fluorescence microscopy, DAPI was excited at 400 nm and detected at 420 nm, FITC was excited at 505 nm and detected between 515 and 555 nm, and rhodamine was excited at 595 nm and detected between 600 and 660 nm. Negative controls were used to determine the appropriate exposure time and prevent the generation of false positives in the samples.

Western blotting

Cell lysates were prepared from non-transfected and siRNA-transfected sorted CCE/R cells, human embryonic kidney cells and MEFs. To obtain sufficient protein, lysates were pooled from separate experiments. Equal amounts of protein (100 μg) were electrophoresed on a 9% SDS-PAGE gel then semi-dry blotted onto a polyvinyl difluoride membrane by applying a current of approximately 0.8 mA/cm^2 of gel for 70 minutes. The membrane was blocked with 5% dried milk powder (Marvel, Premier Brands, UK) in Tris buffered saline solution (TBS) containing 0.1% Tween 20 (Sigma), for at least 1 hour at room temperature. The membrane was then probed with antibodies against either β-actin (1:10,000; Sigma), POLG (1:100; Abcam), OCT4 (1:10,000; Abcam) or brachyury (1:250; R&D Systems) overnight at 4°C. The membrane was washed with TBS/0.1% Tween 20 for 25 minutes with a change of wash solution every 5 minutes. The membrane was probed with secondary antibodies conjugated with horseradish peroxidase (Vector Labs), anti-rabbit (1:5000), anti-mouse (1:5000), anti-goat (1:5000) or anti-rabbit (1:5000), for 1 hour at room temperature. The membrane was washed with TBS/0.1% Tween 20 for 25 minutes with a change of wash solution every 5 minutes and the bands detected by enhanced chemiluminescence reagent (Perbio Science, Cramlington, UK). Between probing with each antibody the blot was stripped using Restore Western Blot Stripping solution (Perbio Science). Densitometry analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij).

Statistical analysis

One-way ANOVA was used to determine differences in *Polg*, *Polg2* and *Tfam* expression, and for mtDNA copy number, in spontaneously differentiated R1 and D3 mESCs and RA-induced D3 mESCs. Differences in the percentages of vimentin-, nestin-, β-tubulin III- and brachyury-labelled cells (vimentin$^+$, nestin$^+$, β-tubulin III$^+$ and brachyury$^+$ cells, respectively) during spontaneously differentiated D3 and RA-induced D3 mESCs, and differences in *Dppa5*, *Pramel7* and *Ndp52l1* expression during spontaneous differentiation of R1 mESCs were also determine by one-way ANOVA. Two-way ANOVA was used to determine differences in *Polg*, *Polg2* and *Tfam* expression, mtDNA copy number, and for the percentages of vimentin$^+$, nestin$^+$, β-tubulin III$^+$ and brachyury$^+$ cells between the spontaneously and RA-induced differentiation protocols. Data were transformed logarithmically where appropriate. Statistical differences were determined using unpaired *t*-tests and groups were considered significantly different when $P<0.05$.

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