The effects of nuclear reprogramming on mitochondrial DNA replication.

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Abstract

Undifferentiated mouse embryonic stem cells (ESCs) possess low numbers of mitochondrial DNA (mtDNA), which encodes key subunits associated with the generation of ATP through oxidative phosphorylation (OXPHOS). As ESCs differentiate, mtDNA copy number is regulated by the nuclear-encoded mtDNA replication factors, which initiate a major replication event on Day 6 of differentiation. Here, we examined mtDNA replication events in somatic cells reprogrammed to pluripotency, namely somatic cell-ES (SC-ES), somatic cell nuclear transfer ES (NT-ES) and induced pluripotent stem (iPS) cells, all at low-passage. MtDNA copy number in undifferentiated iPS cells was similar to ESCs whilst SC-ES and NT-ES cells had significantly increased levels, which correlated positively and negatively with *Nanog* and *Sox2* expression, respectively. During pluripotency and differentiation, the expression of the mtDNA-specific replication factors, *PolgA* and *Peo1*, were differentially expressed in iPS and SC-ES cells when compared to ESCs. Throughout differentiation, reprogrammed somatic cells were unable to accumulate mtDNA copy number, characteristic of ESCs, especially on Day 6. In addition, iPS and SC-ES cells were also unable to regulate ATP content in a manner similar to differentiating ESCs prior to Day 14. The treatment of reprogrammed somatic cells with an inhibitor of *de novo* DNA methylation, 5-Azacytidine, prior to differentiation enabled iPS cells, but not SC-ES and NT-ES cells, to accumulate mtDNA copies per cell in a manner similar to ESCs. These data demonstrate that the reprogramming process disrupts the regulation of mtDNA replication during pluripotency but this can be re-established through the use of epigenetic modifiers.

Introduction

The maternally inherited mitochondrial genome (mtDNA) encodes 13 of the 90+ subunits of the electron transfer chain (ETC) [1]. Within the ETC, the process of oxidative phosphorylation (OXPHOS) takes place, which generates the majority of cellular ATP [2]. Functional maturity of OXPHOS complexes I-V (CI-IV) relies upon the expression of nuclear encoded assembly factors coordinating the assembly of OXPHOS subunits encoded by both the nuclear and mitochondrial genomes [3,4,5]. Mutations, deletions and depletion of mtDNA, as well as nuclear mutations, can render OXPHOS-dependent cells dysfunctional, which in turn, result in either severely debilitating diseases or lethality [3,4,5,6,7]. Transcription and replication of the mitochondrial genome are dependent upon nuclear-encoded transcription and replication factors, which translocate to the mtDNA genome [8]. The key mtDNA replication factors include: the mtDNA specific polymerase, Polymerase Gamma, which consists of a catalytic subunit (POLGA) and its accessory subunit POLGB; mitochondrial transcription factor A (TFAM), which initiates the process of transcription and generates the DNA/RNA hybrid primer that is used to prime mtDNA replication by POLG; the mitochondrial specific helicase, Twinkle; and the mitochondrial single-stranded DNA binding protein (mtSSBP1). Twinkle and mtSSB combine with POLGA and POLGB to generate the minimal mtDNA replisome [9]. Mitochondrial biogenesis, mtDNA gene expression and assembly of the OXPHOS complexes demand coordinated nucleo-mitochondrial communication in order to satisfy cellular demands for ATP [4,10].

MtDNA replication is not initiated during preimplantation development until the final stage, the blastocyst stage, and appears to be restricted to the outer, trophectodermal cells [11,12]. The inner cell mass (ICM) cells, which are pluripotent and have the potential to differentiate into all somatic cells and germ cells, exhibit little or no mtDNA replication [11], and possess a mean mtDNA copy number of 4.83×10^3 per cell [13]. It is likely that the ICM cells continue to restrict mtDNA replication, thus mediating the continual dilution of mtDNA in each newly divided cell, as undifferentiated embryonic stem cells (ESC) possess between 30 and 45 copies of mtDNA/cell [14]. During the early stages of ESC differentiation, mtDNA replication remains at very low levels thus establishing the 'mtDNA set point', which provides all specialised cells with the capacity to acquire the appropriate number of mtDNA copies to meet their specific needs for OXPHOS-derived ATP. For example, fully

differentiated cells, such as skeletal and cardiac muscle cells possess 3650 ± 620 and 6790 ± 920 mtDNA copies/cell, respectively [15] whilst sheep fetal fibroblasts [16] contain 4241 ± 411 copies per cell.

Nevertheless, there is one key mtDNA replication event that takes place on Day 6 of ESC differentiation resulting in a significant increase to ~2370 mtDNA copies/cell [14]. This is then followed by a return to steady state levels of ~13 copies per cell on Day 7. Day 6 of murine ESC differentiation correlates to mouse embryonic day (E) 7.5, an early stage of organogenesis [17], which appears to be a critical checkpoint for mtDNA content, as homozygous *PolgA*$^{-/-}$ knockout mice do not survive passed this stage of development [18]. On the other hand, their heterozygous counterparts exhibit typical mtDNA depletion-like syndromes, such as cardiomyopathy. Furthermore, the regulation of pluripotency and the expression of *PolgA* appear to be finely balanced as demonstrated by partial knockdown of *PolgA*, which results in the loss of pluripotency [14]. This suggests that inconsistencies in this balance and the maintenance of the 'mtDNA set point' will affect a cell's potential to regulate its mtDNA copy number and pluripotent status effectively.

It remains to be determined whether somatic cells that have been reprogrammed to pluripotency are able to establish the 'mtDNA set point' and regulate mtDNA copy number during differentiation [19,20,21]. Reprogramming can be mediated by: i) fusion to ESCs [22,23]; ii) induction with defined factors associated with pluripotency [24,25]; and iii) somatic cell nuclear transfer (NT) [26]. NT embryos display reduced developmental competence due to the incomplete reprogramming of the somatic genome [26,27,28,29]. For example, NT embryos continue to express the mtDNA replication factors during preimplantation development, unlike their in vitro fertilised counterparts [16,30] and can preferentially replicate the somatic mitochondrial genome at the expense of the recipient oocyte's mtDNA [16]. However, ESCs derived from NT embryos appear to be more transcriptionally and epigenetically similar to ESCs than iPS cells [31,32]. Additional reprogramming anomalies are observed in induced pluripotent stem (iPS) cells [33,34], which require continuous passaging [35,36,37], serial reprogramming [32] or chromatin-modifying agents [32,38,39] to acquire ESC equivalence.

In order to determine whether reprogramming can effectively regulate mtDNA copy number during pluripotency and replicate mtDNA at key stages of differentiation, we have investigated these events in low passage somatic cell-embryonic stem (SC-ES) cell hybrids, iPS and NT-ES cells. We have demonstrated that, during pluripotency and the early stages of differentiation, SC-ES cell hybrids and NT-ES cells do not mirror the strict regulation of mtDNA replication exerted on ESCs, although undifferentiated iPS cells do more closely resemble patterns associated with ESCs. Nevertheless, iPS cells do not regulate this effectively during differentiation. Through treatment with 5-Azacytidine, we were able to modulate mtDNA copy number in iPS cells to mimic the early mtDNA replication events of ESCs but not in SC-ES and NT-ES cells.

Materials and Methods

Cell culture

Murine D3 ESCs; iPS 1, 2 and 3 [40], SC-ES 1, 2, 3 and 4 [41], ES-ES [42] and NT-ES [43] cell lines were cultured on gelatin coated flasks (0.1% w/v) in ESC media supplemented with 1000 U/mL (10 ng/ml) leukemia inhibitory factor (LIF; Chemicon, Millipore UK), as previously described [14]. Additional details can be found within the Supplemental Experimental Procedures.

Spontaneous differentiation

Undifferentiated ESCs were induced to differentiate using the hanging drop method [44]. Briefly, ESCs were cultured in ESC media minus LIF as 20 μl droplets (approximately 450 cells per drop) on the lid of an inverted Petri dish (Sterilin, Staffordshire, UK) for 48 hr (Days 1 to 2) to promote the formation of embryoid bodies. Embryoid bodies were then placed into suspension for a further 5 days at 37℃/5%CO2. They were then plated onto gelatin (0.1 %; Sigma)-coated six-well plates (Nunc, Roskilde, Denmark) and cultured up to Day 14 of differentiation.

Treatment with 5-Azacytidine (5-Aza) and Rhodamine 6G (R6G)

Pluripotent stem cells were cultured in ESC media for 24 hr and then cultured in ESC media supplemented with 10 μM 5-Aza for 72 hr. Differentiation was induced as described above. For R6G treatment, pluripotent stem cells were cultured in ESC media for 48 hr and then cultured in ESC media supplemented with 1 μg/μl R6G for 72 hr. For both treatments, the media was changed every 24 hrs.

Immunocytochemistry (ICC)

ICC for 5-Bromo-2'-deoxy-uridine (BrdU; Roche Applied Sciences, Sussex, UK) and MitoTracker Red (Molecular Probes, UK) was performed as previously described [14]. Samples were mounted on slides using mounting medium containing DAPI (Vectashield; Vector Labs, UK). Images were captured using a Carl Zeiss LSM 510 confocal microscope and images analysis was performed using Adobe Photoshop and Zeiss LSM Image Browser (http://www.zeiss.de).

ATP assay

The ATP content of pluripotent and differentiating cells was determined using the ATP-lite bioluminescence luciferase-based assay (Perlin Elmer, Australia), as described by the manufacturer. Luminescence was measured using a FLUOstar Optima plate reader (BMG Labtech, Germany). Samples were examined in triplicate simultaneously and each time point was measured a minimum of three times. The results were expressed as relative luminescence units compared to undifferentiated ESCs.

Blue-Native Polyacrylamide gel electrophoresis (BN-PAGE)

Whole cell extracts (100 µg) were solubilised in 1% Triton X-100 and separated on a 4-13% acrylamide gradient gel with a 4% acrylamide stacking gel, as previously described [45]. After BN-PAGE, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane and probed with primary antibodies against: polyclonal rabbit anti-NADH dehydrogenase (ubiquitin) 1 alpha subcomplex subunit 9 (Complex I) [10]; monoclonal mouse anti-succinate dehydrogenase flavoprotein subunit (Complex II; Molecular Probes, Invitrogen, Australia); monoclonal mouse anti-cytochrome b-c1 complex subunit 1 (Complex III; Molecular Probes, Invitrogen, Australia); and monoclonal mouse anti-cytochrome c oxidase subunit 1 (Complex IV; Molecular Probes, Invitrogen, Australia) overnight at 4℃. Proteins bands were detected using anti- goat or rabbit HRP-conjugated secondary antibodies (Sigma, Australia).

RT-PCR and Real Time PCR

RNA extraction was performed, as previously described in [14], and then reverse transcribed with the BioScript system (Bioline, London, UK). Total DNA was extracted using the Gentra Puregene DNA extraction kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. The samples were then treated with RNase solution (Qiagen) for 5 min at 37°C to obtain RNA-free DNA. Reactions were performed in a Rotorgene-3000 real-time PCR machine (Corbett Research, Cambridge, UK), as previously described in [14]. Additional details can be found in the Supplemental Experimental Procedures.

Statistical analysis

All real-time PCR data were normalised to β-Actin. A one-way ANOVA, in combination with Bonferroni post-hoc tests, was used to determine the differences in mtDNA copy number and expression of the mitochondrial specific replication factors (*Tfam, Peo1, mtSsbp1, PolgA* and *PolgB*) between undifferentiated cell types and between days of differentiation within a particular cell type. A two-way ANOVA in combination with Bonferroni post-hoc tests was used to determine differences in *Tfam, Twinkle, mtSsbp1, PolgA* and *PolgB* expression and mtDNA copy number between cell types during differentiation. The expression of Oct4, Nanog and Sox2 were ranked by statistical significance (1 = increased expression; 0 = non-significant; -1 = reduced expression) and the correlation with mtDNA copy was determined using Pearson's correlation coefficient. Statistical analysis was performed using GraphPad Prism 5.01 (Graph PadSoftware, Inc., San Deigo, CA, http://www.graphpad.com). Data are expressed as mean ± s.e.m.

Results

mtDNA copy number in pluripotent reprogrammed somatic cells

In order to determine whether mtDNA copy number in somatic cells is reset to match that of ESCs following reprogramming, we determined the number of mtDNA copies present in ESCs and mouse embryonic fibroblasts (MEFs), which provided the parental partners for the generation of four somatic-ES cell hybrid lines (SC-ES 1 to 4). The ESCs had low levels of mtDNA copy number per cell, namely 36.56 ± 1.79 whilst the MEFs contained 807.23 ± 21.8 copies ($P<0.001$; Fig. 1A). We then interrogated each of the hybrid lines, which have previously been demonstrated to be pluripotent and capable of differentiating into cell types from all three germ layers [41]. Although each of the lines was generated under the same conditions, we observed that SC-ES 2 and 3 (164.46 ± 4.76 and 164.41 ± 1.44, respectively) and SC-ES 1 and 4 (251 ± 3.06 and 284.96 ± 14.27, respectively) had very similar levels (Fig. 1A). These two sets were significantly different ($P<0.001$) and all four SC-ES hybrids were statistically different to the parent cell lines ($P<0.001$). However, to determine whether the increased copy number following fusion was the result of incomplete reprogramming or the tetraploid nature of the fusions, we analysed the mtDNA content of pluripotent ES-ES hybrid cells (ES-ES) [42]. They also had elevated levels of mtDNA copy number per cell (259.76 ± 6.32; Fig. 1A), which were significantly different to levels in SC-ES 2 and 3 ($P<0.001$), MEFs ($P<0.001$), and the parental cell line, ESCs ($P<0.001$).

To determine whether these outcomes resulted from discrete incomplete reprogramming of the somatic cells, we analysed the mtDNA copy number of three separate iPS cell lines (iPS 1, 2 and 3) produced from the same MEF parental line used to generate SC-ES 1 to 4. In their undifferentiated state, there was little variability between the lines with mean copy numbers per cell being 45.62 ± 3.92, 54.12 ± 6.47 and 47.09 ± 0.62, respectively ($P>0.05$; Fig. 1A), which was not significantly different to the ESCs ($P>0.05$; Fig. 1A), but was significantly different to the parental cell line ($P<0.001$). Finally, we examined undifferentiated NT-ES cells previously shown to be pluripotent and to contribute to all three germ layers [43]. This cell line contained elevated numbers of mtDNA copy (196.93 ± 13.96) compared to ESCs and iPS cells ($P<0.001$) and similar copy number to SC-ES 1 and 4 cells and ES-ES cells (Fig. 1A). These data suggest that iPS cells derived from reprogrammed MEFs can mediate both pluripotent gene expression and mimic levels of mtDNA copy number

associated with undifferentiated ESCs, whilst the cells derived from cell fusion and nuclear transfer fail to reset mtDNA copy number.

The relationship between mtDNA copy number and pluripotency

Using quantitative RT-PCR, we analysed the transcript levels of the pluripotent markers *Oct4* (Fig. 1B), *Nanog* (Fig. 1C) and *Sox2* (Fig. 1D) relative to ESCs in reprogrammed cells (iPS 2, SC-ES 1, ES-ES and NT-ES). Undifferentiated SC-ES 1 and ES-ES cells expressed similar levels of *Oct4* to ESCs while transcripts were elevated in iPS 2 ($P<0.01$) and reduced in NT-ES ($P<0.01$) cells. *Nanog* transcripts in iPS and ES-ES cells were similar to ESCs levels, although SC-ES ($P<0.01$) and NT-ES ($P<0.01$) cells expressed relatively higher levels. Similarly, for *Sox2* expression, SC-ES ($P<0.05$) and NT-ES ($P<0.05$) cells expressed significantly lower levels than observed for ESCs, whilst iPS and ES-ES cells were comparable to ESCs. The similarity in pluripotent gene expression between tetraploid ES-ES and ESCs most likely reflects the absence of nuclear reprogramming upon fusion of ESCs and the elevated mtDNA copy number in ES-ES cells may be attributed to their tetraploid nucleus. To further evaluate the effects of reprogramming on mtDNA copy number, we performed linear regression analysis of pluripotent gene expression ranked by statistical difference against mtDNA copy number (Fig. 1E). These calculations demonstrate their mtDNA copy number is positively and negatively correlated ($R^2 = 0.9039$; $P<0.05$) with *Nanog* and *Sox2*, respectively. These correlations account for the elevated levels of mtDNA copy number observed in SC-ES and NT-ES cells.

ATP content and OXPHOS complex expression in pluripotent stem cells

Due to the differences in mtDNA copy number between the different reprogrammed cell types, we then examined the effects of reprogramming on ATP content (Fig. S1A). All reprogrammed pluripotent cell types and ESCs contained significantly lower levels of ATP than MEFs ($P<0.001$). Although reprogramming by cell fusion (SC-ES and ES-ES) resulted in ATP levels being comparable to ESCs ($P>0.05$), iPS cells had reduced cellular ATP compared to ESCs ($P<0.05$). In order to determine whether the differences in copy number observed in reprogrammed cells influenced ATP content, we depleted reprogrammed somatic cells with the mitochondrial toxin, Rhodamine 6G (R6G; 1 μg/ml), which is a known mtDNA depletion reagent [46, 47]. After 72 hr of R6G treatment, mtDNA copy number of MEFs was depleted to 9.4% of control levels (Fig. S1B) and ATP content was reduced by

33.7% (Fig. S1A; P<0.05). Treatment of ESCs, and iPS 2, SC-ES 1 and ES-ES cells reduced mtDNA copy number to 26.5%, 27.6%, 8.7% and 9.9% of their untreated levels, respectively (Fig. S1B), though no significant effect was observed on ATP content (Fig. S1A). These data suggest that ESCs and reprogrammed cell types do not rely on OXPHOS as their main source of ATP.

In order to determine whether the lower ATP content observed in pluripotent cells was a consequence of low or absent OXPHOS complexes, we analysed the steady state levels of the OXPHOS complexes (CI-IV) by BN-PAGE (Fig. S1C). CI, CII and CIV were present at similar levels in ESCs, iPS, SC-ES and NT-ES cells compared to MEFs, while ES-ES had elevated levels of CIII compared to ESCs and MEFs (Fig. S1C). These findings indicate that the low ATP content in pluripotent cells is not due to the absence of stable OXPHOS complexes.

Expression of mtDNA replication factors in iPS and SC-ES cells

In order to determine whether reprogrammed somatic cells are able to restrict mtDNA replication in their undifferentiated, pluripotent state, we labelled these cells with 5-bromo-2-deoxyuridine (BrdU) and the mitochondrial specific label, MitoTracker Red. iPS cells demonstrated increased incorporation of BrdU into their mtDNA when compared with ESC and SC-ES cells suggesting that they were more actively replicating mtDNA (Fig. 1F). We then investigated whether the differences in mtDNA copy number and BrdU incorporation in reprogrammed cell types were a consequence of variable expression of the mtDNA-specific replication factors. We analysed each of the cell lines for expression of *Tfam*, *Twinkle (Peo1)*, *mtSsbp1*, *PolgA* and *PolgB* mRNA by real time PCR (qPCR) (Fig. S2). For *PolgA*, only ES-ES cells (P>0.05) were not statistically different to undifferentiated ESCs (Fig. S2A). The MEF cells expressed statistically less *PolgA* (P<0.001) than observed for undifferentiated ESCs, while the SC-ES and iPS cells expressed significantly more (P<0.001; see Fig. S2A). *Peo1* levels in MEFs and SC-ES 1, 2 and 4 cells were, however, only significantly different to ESCs (Fig. S2B). When compared to ESCs, SC-ES 2 and iPS 1 and 2 cells expressed significantly less *mtSsbp1* (Fig. S2C). *PolgB* levels were significantly reduced in ES-ES cells (P<0.01), iPS 1, 2 and 3 cells (P<0.01), MEFs (P<0.001) and SC-ES 2 and 4 cells (P<0.01) whilst SC-ES 1 cells expressed significantly more (P<0.01; Fig. S2D). Furthermore, MEF levels were considerably reduced when compared to all cell types (P<0.001). The patterns of expression for *Tfam* demonstrated little

difference between all pluripotent cell types (Fig. S2E), however, they all expressed elevated levels when compared to MEFs ($P<0.001$).

Expression of genes of differentiation in reprogrammed cells

We verified the potential of the reprogrammed cell types to differentiate into all three germ layers (Fig. 2). Each reprogrammed cell type expressed genes characteristic of the mesoderm (*CD31* and *VEGFR*), ectoderm (*Nestin* and *MAP2*) and endoderm (*AFP* and *FoxA2*). Nevertheless, we observed some differences between cell types. Of note, *MAP2* expression was lower in NT-ES cells prior to Day 14 and FoxA2 expression peaked earlier in SC-ES cells than in other cell types (Fig. 2).

Patterns of mtDNA replication in differentiating reprogrammed cells

During the early stages of ESC differentiation, mtDNA replication remains strictly regulated with a key replication event taking place on Day 6 of differentiation resulting in a significant increase in the number of mtDNA copies/cell [14]. To assess whether reprogrammed cells were able to regulate their mtDNA copy number in a manner similar to ESCs during the process of differentiation, we generated embryoid bodies and analysed their mtDNA replication events at 24 hr intervals during Days 1 to 7 and Day 14 of differentiation. Firstly, we focused on mtDNA copy number during differentiation. After an initial increase in mtDNA copy number in SC-ES 1 cells at 24 hr of differentiation ($P<0.001$), levels dropped to lower than those of the undifferentiated cells on Days 2, 4 and 5 ($P<0.001$) whilst levels were not significantly different on Days 3, 6 and 7 ($P>0.05$) (Fig. 3A). We then interrogated iPS 2 cells and detected a completely different pattern (Fig. 3B), where following an initial burst of mtDNA replication on Day 1, mtDNA copy number returned to steady state levels on Day 2. However, there were significant increases on Days 3 and 5 ($P<0.001$), followed by a return to low levels of copy on Days 4 and 6. Consequently, neither cell type exhibited the large increase in mtDNA copy number observed in ESCs on Day 6 [14]. Whilst both sets of reprogrammed lines had approximately 200 copies of mtDNA per cell on Day 7, there were significantly more copies present on Day 14 for the hybrid cells ($P<0.001$), as would be anticipated in cells committing to various fates. Again dual-staining with BrdU and MitoTracker confirmed that mtDNA replication was active during the early stages of differentiation (Fig. 3C) and that SC-ES 1 and iPS 2 cells had extended cytoplasms.

In order to determine whether discordant mtDNA replication events during differentiation were a consequence of nuclear reprogramming or ploidy, we analysed mtDNA copy number for ES-ES cell fusions and NT-ES cells. After 24 hr of differentiation, no difference was detected for ES-ES cells (Fig. 3D), whilst NT-ES cells (Fig. 3E) displayed a significant increase ($P<0.001$), similar to events reported for R1 ESCs [14], but at far lower levels. On Days 6 and 7 of ES-ES differentiation, a progressive increase in copy number was evident, with a considerable increase by Day 14 ($P<0.001$). At the same time points, NT-ES cells had a copy number similar to their undifferentiated cells (Fig. 3E).

We then determined if mtDNA turn over on Day 6 of differentiation was associated with the loss of pluripotency, specifically the levels of expression of Oct 4, Nanog and Sox2 on Days 6 and 7 of differentiation. On Day 6 of differentiation, *Oct4* transcript levels were reduced compared to undifferentiated ESCs for all cell types (Fig. 1B) and no differences were observed between the cell types. Similar levels of *Oct4* transcript were observed on Day 7 although the ES-ES cell line demonstrated a further reduction between Days 6 and 7 (Fig. 1B; $P<0.001$). *Nanog* expression for ESCs remained statistically similar throughout differentiation although iPS cells expressed high levels on Day 6 ($P<0.01$) and extremely low levels on Day 7 ($P<0.01$; Fig. 1C). SC-ES ($P<0.01$) and NT-ES ($P<0.001$) cells contained fewer *Nanog* transcripts on Day 6 compared to their undifferentiated states, and these levels were similar to those observed for ESCs. Expression of *Sox2* in iPS cells was reduced on Days 6 and 7 of differentiation compared to the undifferentiated state, whilst ES-ES cells demonstrated a decrease on day 7 ($P<0.01$; Fig. 1D). Both SC-ES ($P<0.01$) and NT-ES ($P<0.01$) cells expressed higher levels of *Sox2* on Day 7 compared to their undifferentiated counterparts and ESCs. Linear regression analysis of mtDNA copy number and pluripotent gene expression determined that mtDNA copy number was not a function of pluripotent gene expression during differentiation (data not shown).

Expression of mtDNA replication factors during differentiation of pluripotent stem cells

As BrdU staining and variability in mtDNA copy number indicated that diverse mtDNA replication events were taking place during differentiation, we determined the levels of transcriptional activity for *Tfam* (Fig. 4A), *Peo1* (Fig. 4B), *mtSsbp1* (Fig. 4C), *PolgA* (Fig. 4D) and *PolgB* (Fig. 4E) at several key stages of mtDNA replication during differentiation. It was evident that there were considerably lower

levels of expression for *Peo1* on Days 1, 7 and 14 in the reprogrammed somatic cells when compared to ESCs at the same time point (Fig. 4B; P<0.01 to 0.001). There was significantly less *Tfam* (Fig. 4A) and *PolgA* (Fig. 4D) on Days 1, 7 and 14 when compared to their undifferentiated counterparts. However, the increased levels of *PolgA* (Fig. 4D) in SC-ES 1 cells on Days 7 and 14, compared to Day 1, suggest that they were primed for mtDNA replication.

ATP content and the steady state levels of the OXPHOS complexes during differentiation

We then determined whether the disparities in mtDNA copy number and mtDNA replication factor expression in the reprogrammed somatic cells during differentiation would affect their capacity to produce ATP during differentiation. We observed on Day 7 of differentiation that iPS cells contained significantly less ATP than ESCs (P<0.01) whilst SC-ES had elevated levels of ATP (P<0.05; Fig. S3A). Subsequently, ATP levels on Day 14 were not significantly different between ESC, iPS, SC-ES and ES-ES cells (P>0.05; Fig. S3B) though these levels were statistically elevated compared to their undifferentiated counterparts (P<0.001: Fig. S1A cf Fig. S3B).

Analysis of steady state levels for the OXPHOS complexes after resolving by BN-PAGE revealed that iPS cells, on day 7 of differentiation, contained higher levels of CI, CIII and CIV than ESCs and MEFs (Fig. S3C). At the same time point, ESCs, ES-ES and NT-ES cells all contained similar levels of CI-IV whilst SC-ES cells contained lower steady state levels of CIV compared to ESCs (Fig. S3C).

The effects of 5-azacytidine (5-Aza) on mtDNA replication and copy number

As it has been previously shown that treatment of low-passage iPS cells with chromatin modifying drugs improves reprogramming outcomes [32,38,39], we hypothesized that resetting the epigenome of ESCs and reprogrammed somatic cells with the DNA methyltransferase inhibitor, 5-Azacytidine (5-Aza), would directly influence mtDNA copy number. Firstly, we treated ESCs with 5-Aza ($ESC^{5\text{-}Aza}$) for 72 hrs prior to embryoid body formation and differentiation. In undifferentiated cells, this increased $ESC^{5\text{-}Aza}$ mtDNA copy number from 36.56 ± 1.79 to 61.23 ± 5.59 (Fig. 5A), whilst 5-Aza treatment had no effect on iPS ($iPS^{5\text{-}Aza}$) cell mtDNA copy number (P>0.05; Fig. 5B) but resulted in an increase in ATP levels in iPS cells, similar to ESCs (P<0.05; Fig. 5F). Following 6 days of differentiation, ESC mtDNA copy number decreased from 2370 to 420.92 ± 53.65 with a similar pattern being observed on

Day 14 (Fig. 5A). On the other hand, iPS$^{5\text{-Aza}}$ cells were able to mediate the significant increase in mtDNA copy number on Day 6 (2112.82 ± 111.53) and Day 14 (7228.45 ± 141.97) with a concurrent reduction observed on Day 7 (579.15 ± 43.87; Fig. 5B cf Fig. 3B). Conversely, undifferentiated SC-ES$^{5\text{-Aza}}$ (Fig 5C cf Fig. 3C) and ES-ES$^{5\text{-Aza}}$ (Fig. 5D cf Fig. 3D) cells contained significantly fewer copies of mtDNA (195.79 ± 13.87; $P<0.05$ and 186.96 ± 6.03, respectively; $P<0.001$) after 5-Aza treatment. SC-ES$^{5\text{-Aza}}$ (Fig. 5C) cells were unable to increase mtDNA copy on Day 6 (876.91 ± 101.49) and 14 (883.8 ± 79.94) to match ESCs though numbers were increased when compared to their untreated counterparts (Fig. 3A). In ES-ES$^{5\text{-Aza}}$ cells, an increase to 1896.37 ± 128.79 and a decrease to 363.31 ± 12.78 mtDNA copies/cell were also detected on Days 6 and 7, respectively. This was followed by an increase on Day 14 (1182.82 ± 47.74 mtDNA copies/cell; Fig. 5D), which, although dissimilar to ESCs, resulted in a different mtDNA copy number profile during differentiation when compared to untreated cells (Fig 3D). Treatment of NT-ES cells with 5-Aza (NT-ES$^{5\text{-Aza}}$; Fig 5E cf Fig. 3E) had no effect on the undifferentiated state (203.23 ± 17.32 mtDNA copies/cell). However, during differentiation, mtDNA copy number decreased on Day 1 (158.82 ± 17.81) and significantly increased on Day 7 (1155.12 ± 137.28). The outcomes show that treatment with 5Aza results in iPS, but not SC-ES and NT-ES, cells being able to mediate their mtDNA replication events in a manner similar to ESCs.

Discussion

The data presented here demonstrate that somatic cell reprogramming by cell fusion (SC-ES cells) and NT results in a 5- to 9-fold higher mtDNA copy number per pluripotent stem cell than observed in ESCs, whilst undifferentiated iPS cells have similar mtDNA copy number to ESCs. Nevertheless, none of the reprogrammed cell types were able to modulate the key mtDNA replication events that take place in ESCs during differentiation. However, the culture of undifferentiated reprogrammed cells in 5-Aza demonstrated that iPS cells, but not SC-ES and NT-ES cells, could mimic the key mtDNA replication events of the ESCs during differentiation.

We have previously hypothesised that pluripotent stem cells establish the 'mtDNA set point' [19,20], which ensures that they maintain low numbers of mtDNA during pluripotency and early differentiation. In this instance, mtDNA replication is restricted to replenishing the mitochondrial genome following cell division or when cells reach precariously low levels of mtDNA. ESCs maintain this pattern during the early stages of differentiation except for one major replication event that takes place on Day 6 of differentiation. MtDNA copy number then returns to low levels until Day 11 [14, 21]. Consequently, the establishment of the mtDNA set point is an essential part of cellular differentiation as it enables differentiating cells to acquire the appropriate numbers of mtDNA to meet the OXPHOS specific requirements of the specialised cell and its function once commitment has taken place [21].

Our data suggest that the establishment of the mtDNA set point is dependent on the degree of pluripotency within cells. The elevated mtDNA copy number observed in undifferentiated SC-ES and NT-ES cells correlated with the variable levels of expression of the pluripotent transcription factors, *Sox2* and *Nanog*, whereby they exhibited elevated levels of *Nanog* and decreased levels of *Sox2* when compared with ESCs. Furthermore, *Oct4* expression was reduced in NT-ES cells, while iPS cells displayed elevated *Oct4* expression and low copy number. Whilst upregulation of *Nanog* has been proposed as an essential requirement for establishing and maintaining pluripotency [48,49], variation within the OCT4-SOX2-NANOG network alters the expression of their target genes, including their own expression and triggers the onset of differentiation [50,51,52]. We argue that the disparity in expression of the genes of the OCT4-SOX2-NANOG network could be attributed to incomplete reprogramming of the somatic genome, as described by others [32,53] resulting in the failure to fully

establish pluripotency and thus the mtDNA set point. Indeed, undifferentiated iPS cells were also primed for mtDNA replication, as demonstrated by increased levels of labelling for BrdU coupled to the increased expression of PolgA. Consequently, reduced mitochondrial expansion and mtDNA copy number may be attributed to mitochondrial regulatory processes, such as mitophagy, as observed in ESCs [53]. Nevertheless, iPS cells appear to be unable to exert strict control on mtDNA replication, hence the 2 to 3 fold increase in mtDNA copy number and expanded cytoplasms observed on Day 1 of differentiation. Although undifferentiated ES-ES fusions displayed similar levels of expression for *Oct4*, *Nanog* and *Sox2* to ESCs, they have elevated mtDNA copies. This suggests that the regulation of mtDNA copy number in ES-ES cells is influenced by their tetraploid nature. In the case of the undifferentiated SC-ES cells, their partial reprogramming and tetraploidy is likely to further complicate their ability to regulate mtDNA copy number.

The copy number present in the SC-ES and NT-ES cells is very similar to somatic progenitor cells, which tend to have approximately 200 copies of mtDNA per cell [54], as do primordial germ cells (PGCs) [55]. In terms of their mtDNA profiles, SC-ES and NT-ES cells are possibly further down the pathway of differentiation than ESCs. Nevertheless, this phenomenon is not unique as PGCs re-express key pluripotent genes during differentiation [56] as do some cancer stem cells [57], suggesting that reestablishment of pluripotent gene expression does not necessarily reduce mtDNA copy number to mtDNA set point levels. Not only do PGCs re-establish pluripotency, they express other cell specific transcriptional regulators [58,59,60], which, as for other progenitor cells, will ensure that mtDNA copy number is tightly controlled. However, variations of this process do not seem to have been acquired by reprogrammed somatic cells.

In support of our hypothesis that incomplete reprogramming fails to establish the mtDNA set point, we have observed, as have others [61], that the expression of pluripotent genes, such as *Oct4*, *Nanog*, *Dppa5*, *Pramel7* and *Ndp52116* [14,21], fluctuate as differentiation is initiated. The patterns of pluripotent gene expression we observed in the undifferentiated reprogrammed somatic cells is similar. Consequently, none of the reprogrammed somatic cell types, were able to maintain the low numbers of mtDNA copy during the early stages of differentiation, as demonstrated by differentiating ESCs. Most critically, none of the reprogrammed cell types were able to mediate the key mtDNA

replication event that takes place on Day 6 of differentiation, and exhibited quite distinct patterns of gene expression for Nanog and Sox2 on Days 6 and 7. However, it appears that iPS cells attempt to compensate through a small increase in mtDNA copy number on Day 7, though they also failed to mediate the increase in mtDNA copy number on Day 11. Therefore, we propose that, by not having clearly established pluripotency, reprogrammed somatic cells do not establish the 'mtDNA set point', which further reflects their inability to coordinate the assembly of the electron transfer chain, as shown on Day 7 in iPS cells.

The effects of reprogramming are likely to directly influence mtDNA replication, as this process is highly dependent on the expression of nuclear-encoded genes that, once transcribed and translated, translocate to the mitochondrion. Indeed, the mitochondrial-specific polymerase, PolgA, appears to be an important sensor for both pluripotency and mtDNA proliferation during early development. It has previously been shown that partial knockdown of PolgA in undifferentiated ESCs results in the decrease in Oct4 expression and forces differentiation into early lineages [14]. The failure to initiate mtDNA replication through the expression of PolgA at specific stages during early development has undesired effects whereby PolgA$^{+/-}$ mice exhibit mtDNA depletion syndromes, which result in serious effects on organ function, and, in particular, triggers the onset of cardiomyopathy [18]. On the other hand, PolgA$^{-/-}$ mice are embryonic lethal from E7.5, a time point that is comparable to Day 6 of ESC differentiation [17]. Furthermore, Tfam$^{+/-}$ mice also exhibit mtDNA depletion and Tfam$^{-/-}$ mouse embryos do not survive after E8.5 to 10.5. [62], which is equivalent to Day 7+ of differentiation. Consequently, it is likely that failure to mediate the key mtDNA turnover event at Day 6 would result in differentiated cells possessing few copies of mtDNA, and thus exhibit mtDNA depletion-like syndromes [63,64].

Whilst the differences in the regulation of mtDNA copy number might be an indirect effect of cellular ploidy in SC-ES and ES-ES cells, thus altering the expression of the nuclear-encoded mtDNA replication factors and, in turn, mtDNA copy number, the observed outcomes are likely to arise from aberrant epigenetic regulation. Indeed, recent data demonstrate that reprogrammed cells are not equivalent to ESCs [35,38,39,65,66]. ESC equivalence is acquired in iPS cells when cultured continually [26, 29, 31], serially reprogrammed [26] or treated with epigenetic modifying agents

[32,38,39]. The treatment of partially reprogrammed iPS cells with 5-Aza [32,39] and valproic acid [38] induces epigenetic changes that improve the reprogramming outcomes of iPS cells. In agreement with these studies, we find differential expression of genes involved in mitochondrial biogenesis (*PolgA and Peo1*) characteristic of differentiated cells, and a consequent failure to accumulate thousands of mtDNA copies per cell. The alterations in mtDNA copy number, and ATP generation, in 5-Aza treated iPS cells validates the above conclusions and suggests that the regulation of mtDNA replication may be either directly or indirectly related to the epigenetic regulation of one or more of the nuclear-encoded mtDNA-specific replication factors or the downstream effects of general reprogramming.

The ability of a cell to regulate mtDNA replication effectively is perhaps inherent of it having undergone a consistent process of regulation. As ESCs are derived from the ICM cells of the blastocyst [73], they will have undergone a process of mtDNA reduction mediated by the preimplantation embryo with each newly divided cell having fewer copies of mtDNA [12]. Although mtDNA replication is triggered at the blastocyst stage in the trophectoderm, the 4.83×10^3 mtDNA copies present in the ICM cells continue to be reduced until the onset of gastrulation [13], as demonstrated by 30 and 45 copies in mouse ESCs [14,21]. It is this continual reduction in mtDNA copy number that establishes the mtDNA set point, which is maintained during the early stages of differentiation. This allows cells during the later stages of differentiation to acquire the appropriate numbers of mtDNA to support their specific OXPHOS-derived ATP requirements that are suited to their specialisation [21]. Our data would indicate that none of the reprogrammed somatic cells have acquired the mtDNA set point as evidenced by their failure to reduce their mtDNA copy number in the undifferentiated state (SC-ES and NT-ES cells) and maintain this state during the early stages of differentiation (SC-ES, NT-ES and iPS cells).

The differential regulation of mtDNA replication in differentiating reprogrammed cells would probably generate outcomes similar to those associated with NT where mtDNA replication is not controlled in the preimplantation embryo with persistent upregulation of the mtDNA replication factors [16,30]. Many of these offspring do not survive post-parturition because, once ex-utero, they are no longer able to support themselves metabolically [67]. Our data suggest that ESCs derived from NT embryos display aberrant reprogramming during *in vitro* differentiation consistent with the inefficient production of

cloned offspring. However, recent reports suggest NT-ES cells are phenotypically similar to fertilised ESCs [32]. These differences may be attributed to the use of a MEK1 inhibitor during NT-ESC derivation [27,32,49,52], which promotes reprogramming and establishment of ESCs [49,52,76,68]. This failure of NT-ES cells to regulate mtDNA copy during differentiation suggests that the influence of control on mtDNA copy number may arise from the maturing oocyte, which modifies its mtDNA content up to the metaphase II stage, so that under normal conditions mtDNA replication is restricted and species-specific changes in mtDNA copy number occur during preimplantation development [11]. Consequently, any reprogrammed somatic cell will have escaped these effects and, in combination with partial modulation of the mtDNA replication factors, a nucleus transferred into an enucleated oocyte will elicit aberrant patterns of mtDNA replication providing further distortion to early mtDNA replication events.

Previous reports of human pluripotent cells have demonstrated a similarity in mtDNA copy number between differentiated ESCs and iPS cells [69,70]. In this context, the deregulation of mtDNA copy number presented here may be attributed to variations between human and mouse mtDNA turn over during differentiation [14,71] or the heterogeneous nature of the reprogramming process [32,33,34,52]. The variability in mtDNA copy number during the early stages of differentiation impacts on the ability of reprogrammed cells to generate appropriate levels of ATP at these stages, as demonstrated by iPS cells containing significantly less ATP and SC-ES cells producing significantly more on Day 7 than ESCs. Contrastingly, on day 7 of iPS differentiation the steady state levels of CI, III and IV were considerably higher than all other cell types analysed. The levels of ATP generated on Day 14 and levels of steady state complexes for iPS and SC-ES were similar to ESCs while mtDNA copy number was considerably lower. These discrepancies between ATP and mtDNA copy number in iPS cells may reflect a compensatory mechanism whereby expression of the OXPHOS subunits is increased to counteract low levels of ATP. Indeed, increased subunit expression is observed in some mitochondrial-type diseases to compensate for defects in the ETC [72,73]. However, the inability of reprogrammed cells to produce mtDNA copy number characteristic of differentiated cells was not reflected in the levels of ETC complexes or the ATP content on day 14. The lack of correlation between ATP content and mtDNA copy number during later stages of differentiation mimics mtDNA disease phenotypes in which debilitating mutations in the mtDNA manifest as clinical symptoms only

once a threshold of mutant vs wild-type is breached in a stage specific manner [74]. In some instances, mutant threshold correlates with reduced OXPHOS capacity only upon high energy demand, for example during exercise [75].

The importance of the continued upregulation of mtDNA copy number to the generation of ATP has been demonstrated in cybrid models of mtDNA disease [76]. These anomalies are of concern as there is a number of ATP-mediated developmental events that take place during these early stages. These include the epigenetic regulation of gene expression [77], which will impact on cellular differentiation and function with the likelihood that ATP levels determine the extent to which these epigenetic modifications can take place [78]. It has been accepted that the mitochondrial genome is not methylated [79,80], although a recent study has reopened this debate [81]. Since the mtDNA replication factors are nuclear encoded, it is likely that ATP-mediated epigenetic regulation of mtDNA will function through nuclear factors. There is some evidence suggesting that the expression of PolgA is mediated by epigenetic modification, namely an increase in DNA methylation of exon-2 of this gene [82]. Such regulation of mtDNA appears to be vital during spermatogenesis as those sperm cells capable of fertilisation have significantly lower numbers of mtDNA copy compared to those that fail to fertilise [82]. This mechanism would also account for the cell specific regulation of mtDNA copy number where there is large variability between cell types [15].

In conclusion, we propose that the control of mtDNA replication following somatic cell reprogramming is dependent on the complete reestablishment of pluripotency including the OCT4-SOX2-NANOG network. This would enable the mtDNA set point to be established so that mtDNA copy number is strictly regulated during pluripotency and differentiation. It is apparent from our data that this process is better achieved by cells undergoing induced pluripotency, since they adopt mtDNA replication and ATP profiles similar to ESCs during pluripotency and differentiation, following treatment with an epigenetic modifier. Nevertheless, the epigenetic control of mtDNA replication still requires elucidation, which is most likely a phenomenon that is inherited or set in the oocyte just prior to fertilisation.

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Figure Legends

Figure 1. The relationship between mtDNA copy number and pluripotency in reprogrammed cells. (A): Real- time PCR was performed to calculate the mtDNA copies/cell in undifferentiated iPS cells (lines 1-3), SC-ES cells (lines 1-4), ES-ES cells and their parental cell lines, MEFs (QS/Rosa26) and D3 ESCs (ESC); and NT-ES cells. All samples were normalised to β-actin. Bars represent means ± s.e.m; significant differences between cell types are indicated (*** P<0.001). Expression of *Oct4* (B), *Nanog* (C) and *Sox2* (D) was analysed by quantitative real-time PCR in mouse ESCs, iPS 2, SC-ES 1, ES-ES and NT-ES cells in their undifferentiated states and on Day 6 and 7 of differentiation. All samples were normalised to β-actin. Bars represent means ± s.e.m; significant differences between cell types are indicated (* P<0.05, ** P<0.01, *** P<0.001). (E) Altered pluripotent gene expression is correlated with elevated mtDNA copy number in reprogrammed cells. Pluripotent gene expression in ESCs, iPS, SC-ES and NT-ES was ranked (increased or decreased ranking based on relative expression against control ESCs) and plotted against mtDNA copy number. The correlation coefficient signifies the positive relationship between mtDNA and pluripotent gene expression in SC-ES and NT-ES. (F) Mitochondrial replication was examined by dual-labelling of BrdU (green) and MitoTracker (red) in undifferentiated (Und) ESCs, and SC-ES 1 and iPS 2 cells. ESCs and SC-ES cells demonstrated little or no replicating mtDNA while iPS cells contained large amounts of replicating mtDNA. Cell nuclei were visualised by DAPI (blue) staining. Scales bars 10 μm.

Figure 2. Expression of genes of differentiation in reprogrammed cells. ES and iPS 2, SC-ES 1, ES-ES and NT-ES cell differentiation was confirmed by RT-PCR analysis of germ layer markers: mesoderm (CD31 and VEGFR), ectoderm (Nestin and MAP2) and endoderm (AFP and FoxA2). β-actin was used to indicate equal loading.

Figure 3. MtDNA replication during in vitro differentiation. Embryoid bodies from (A) SC-ES 1 and (B) iPS 2 cells were analysed using real time PCR to determine the mtDNA copy number per cell during differentiation: Days 1 to 7 and 14 after the removal of LIF. All samples were normalised to β-actin and bars represent means ± s.e.m. Significant differences between cell types are indicated (* P<0.05, ** P<0.01, *** P<0.001). (C) Dual-labelling with BrdU (24 hr; green) and MitoTracker (Red) on Day 1 of differentiation showed increased mtDNA replication and cytoplasmic expansion in iPS and

SC-ES cells. Cell nuclei were visualised by DAPI (blue) staining. Scales bars 10 μm. Differentiating cells from (D) ES-ES (E) and NT-ES cells were analysed using real time as described in (A) on Days 1, 6, 7 and 14 of *in vitro* differentiation. All samples were normalised to β-actin and bars represent means ± s.e.m. Significant differences between cell types are indicated (*** P<0.001).

Figure 4. The expression of the mtDNA-specific replication factors during in vitro differentiation of reprogrammed somatic cells. Expression of (A) *Tfam,* (B) *Peo1,* (C) mt*Ssbp1,* (D) *PolgA* and (E) *PolgB* in ESC, SC-ES and iPS cells was analysed by quantitative real-time PCR on Days 1, 7 and 14 of differentiation. All samples were normalised to ***β-actin*** and values are expressed as a ratio of undifferentiated (Und) ESCs. Bars represent means ± s.e.m. The significant differences between cell types and between days of differentiation within a particular cell line are indicated (* P<0.05, ** P<0.01, *** P<0.001).

Figure 5. Treatment of pluripotent iPS cells with 5-Aza improves the regulation of mtDNA copy number during differentiation. Undifferentiated (und) (A) ESC cells and (B) iPS 2, (C) SC-ES 1, (D) ES-ES and (E) NT-ES cells were treated with 10 μM 5-Aza for 72 hr prior to differentiation and the effects on mtDNA copy were analysed by real time PCR on undifferentiated cells and Days 1, 6, 7 and 14 of *in vitro* differentiation. Significant differences between cell types are indicated (* P<0.05; ** P<0.01; *** P<0.001). ATP levels were analysed in ESC, iPS 2, SC-ES 1, ES-ES, and NT-ES pluripotent stem cells in normal ESC media or media supplemented with 10 μM 5-Aza for 72 hr (F). The values are relative luminescent units (RLU) normalised to ESCs from three independent experiments. Bars represent means ± s.e.m; significant differences between treatments for each cell type are indicated (* P<0.05).

Figure 1

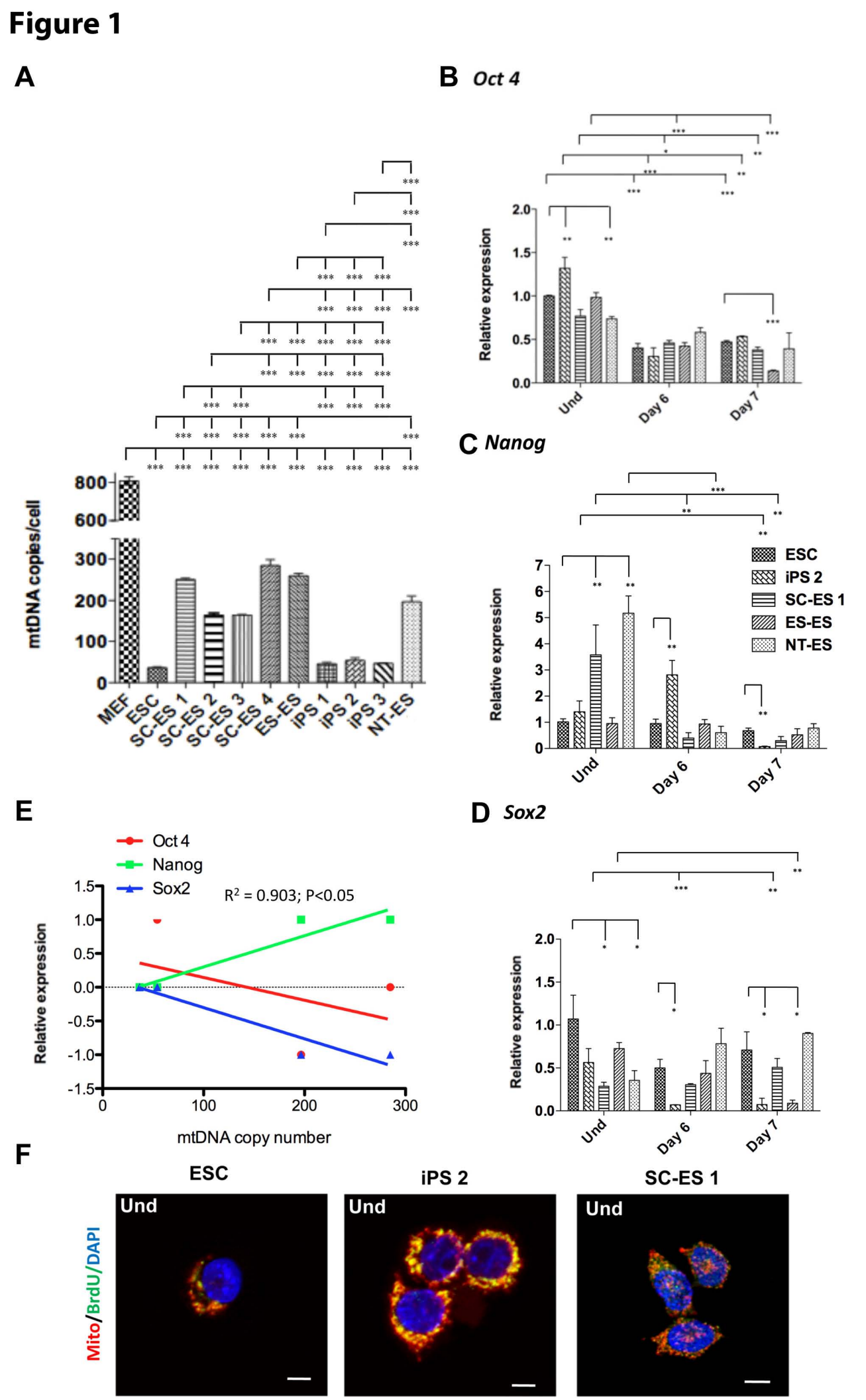

A
B Oct 4
C Nanog
D Sox2
E
F
mtDNA copies/cell
MEF
ESC
SC-ES 1
SC-ES 2
SC-ES 3
SC-ES 4
ES-ES
iPS 1
iPS 2
iPS 3
NT-ES
Relative expression
Und
Day 6
Day 7
ESC
iPS 2
SC-ES 1
ES-ES
NT-ES
Oct 4
Nanog
Sox2
R² = 0.903; P<0.05
mtDNA copy number
Mito/BrdU/DAPI

Figure 2

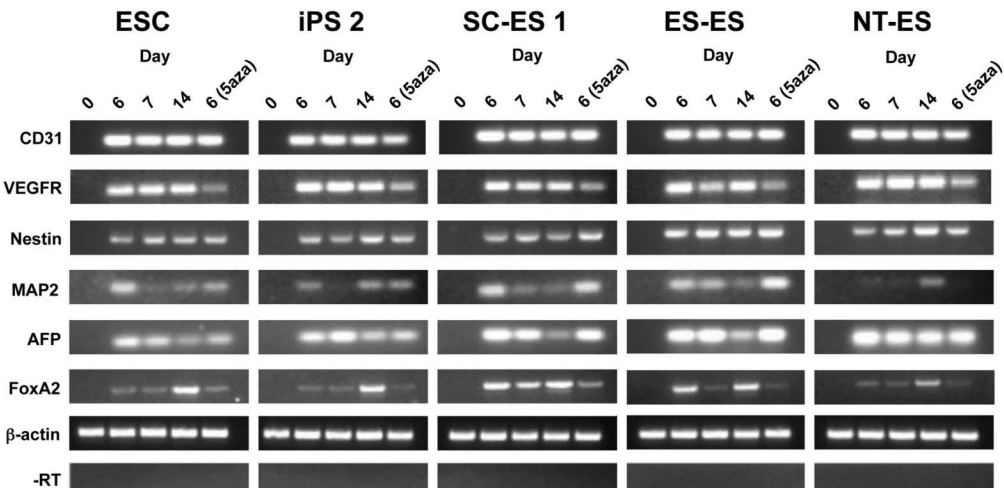

ESC
iPS 2
SC-ES 1
ES-ES
NT-ES
Day
0 6 7 14 6 (5aza)
CD31
VEGFR
Nestin
MAP2
AFP
FoxA2
β-actin
-RT

Figure 3

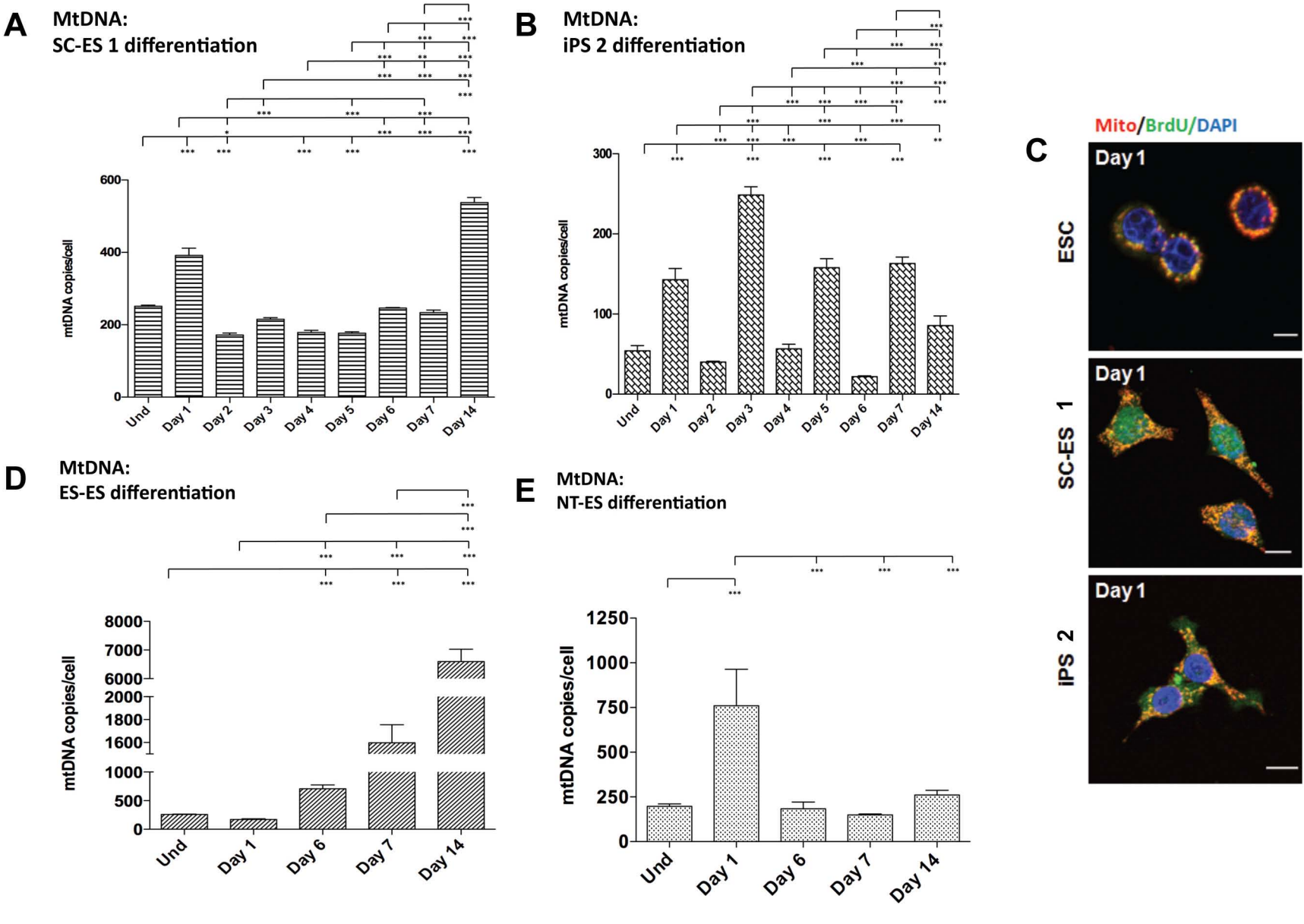

A
MtDNA:
SC-ES 1 differentiation
mtDNA copies/cell
Und
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 14
B
MtDNA:
iPS 2 differentiation
mtDNA copies/cell
C
Mito/BrdU/DAPI
ESC
SC-ES 1
iPS 2
Day 1
D
MtDNA:
ES-ES differentiation
mtDNA copies/cell
E
MtDNA:
NT-ES differentiation
mtDNA copies/cell

Figure 4

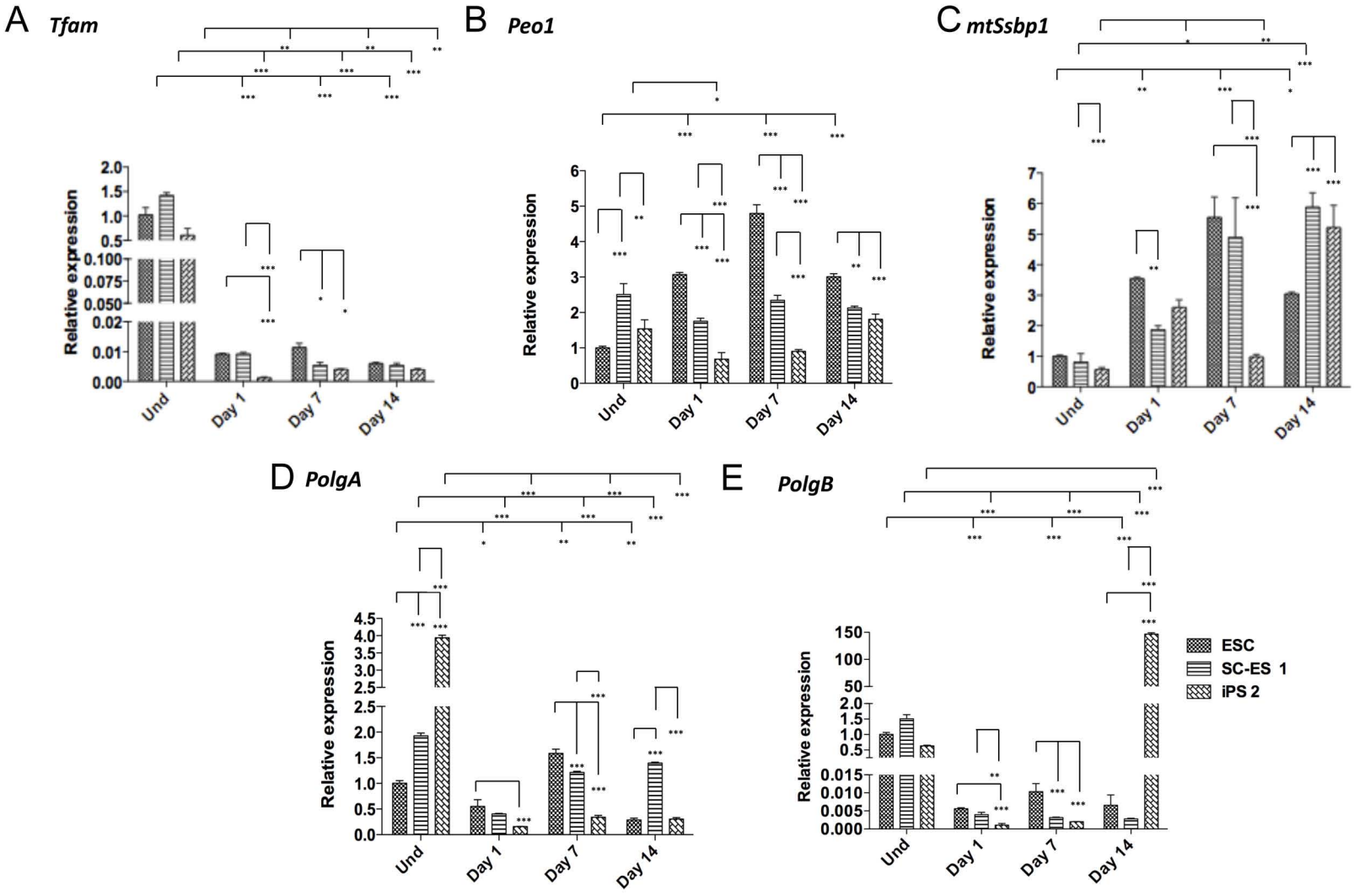
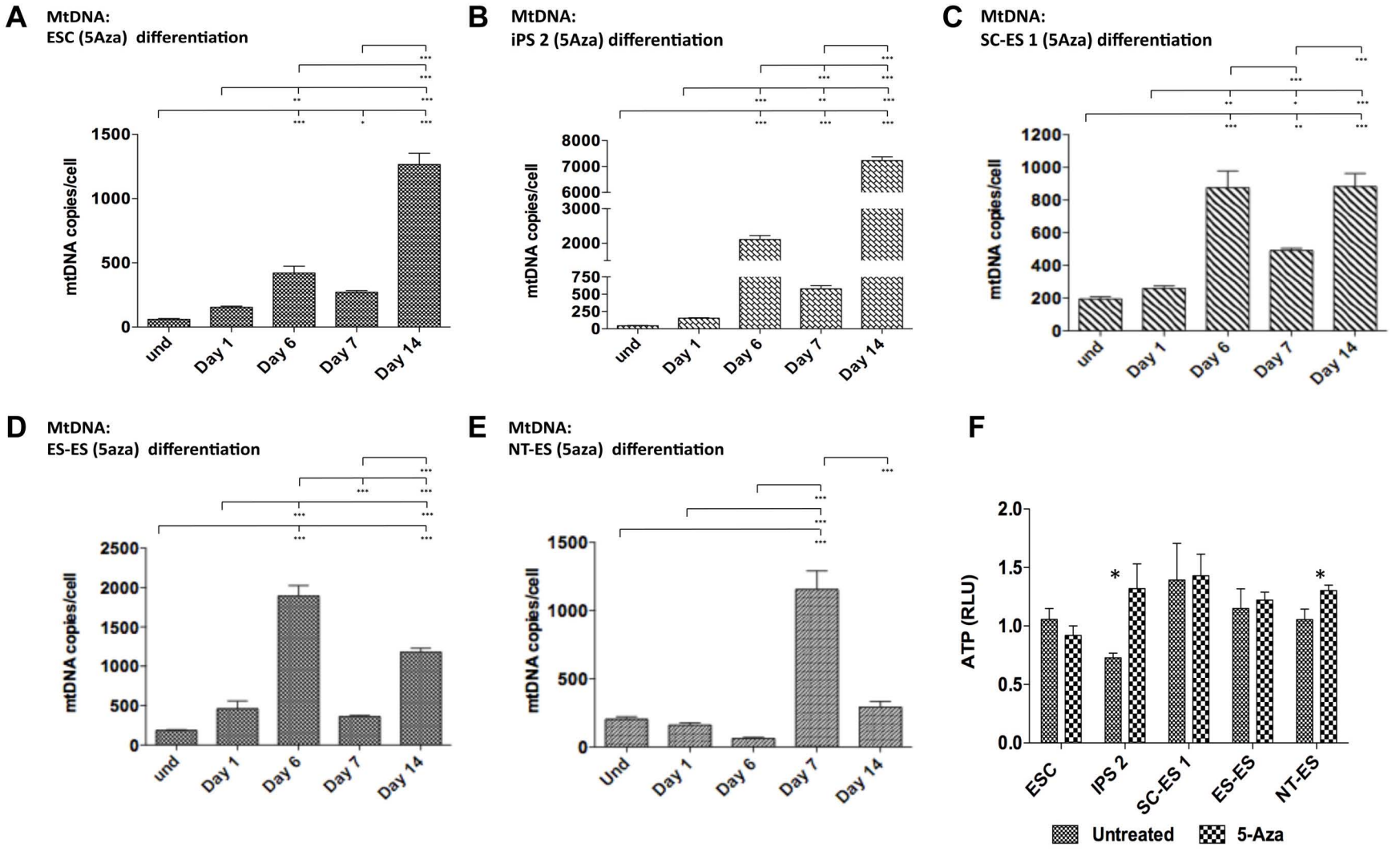

A Tfam
B Peo1
C mtSsbp1
D PolgA
E PolgB
Relative expression
Und
Day 1
Day 7
Day 14
ESC
SC-ES 1
iPS 2

Figure 5


A
MtDNA:
ESC (5Aza) differentiation
mtDNA copies/cell
1500
1000
500
0
und
Day 1
Day 6
Day 7
Day 14
B
MtDNA:
iPS 2 (5Aza) differentiation
mtDNA copies/cell
8000
7000
6000
3000
2000
750
500
250
0
und
Day 1
Day 6
Day 7
Day 14
C
MtDNA:
SC-ES 1 (5Aza) differentiation
mtDNA copies/cell
1200
1000
800
600
400
200
0
und
Day 1
Day 6
Day 7
Day 14
D
MtDNA:
ES-ES (5aza) differentiation
mtDNA copies/cell
2500
2000
1500
1000
500
0
und
Day 1
Day 6
Day 7
Day 14
E
MtDNA:
NT-ES (5aza) differentiation
mtDNA copies/cell
1500
1000
500
0
Und
Day 1
Day 6
Day 7
Day 14
F
ATP (RLU)
2.0
1.5
1.0
0.5
0.0
ESC
IPS 2
SC-ES 1
ES-ES
NT-ES
Untreated
5-Aza

Author/s:
Kelly, RDW; Sumer, H; McKenzie, M; Facucho-Oliveira, J; Trounce, IA; Verma, PJ; St John, JC

Title:
The Effects of Nuclear Reprogramming on Mitochondrial DNA Replication

Date:
2013-02-01

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