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The Expression of Mitochondrial DNA Transcription Factors during Early Cardiomyocyte *In Vitro* Differentiation from Human Embryonic Stem Cells

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ABSTRACT

Mitochondrial biogenesis and activation of both oxidative phosphorylation, as well as transcription and replication of the mitochondrial genome, are key regulatory events in cell differentiation. Mitochondrial DNA transcription and replication are highly dependent on the interaction with nuclear-encoded transcription factors translocated from the nucleus. Using a human embryonic stem cell line, HSF 6, we analyzed the proliferation of mitochondria and the expression of mtDNA-specific transcription factors in undifferentiated, migratory embryonic stem cells and spontaneously derived cardiomyocytes. Mitochondrial proliferation and mtDNA transcription are initiated in human embryonic stem cells as they undergo spontaneous differentiation in culture into beating cardiomyocytes. Undifferentiated, pluripotent human embryonic stem cells have few mitochondria, and, as they differentiate, they polarize to one extremity of the cell and then bipolarize the differentiating cell. The differentiated cell then adopts the cytoplasmic configuration of a somatic cell as evidenced in differentiating cardiomyocytes. Transcription and replication of the extranuclear mitochondrial genome is dependent on nuclear encoded factors exported to the mitochondrion. However, the differentiating cardiomyocytes have reduced or absent levels of these transcription and replication factors, namely mitochondrial transcription factors A, B1, B2, and nuclear respiratory factor 1 and polymerase γ. Therefore, final embryonic stem cell commitment may be influenced by mitochondrial proliferation and mtDNA transcription. However, it is likely that differentiating cardiomyocytes are in mitochondrial arrest, awaiting commitment to a final cell fate.

INTRODUCTION

CONSIDERABLE ADVANCE has been made in understanding gene and protein expression associated with the maintenance of the undifferentiated state of embryonic stem cells (ESCs) and with the molecular mechanisms that underlie their commitment to distinct adult cell types. Ex-

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amples of stem cell markers include SSEA-1, SSEA-3, SSEA-4, Tra-l-81, Tra-l-60, OCT-4, Sox-2, and FGF-4 (Henderson et al., 2002). However, ESC commitment and successful differentiation necessitates changes other than differential expression of nuclear genes. The development and differentiation of nearly every eukaryotic cell can be characterized by the cell's own mitochondria, including the number of mitochondria, their metabolic states, their RNA and protein synthesis machineries, their replication, and the mitochondria's own state of differentiation.

Since different cell types have very distinct metabolic requirements, differentiation of ESCs will also require appropriate changes to mitochondria, and mitochondrial DNA (mtDNA). Indeed, expression of mitochondrial genes is vital to cellular function, especially as the electron transfer chain (ETC) is the cell's major generator of ATP. Muscle cell type can be characterized by its number of mitochondria and the number of mtDNA genomes per mitochondrion, a specificity that relates to ATP requirement (Moyes et al., 1998). However, other reports suggest that there is no direct relationship between oxidative capacity and mtDNA content (Wiesner et al., 1992). It is therefore important that the involvement of mitochondria and mtDNA in stem cell fate be more fully investigated and understood. Of the limited data available, ultrastructural analysis of undifferentiated human (h) ESCs by transmission electron microscopy (TEM) shows the presence of elongated mitochondria similar to those of the inner cell mass. Those cells at the onset of cellular differentiation contained both oval and tubular mitochondria (Sathananthan et al., 2002) and, in mouse ESCs, also possess both spherical and oval mitochondria (Baharvand et al., 2003). Furthermore, a recent report suggests a role for Complex III of the ETC in cardiomyocyte differentiation (Spitkovsky et al., 2004).

Both mammalian gametes and somatic cells have mtDNA, a 16.6-kb maternally inherited genome (Birky, 1995), attached to the inner membrane of the mitochondrion. It encodes 13 of the subunits of the ETC complexes, associated with the process of oxidative phosphorylation (OXPHOS), along with 22 tRNAs and two rRNAs. Mutation or depletion of mtDNA can result in compromised cellular function and the onset of severely debilitating or lethal mtDNA diseases due to an incomplete ETC failing to generate sufficient levels of ATP (Wallace, 1999).

Transcription and replication of mtDNA require interaction between the nucleus and the mitochondria. The nuclear-encoded mitochondrial transcription factor A (TFAM; Fisher and Clayton, 1988) regulates the number of mtDNA transcripts (Clayton, 1998). Low levels of TFAM are associated with mtDNA depletion and several severe mtDNA diseases, such as infantile mitochondrial myopathy (Poulton et al., 1994). TFAM expression is associated with key stages of development. For example, murine heterozygous TFAM knockout embryos have reduced mtDNA copy number, and phenotypically the offspring have respiratory chain deficiency of the heart. In addition, homozygous TFAM knockout embryos suffer from severe mtDNA depletion and abolished OXPHOS. Most interestingly, these $TFAM^{-/-}$ embryos proceed through implantation and gastrulation, but die prior to embryonic day 10.5 (Larsson et al., 1998; Li et al., 2000). This arises as mtDNA copy number remains unchanged until at least the blastocyst stage (Larsson et al., 1998; Piko and Matsumoto, 1976; Ebert et al., 1988; Piko and Taylor, 1987), with each newly divided blastomere possessing fewer copies of the genome following each stage of embryonic division (Jansen and de Boer, 1998), since there is no active mtDNA replication. Those blastomeres giving rise to the inner cell mass are those that generate fetal tissue and are, most likely, harvested to generate undifferentiated hESC lines.

TFAM function necessitates interaction with other factors such as mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M), which regulate basal transcription of mammalian mtDNA (Falkenberg et al., 2002) and polymerase γ (PolG). Anti-retroviral drugs used to reduce HIV load produce a steady decline in mtDNA wild type (Nelson et al., 1997; White et al., 1997) and can increase the number of multiple mtDNA deletions observed (White et al., 1997) through the inhibition of PolG (Dalakas, 2001). Furthermore, TFAM activity is regulated by nuclear respiratory factor–1 (NRF-1) by binding to the promoter region of TFAM (Choi et al., 2002). Disruption to NRF-1 leads to reduced expression of TFAM and mtDNA copy number (Virbasius and Scarpulla, 1994).

We have analyzed the levels of these mtDNA transcription and replication regulators, and observed that early differentiation seems to mirror mitochondria and mtDNA dynamics that take place in the embryo. However, cells partially committed to a specific fate, for example, car-

diomyocytes differentiating in cell culture, display reduced expression of key mtDNA transcription regulators, similar to those of cells depleted of their mtDNA content, thus raising questions related to their potential effectiveness in maintaining consistent functional activity.

MATERIALS AND METHODS

Culturing of human embryonic stem cells

Undifferentiated Human University of San Francisco 6 (HSF6) cells, at passages 50–60, were cultured on mitomycin C–treated CF-1 murine embryonic fibroblasts in 80% DMEM high glucose (Invitrogen Inc., Carlsbad, CA), 20% knockout serum replacement (Invitrogen Inc.), 1 mM L-glutamine (Invitrogen Inc.), 0.1 mM MEM non-essential amino acids (Invitrogen Inc.), 0.1 mM beta-mercaptoethanol (Sigma, Milwaukee, WI), 4 ng/mL human recombinant bFGF-2 (Invitrogen Inc.). Cells were disassociated with either trypsin/EDTA (Invitrogen Inc.) or collagenase IV (Worthington Biochemicals, Lakewood, NJ). The HSF6 cell line is officially approved by the National Institutes of Health and is listed on their Stem Cell Registry.

Spontaneous differentiation of HSF-6 hESCs into cardiomyocytes

Creation of embryoid bodies by suspension culture: Undifferentiated and partially differentiated (mounded) aggregates of cells were mechanically dissected. Large clumps were dissociated by pipetting with a narrow bore tip and/or digestion in collagenase IV. After settling in the incubator with fresh media, all but 2 mL were aspirated, and the resulting aggregates were transferred to 60-mm Petri dishes. Clumps of cells indicative of embryoid bodies that adhered to Petri dishes during the suspension stage were mechanically scraped or collagenase IV–treated, and transferred to gelatin-coated or Matrigel-coated dishes consisting of 0.3 mg/mL Matrigel (BD BioSciences, Bedford, MA), 40 μg/mL fibronectin from bovine plasma (Sigma), and 10 μg/mL laminin (Sigma). Type B gelatin from bovine skin (0.1%, Sigma) was also used.

MtDNA depletion

hESC cultures were depleted of mtDNA according to the protocol of King and Attardi (1996). The cultures were grown in DMEM (Sigma) containing 4500 mg/L glucose and 1 mM pyruvate (Sigma), and supplemented with 5% FBS (v/v), 50 μg/mL uridine (Sigma), and penicillin and streptomycin (Rho^+). hESCs were grown in the presence of 50 ng/mL ethidium bromide (EB, Sigma) for 2–4 weeks to isolate cells completely lacking mtDNA. After EB treatment, the cells were maintained in DMEM supplemented with 5% FBS and 50 μg/mL uridine.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by labeling cells with 5,5′,6,6′-tetrachloro–1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, OR). Cells were incubated in 0.3 μg/mL JC-1 and 2 ng/mL Hoechst 33342 for 15 min, rinsed, and observed with a Leica TCSSP2 confocal microscope. Cells were excited at 485 nm and emission detected in both the red and green channels. Red fluorescence indicates active mitochondria with high membrane potential through the formation of J-aggregates. Green fluorescence is representative of JC-1 remaining in its monomeric form in less active mitochondria.

Assessment of the specific proteins encoded by the mitochondrial genome

Monoclonal antibodies (Molecular Probes) were used to identify subunits of complex IV–cytochrome c oxidase (COX) I, COXII, and COXIII of the ETC. Additionally, COXVIc, a nuclear encoded gene for this complex, was also analyzed. Cells were fixed in 2% formaldehyde for 1 h, permeabilized with 1% (v/v) Triton X-100 (Sigma), and blocked with 10% (v/v) normal goat serum (Sigma) and 2.5% BSA in PBS. Monoclonal antibody (5 μg/mL) was added and the cells incubated at 37°C for 1 h. Cells were rinsed with 0.1% Triton X-100, labelled with appropriate Alexa 488 secondary antibodies (Molecular Probes), counterstained with DAPI (Vectashield, Vector Laboratories, Burlingame, CA), and viewed using a Nikon E1000 fluorescence microscope.

Assessment of embryoid body and cardiomyocyte protein expression

Segments of embryoid bodies were digested with trypsin, plated on either laminin or fibronectin-coated coverslips, and cultured for 1–3

days. Coverslips were fixed with either cold methanol or 2% paraformaldehyde, and stained with antibodies against α-fetoprotein (Santa Cruz Biotechnology, Santa Cruz, CA), BMP-4 (Chemicon, Temecula, CA), human chorionic gonadotropin (Chemicon), brachyury (clone C19; Chemicon), and c-kit (Chemicon) to determine the presence of cells with different origins. Contractile regions were carefully microdissected, plated on coverslips, and stained with cardiomyocyte-specific antibodies, including atrial natriuretic factor (Cymbus Biotechnology, Eastleigh, NH) and cardiac troponin I (Chemicon), as well as with an antibody to nebulin (clone N-19; Santa Cruz Biotechnology), a skeletal muscle-specific protein. Coverslips also were stained with antibodies to desmin (Chemicon) and MyoD1 (Novocastra, Newcastle upon Tyne, UK), to assay embryoid bodies that were muscle-specific. MF20 (Developmental Studies Hybridoma Bank, University of Iowa) was used to analyze sarcomere development. For comparison, staining of ESC-derived cardiomyocytes was compared with the established rat cardiomyocyte cell line h9c2 (2-1), using many of the same markers. Secondary antibodies were obtained from Molecular Probes, DAPI was used as a nuclear counterstain, and samples were visualized with a Nikon E1000 fluorescence microscope.

Transmission electron microscopy

Undifferentiated HSF-6 ESCs were fixed in 2.5% glutaraldehyde (Sigma), washed in PBS, and incubated in 1% OsO_4 (Osmium; Sigma) with potassium ferricyanide (Sigma) for 1 h at 4°C. The sample was washed in PBS, dehydrated through an ethanol series, washed in propylene oxide, and incubated in 1:1 Epon/p. oxide (Sigma). After overnight incubation at 4°C in 100% Epon (Sigma), the pellet was cured at 37°C for 24 h, followed by 48-h incubation at 60°C. The sample was sectioned, mounted on 200-mesh copper grids, heavy metal counterstained with uranyl acetate (Sigma) and lead citrate (Sigma), and visualized using TEM (JEOL JEM-1210).

RT-PCR

RNA was isolated from undifferentiated (U), migratory (M), differentiated, and EB-depleted hESCs, and TOV (an ovarian tumor cell line) cells using the RNAqueous–4PCR Kit (Ambion, TX) according to the manufacturer's instructions. RNA from human heart was obtained from Clontech. 800 ng/μL of RNA was used as template for the generation of cDNA using the RT components from the Cells to DNA Kit (Ambion) according to the manufacturer's instructions. RT was performed for 1 h at 42°C, followed by 15 min at 72°C. cDNA was amplified for β-actin, TFAM, TFB1M, TFB2M, NRF-1, NRF-2α, NRF2β, PolG, COXI, COX II, COX III, Oct-4, FGF4, Rex-1, SOX1, ANF, Nkx2.5, and α-MHC. Primer sequences, annealing temperatures, and product sizes are listed in Table 1. All primer sets designed for nuclear-encoded genes span at least one intron. However, as the mtDNA genome does not possess introns, we ensured that all RNA templates were purified from DNA contamination and appropriate no-enzyme controls in the RT-PCR were run to demonstrate that no DNA contamination was present. Reactions were run in 50 μL volumes consisting of 1 $\times$ PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$ (Bioline), 200 μM Nucleotide Mix (Roche, USA), and 0.5 μM each primer and 2.5 U Biolase (Bioline). Reaction conditions were initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing (Table 1) for 30 sec and extension at 72°C for 45 sec, with a final extension of 72°C for 5 min. The products were resolved on 2% agarose gels.

Due to the amplification of both high (B-Actin and COXI) and low (TFAM, PolG, NRF, TFB1M, and TFB2M) copy number genes, we also ran preliminary experiments where we increased rounds of amplification from 25 to 45 cycles. This was performed to determine whether the amplicons for the high copy number genes to be analyzed were in the exponential and not the saturation phase of PCR. We were also satisfied that these increases in cycle number did not generate amplicons from very low levels of target DNA, which might have otherwise been excluded from our analysis. Furthermore, all reactions were performed on three separate occasions to ensure accuracy. As a result, 35 cycles of PCR were deemed as being appropriate for the presentation of our data.

DNA sequencing

PCR product specificity was confirmed through DNA sequencing using the Big Dye Terminator Version 3 (Applied Biosystems) and half BD Dye Terminator Sequencing Reagent

Table 1. Primer Pairings Used for RT-PCR to Determine Transcript Levels

Gene	*Forward primer (5′→3′)*	*Reverse primer (5′→3′)*	*Annealing temperature (°C)*	*Product size (bp)*
COX I	tca taa tcg gag gct ttg gc	caa cct caa cac cac ctt ct	55	421
COX II	atc atc cta gtc ctc atc gc	aga cgt ctt gca ctc atg ag	55	280
COX III	cgc gat gta aca cga gaa ag	tca gag tac ttc gag tct cc	55	379
NRF1	gga gtg atg tcc gca cag aa	cgc tgt taa gcg cca tag tg	58	584
Tfam	tat caa gat gct tat agg gc	act cct cag cac cat att tt	55	441
NRF2α	tag acc tca cca cac tca ac	gtg acc aaa cgg ttc aac tc	58	627
NRF2β	gag ctc cct tta cta cag ac	aac tgt ggt gtt gca gca tg	58	462
PolG	cat tgg aca tcc aga tgc tc	cct gat atg agc tcg gtc aa	58	679
TFB1M	tct gca atg ttc gac aca tc	acc tat ata aga agc tcc ac	49	501
TFB2M	aga agc agt tcc ttg gac ag	agt ggt cta tta cag tgg cg	57	463
β-ACTIN	tgg cac cac acc ttc tac aat gag c	gca cag ctt ctc ctt aat gtc acg c	55	400
Oct-4	cga cca tct gcc gct ttg ag	ccc cct gtc ccc cat tcc ta	60	241
α-MHC	gga gga gca agc caa cac caa	gca gtg agg ttc ccg tgg ca	59	179
ANF	tag gga cag act gca aga gg	cga gga agt cac cat caa acc ac	59	577
Nkx2.5	tgg cta cag ctg cac tgc cg	gga tcc atg cag cgt gga c	60	165
Rex-1	gcg tac gca aat taa agt cca ga	cag cat cct aaa cag ctc gca gaa t	56	306
SOX-2	ccc ccg gcg gca ata gca	tcg gcg ccg ggg aga tac at	55	448
FGF-4	cta caa cgc cta cga gtc cta ca	gtt gca cca gaa aag tca gag ttg	55	370

(Sigma) on a 3100-Avant Genetic Analyzer (Applied Biosystems), using both the respective forward and reverse primers. Both primers and sequences were compared for specificity using Blast Search ⟨http://www.ncbi.nlm.nih.gov/entrez/BLAST/⟩.

RESULTS

Using standard markers of hESC gene expression (Henderson et al., 2002), we have harvested hESCs, derived from the HSF6 cell line, at varying stages of differentiation. These included those cells that had begun to migrate (migratory) from the undifferentiated colony and those from several colonies representative of beating cardiomyocytes. Migratory ESCs are defined as those cells that are located to the periphery of the core of the undifferentiated cells and grow away from the tightly packed colony. Our TEM observations consistently show that few mitochondria are present in individual cells from undifferentiated colonies (Fig. 1), and they tend to localize in small perinuclear groups. This has been confirmed by immunocytochemistry (ICC) staining using antibodies specific to Complex IV genes encoded by both the mtDNA genome (COX I, Fig. 2A) and a nuclear encoded gene (COX VIc, Fig. 2B) and compared with TOV cells, arising from a fully differentiated human ovarian tumor cell line (Fig. 2C,D). It is possible that the number of mitochondria found is related to the scant cytoplasmic volume present in undifferentiated hESCs.

The use of JC-1, a specific marker of mitochondrial membrane potential, to identify mitochondrial proliferation by confocal microscopy clearly indicates a differentiated cytoplasm marked by an increase in mitochondrial content as the cells progress through the process of differentiation (Fig. 3A–C). The migratory stem cell exhibits a cluster-like architecture by partial polarity being established at one end of the cell. It also begins to define the border of the nuclear envelope (Fig. 3A), which is then followed by complete encapsulation (Fig. 3B). In the more advanced differentiated cell, the mitochondria establish "mitochondrial bipolarity," whilst the adjacent regions maintain their previous form (Fig. 3C). This is further highlighted in cardiomyocytes, which have a clearly defined bipolar cytoplasm (Fig. 3D). Throughout the process of cellular expansion, the mitochondria possess an elongated tubule structure typical of those cells respiring through OXPHOS (Gilkerson et al., 2000). Furthermore, the presence of both high (red fluorescence) and low (green fluorescence) membrane potential in the merged images (orange/yellow fluorescence) indicates that these cells are both viable and respiring, but these are likely to be different types of mitochondria in different metabolic states.

Specific mitochondrial transcription factors are differentially expressed during hESC differentiation (Table 2). As the hESCs migrate from the

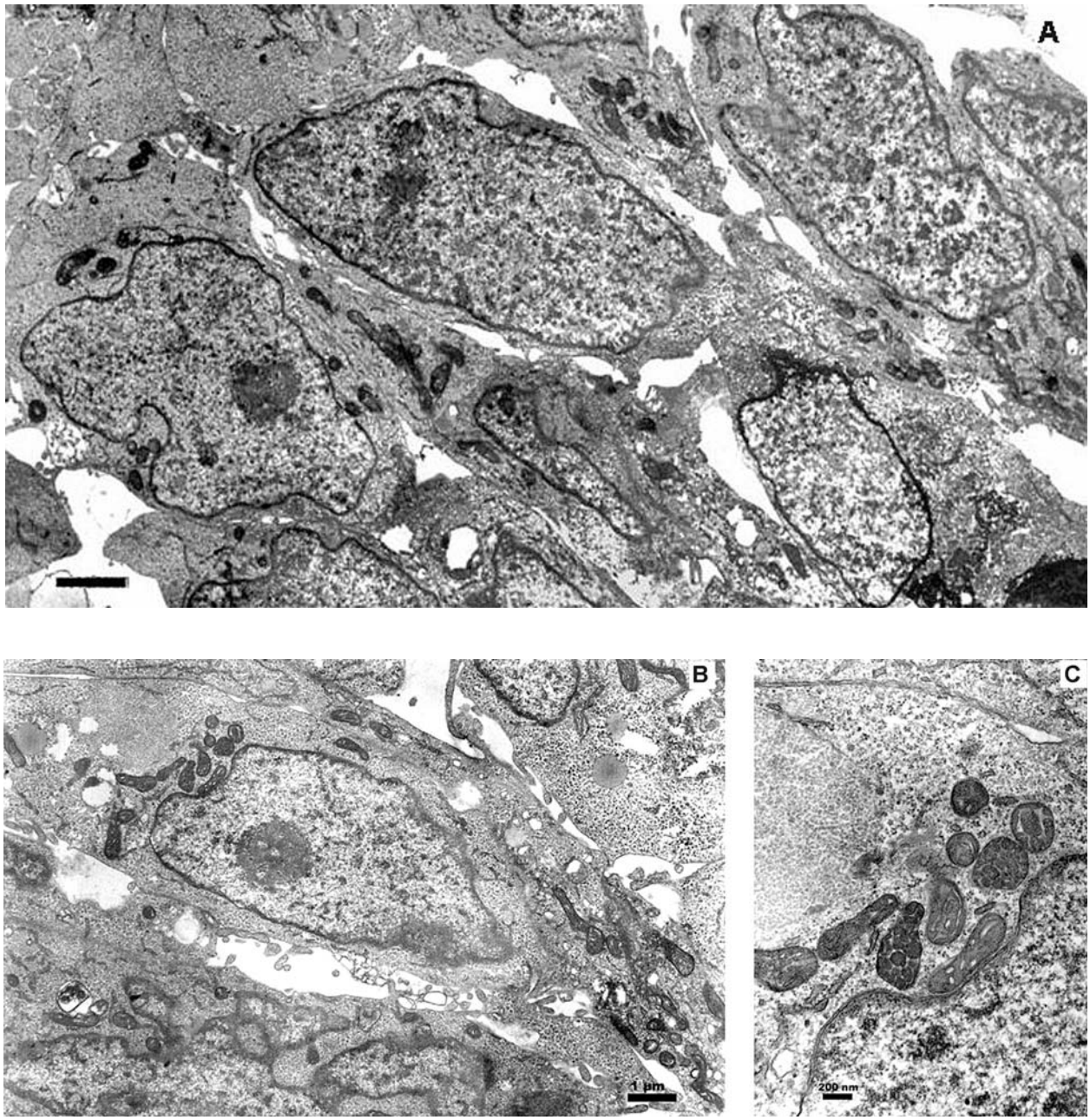

FIG. 1. Electron microscopy of hESC colony structure. (**A**) Transmission electron microscopy (TEM) of undifferentiated hESCs shows mostly a compact colony with cells displaying the typical packing and prominent nucleus–cytoplasmic ratios. (**B**) Individual cells with large nuclei containing reticulated nucleoli, and clusters of mitochondria. (**C**) Detailed definition of mitochondria in a scanty, undifferentiated hESC cytoplasm showing a perinuclear cluster containing both elongated and tubular mitochondria. Bar = 2 μm (A), 1 μm (B), 200 nm (C).

cluster of clonally expanding undifferentiated cells, they show little increase in mitochondrial protein. However, there is an increase in gene expression in the migratory cells for both mtDNA encoded genes and for some mtDNA transcription factors (Table 2; Fig. 4), though at relatively reduced rates. This is most likely to occur in synchrony with their cluster-like appearance and the subsequent establishment of mitochondrial bipolarity and mitochondrial expansion.

The differentiated cardiomyocyte colonies were carefully microdissected, and displayed characteristic and well-synchronized beating frequency. The commitment to a cell-specific fate

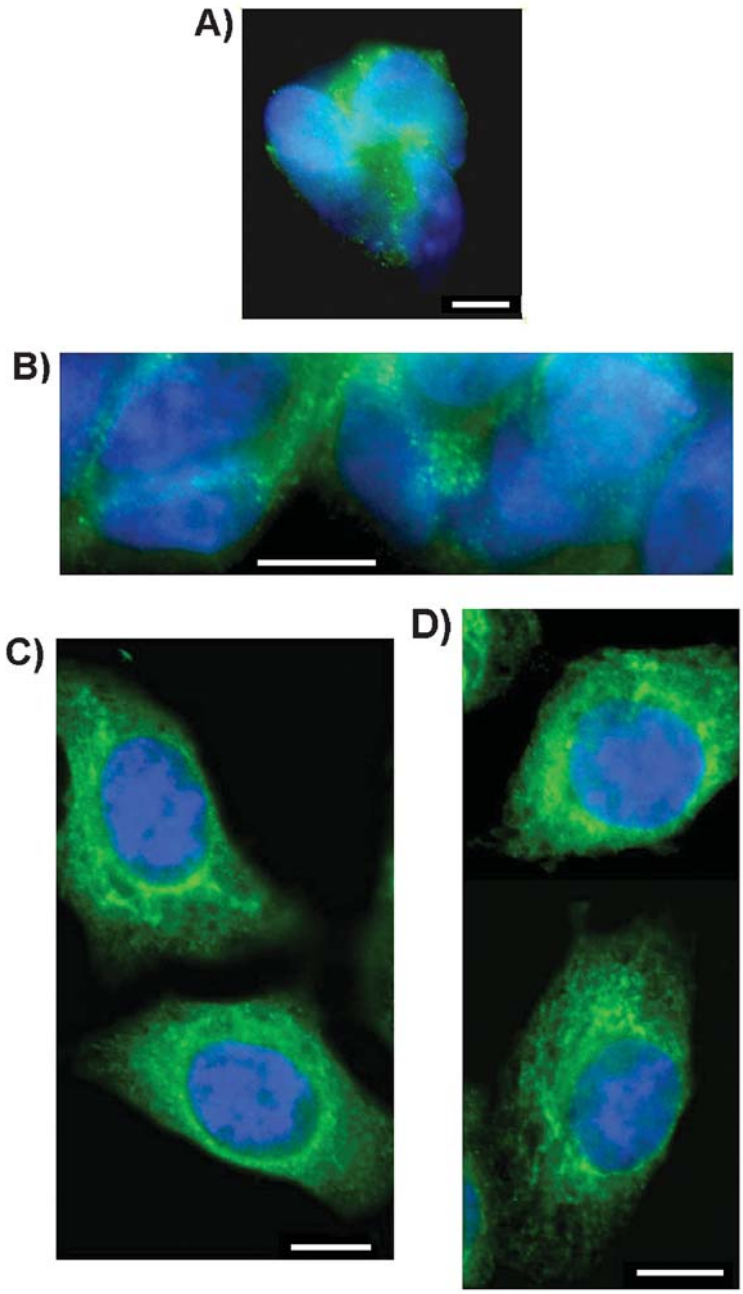

FIG. 2. Immunocytochemistry using antibodies associated with OXPHOS specific to the mitochondrial (COXI, COX III) and nuclear genomes (COXViC) was carried out in undifferentiated hESCs (**A**,**B**) and TOV cells (**C**,**D**). Mitochondria, as detected by both types of encoded proteins, are in green. The cells were counterstained with DAPI (blue) to show DNA content and demonstrate the large nuclear/cytoplasmic ratio present in undifferentiated hESCs (A, COX I; B, COX ViC). This is in contrast to TOV cells, which exhibit a large cytoplasm containing large numbers of mitochondria (C, COX III; D, COX ViC). Bar = 5 μm.

was initially determined by positive staining of these beating colonies with antibodies against desmin, cardiac troponin I, ANF, α-MHC, and MF20, an embryonic myosin heavy chain found in cardiac and skeletal muscle (data not shown). The cardiomyocytes also showed a lack of skeletal muscle-specific proteins, such as nebulin, and were negative for NCAM, a neurectodermal marker (data not shown). Similar results for all these probes were obtained using the rat cardiomyocyte cell line h9c2(2-1) (data not shown).

Cellular fate was confirmed by RT-PCR using specific markers for this lineage, namely α-MHC (Table 2, Fig. 4), ANF, and Nkx2.5 (Table 2). Levels of transcript for α-MHC were similar to those of adult heart RNA, whilst ANF and Nkx2.5 levels were lower or considerably decreased, respectively. Indeed, the decreased levels of ANF transcripts match the moderate ANF staining observed from ICC (data not shown). Furthermore, these cells did not exhibit markers of non-differentiation, as determined by lack of expression for

Oct-4 and FGF-4, whilst expression for Sox-2, also a marker of non-differentiation, was absent in the cardiomyocytes but present at reduced levels in adult heart. Taken together these data confirm that these hESC-derived cells were committed to the cardiomyocyte pathway. However, these cells also exhibited unusual mtDNA transcription factor activity. Although the cardiomyocytes had extensive mitochondrial gene (Table 2, Fig. 4) and protein content, as evidenced by their large cytoplasmic content (Fig. 3D), they showed either decreased or no gene expression for the mtDNA transcription factors and PolG (Table 2, Fig. 4). However, PolG transcripts were also absent in adult heart, suggesting that there was no active mtDNA replication taking place in either the cardiomyocytes or the adult heart. Furthermore, TFAM was not expressed in the cardiomyocytes (Fig. 4) nor were TFB1M, and TFB2M, transcription factors specific to maintenance of basal levels of mtDNA transcription. However, it appears that NRF-2α and 2β are expressed at basal levels, similar to undifferentiated cells (Table 2, Fig. 4). Interestingly, of the hESCs, only the migratory cells express NRF1 (Fig. 4), the protein that binds to the promoter of TFAM, thus facilitating transcription and replication (Virbasius and Scarpulla, 1994; Choi et al., 2002).

In order to determine whether the levels of expression in these cardiomyocytes were aberrant or indicative of cells transcribing and replicating little or no mtDNA, we depleted undifferentiated (EBU) and migratory (EBM) hESCs of their mtDNA through treatment with ethidium bromide (EB), a known mtDNA depletion agent (King and Attardi, 1996), which also reduces mtDNA transcription and replication factor protein levels in some differentiated cell types (Seidel-Rogol and Shadel, 2002), but TFAM mRNA levels are not normally affected (Larsson et al., 1994; Poulton et al., 1994). This therefore provided a baseline from which to assess the level of minimal mtDNA transcription and replication activity. Both EBU and EBM hESCs matched the reduced activity observed in cardiomyocytes (Table 2, Fig. 4), except that the very low levels of TFAM expression present in the EBU and EBM cells were not present in the cardiomyocytes.

DISCUSSION

We show that few mitochondria are found in undifferentiated hESCs, and this is mirrored by low levels of mtDNA transcription factor activity. The timing of mtDNA transcription and replication are key processes involved in fetal development and are thus key markers of development post-implantation. As demonstrated in partial or complete TFAM knockout animals, loss or inconsistent timing of such events can be either lethal for embryo survival or contribute to the onset of mtDNA type depletion syndromes (Larsson et al., 1998; Li et al., 2000). Consequently, the low number of mitochondria present in hESCs is also indicative of the critical timing related to the onset of mtDNA replication and marks the phase just prior to its initiation.

As anticipated, migratory hESCs exhibit an increase in gene expression for mtDNA-encoded genes reflected in increased numbers of mitochondria. These migratory cells also demonstrate an increase in some mtDNA transcription and replication factor gene expression. However, the differentiated hESCs that are representative of cardiomyocytes, by exhibiting varying levels of cardiomyocyte-specific gene and protein expression and with the propensity to beat in culture, have reduced mtDNA transcription and replication factor gene expression (similar to EB-treated cells), but maintain high levels of mitochondria and exhibit expanded bipolarity. It is certainly evident from JC-1 labelling that the mitochondria from these differentiating cardiomyocytes are active and appear to survive in non-supplemented media. OXPHOS function is highly dependent on a complete ETC (Moyes et al., 1998), and those somatic cells depleted of mtDNA are maintained in culture through supplementation with metabolic substrates (King and Attardi, 1996), such as the EBU and EBM cells we generated. Consequently, they have a lower membrane potential since they are only able to generate ATP through anaerobic metabolism. Furthermore, although these results will benefit from further research on other hESC lines and differentiation protocols, it is apparent that our differentiating cells are morphologically similar to normal, viable differentiated cells with their long tubal mitochondrial structures and propensity to exhibit both high and low mitochondrial membrane potentials (Gilkerson et al., 2000). These increased levels of mitochondria in our cardiomyocytes reflect those observed in murine ESC-derived cardiomyocytes (Paquin et al., 2002).

However, it is likely that our cardiomyocytes have not fully committed to their final fate due

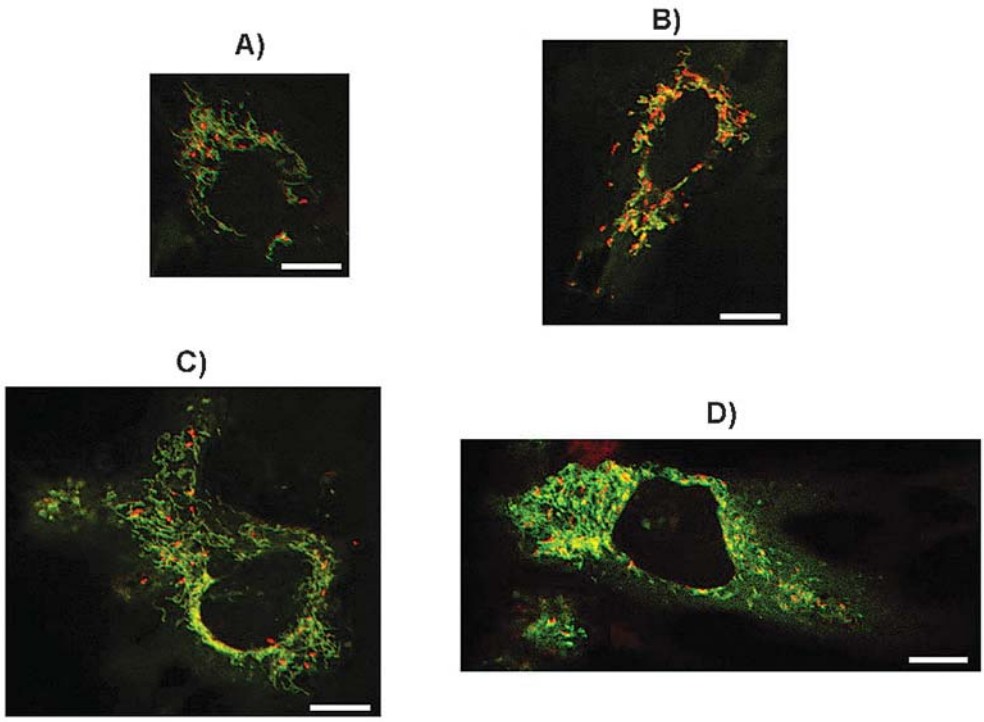

FIG. 3. Confocal microscopy on migratory hESCs and developing cardiomyocytes. Labelling with JC-1 reveals that early migratory cells exhibit mitochondrial polarity with clustering at one end of the cell (**A**). Mitochondria then further proliferate to populate the opposite end of the cell and encompass the nucleus (**B**). As the cell continues to differentiate it becomes bipolar in respect of its mitochondrial content (**C**). The cardiomyocyte has an expansive cytoplasm (**D**). The persistence of both red and green fluorescence in the mitochondrial populations demonstrates the presence of both high (red) and low (green) membrane potentials. In the early migratory cells (A), only a few mitochondria appear to have high membrane potentials, though this population increases with proliferation (B). In the differentiating cells (C) and cardiomyocytes (D), it is apparent that there is an abundance of mitochondria with high and low levels of membrane potential, as represented by the orange/yellow fluorescence in these merged images. Bar = 5 μm.

to (i) reduced expression for Nkx2.5 and moderate expression of ANF; and (ii) the higher levels of mtDNA transcription factor gene expression observed in both the TOV cells and in adult human heart, indicating that mtDNA transcription is an active process persisting in fully differentiated adult cells. Consequently, we would predict that these hESCs have sufficient mitochondria present to sustain cellular expansion and maintain homeostasis.

The generation of active mitochondria within the cytoplasm appears to be a series of organized events originating from their localized perinuclear clustering in the undifferentiated state, followed by circling of the nucleus, resulting in subsequent bipolarity. The mitochondria present in the undifferentiated cytoplasm are structurally similar in their elongated and oval forms (Figs. 1A and 2A) to those previously reported (Sathananthan et al., 2002). There are also likely to be very few copies of the mtDNA genome present. This is also similar to those observations noted in primordial germ cells, where the "founder" population of mtDNA is subsequently clonally amplified during oogenesis and determines the mtDNA status of the subsequent offspring following fertilization (Marchington et al., 1997). In a similar manner, our observations indicate that a clonal expansion ensues in hESCs as they undergo differentiation into specific somatic cell types.

NRF-1 is expressed in all tissues and in exponentially growing cells (Scarpula, 2002). It can be induced following electrical stimulation of neonatal cardiomyocytes (Xia et al., 1997) or by proliferator-activated receptor gamma coactivator–1 (PGC-1). Over-expression of PGC-1 in cultured cardiomyocytes results in nuclear and mitochondrial respiratory gene expression, increased cellular mitochondrial number, and stimulated coupled respiration (Lehman et al., 2000). However, targeted disruption of NRF-1 can be lethal, causing mtDNA depletion in oocytes and their failure to

Table 2. Differential Gene Expression in Human Embryonic Stem Cells

Gene	*U*	*M*	*EBU*	*EBM*	*C*	*Heart*	*TOV*
β-Actin	√	√	√	√	√	√	√
Oct-4	√	√	L	X	X	X	√
α-MHC	X	X	X	X	√	√	X
Nkx2.5	X	X	X	X	VL	√	X
ANF	X	X	X	X	L	√	X
FGF-4	X	X	N/A	N/A	X	X	X
Sox-2	√	√	N/A	N/A	X	L	√
Rex-1	VL	√	N/A	N/A	VL	L	√
COXI	√	√	X	X	√	√	√
NRF-1	X	L	X	X	X	√	√
NRF-2α	VL	L	VL	VL	VL	√	√
NRF-2β	L	√	X	X	L	√	√
TFAM	L	L	VL	VL	X	√	√
TFB1M	X	X	X	X	X	√	√
TFB2M	VL	L	X	X	X	√	√
PolG	X	L	X	X	X	X	√

The analysis of each gene by RT-PCR was performed on the following: U, undifferentiated stem cells; M, migratory stem cells; EBU, ethidium bromide undifferentiated stem cells; EBM, ethidium bromide–treated migratory stem cells; C, beating cardiomyocytes; heart, RNA from human heart (Clontech, USA); TOV, an ovarian tumor cell line.

√, Gene expressed with approximately the same level of transcript as for β-actin; L, low expression of the gene wih regard to β-Actin; VL, very low level of expression in relation to β-Actin; X, gene not expressed; N/A, gene or cell type not analyzed.

implant (Huo and Scarpulla, 2001). Consequently, it appears that embryogenesis has at least two key mitochondrially related developmental triggers, NRF-1 and TFAM. The early expression of NRF-1, NRF2α, and NRF2β, as observed in the undifferentiated and migratory hESCs, would suggest that those nuclear-encoded genes might be involved in mitochondrial proliferation. Furthermore, expression of TFAM, TFB1M, and TFB2M would be indicative of mtDNA transcription being initiated in conjunction with one of the NRF-related genes (Gleyzer et al., 2005). Our data demonstrate that this activity is most likely to occur as hESCs start to migrate, given the increased expression in NRF-1 and -2, and the continued expression of TFAM. Although NRF-1 is not found in the cardiomyocytes we analyzed, it is anticipated that cardiomyocytes would express NRF-1 due to its presence in neonatal cardiomyocytes (Xia et al., 1997) and the presence of its transcripts in the adult heart RNA that we analyzed. This might well arise through adult heart being electrically stimulated during normal cardiac conductance. NRF-2α and -2β were also expressed in our cardiomyocytes. Whilst they interact with NRF-1 to initiate the catalysis of transcription in proliferating cells, they also appear to act independently of NRF-1 (Scarpula, 2002). This would suggest that they are at least recruiting nuclear-encoded mitochondrial protein to support anaerobic metabolism, which also appears to be characteristic of early cardiomyocyte differentiation from murine ESCs (Spitkovsky et al., 2004).

By treating hESCs with EB, we were able to determine the baseline levels of activity of the various mitochondrial transcription factors when no mtDNA was present, as determined by the absence of COXI in these cells. Indeed, those cells treated with EB do not express NRF-2β but, in contrast, express NRF2α and TFAM, suggesting that NRF-2β is recruited during proliferation and thereafter. Otherwise, in terms of TFB1M, TFB2M, and PolG, the EB-treated cells match those transcripts levels for the cardiomyocytes. This would again suggest the importance of anaerobic metabolism for both sets of cells.

The expression of PolG is necessary for mtDNA replication to ensue. It is evident that low levels of PolG are present in migratory cells, and this is in contrast to TOV cells, which express much higher levels. The regulation of the number of mitochondria per cell type and the number of mtDNA copies per mitochondrion are characteristic of individual cell types (Miller et al., 2003; Gahan et al., 2001; Zhang et al., 1994), though this is still controversial in light of other studies (van den Bogert et al., 1993; Wiesner et al., 1992). If

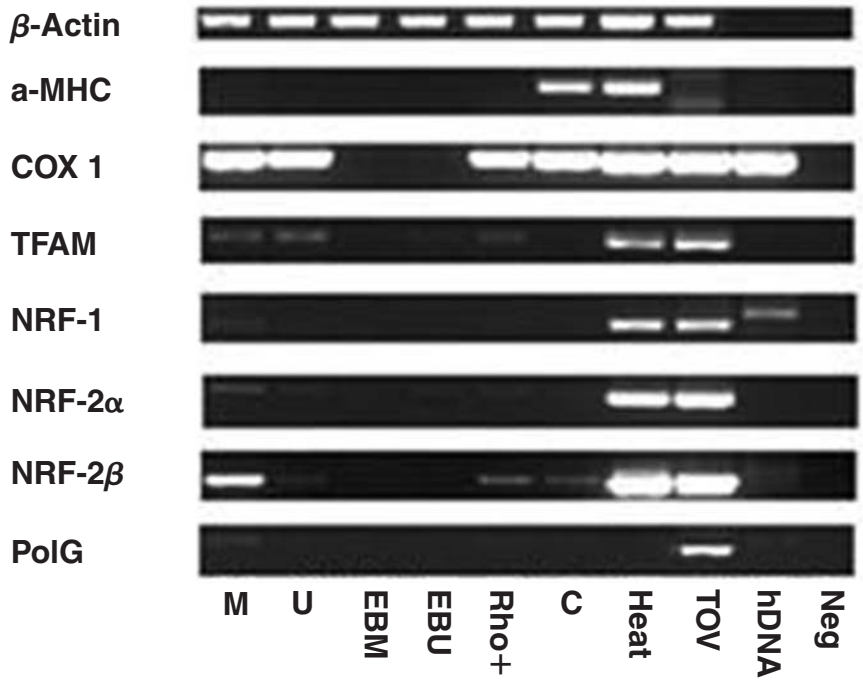

FIG. 4. Regulation of mtDNA transcription and replication factors. Both the *in vitro* developing cardiomyocytes and adult human heart RNA exhibit α-MHC, a marker of cardiomyocyte differentiation. All stages of differentiation express COXI, an mtDNA encoded gene, though only TOV's and migratory cells express PolG, the polymerase specific to mtDNA. However, NRF-1 is expressed by both adult heart and TOVs and at lower levels in migratory cells but not in cardiomyocytes. TFAM is expressed in undifferentiated and migratory ESCs, adult heart and TOV's but no expression appears to be present in the cardiomyocytes. However, basal levels of NRF-2α and NRF-2β are expressed in the cardiomyocytes. M, migratory hESCs; U, undifferentiated; EBM, ethidium bromide–treated M; EBU, ethidium bromide–treated U; Rho+, ESCs cultured in mtDNA depletion media without ethidium bromide; C, hESC-derived cardiomyocytes; heart, adult human heart; TOV, an ovarian tumour cell line; hDNA, human genomic DNA; Neg, negative control reaction.

such a phenomenon were indeed the case, then the final commitment to a cellular phenotype would have to include this subtle distinction. This would further explain the loss of PolG activity observed in the cardiomyocytes, which for active replication would require recruitment of TFAM (Ekstrand et al., 2004), which is not present in the cardiomyocytes but is at very low levels in EB-treated cells. It will be of interest to establish if similar shifts in mtDNA transcription occur when undifferentiated hESCs are directed towards other cell fates, with distinct metabolic requirements.

This incomplete commitment to cardiomyocyte fate is unlikely to arise from the cell culture environment. Spontaneous differentiation of stem cells in culture is standard practice, often resulting in the formation of embryoid bodies that consist of differentiated ESCs from the three embryonic germ layers (Lavon and Benvenisty, 2003; Doevendans et al., 2000; Talbot et al., 2002; Chen et al., 1999). Approximately 8% of embryoid bodies have contracting areas indicative of early cardiomyocytes, verified by antibody staining and RT-PCR (Kehat et al., 2001). However, the clinical use of such cell types remains to be questioned, though considerable work has already been performed to characterize them. The partial commitment to a cardiomyocyte fate would suggest that these cell types are still potentially capable of further cytoplasmic differentiation, indicating their ability to regulate organelle and mtDNA copy per mitochondrion to their appropriate numbers. Consequently, these mitochondria would be able to adapt to the metabolic environment they are destined for. Such a fate would suggest that those cells targeted to an injured or diseased tissue would still be sufficiently pluripotent to differentiate to compensate for the loss of function and would not be required to initiate molecular and cellular plasticity. However, future studies analyzing hESC commitment to a

certain pathway of differentiation, for both experimental and therapeutic purposes, should also include careful analysis of mtDNA content and replication in a series of hESCs, to ensure that those cells are equipped to handle the specific metabolic requirements of the differentiated tissue for extended periods of time. It is likely that this metabolic fitness might not be correctly estimated if only nuclear markers for differentiation are employed.

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REFERENCES

Baharvand, H., and Matthaei, K.I. (2003). The ultrastructure of mouse embryonic stem cells. Reprod. Biomed. Online 7, 330–335.

Birky, C.W., Jr. (1995). Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proc. Natl. Acad. Sci. USA 92, 11331–11338.

Chen, L.R., Shiue, Y.L., Bertolini. L., et al. (1999). Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology 52, 195–212.

Choi, Y.S., Kim, S., Kyu Lee, H., et al. (2004). *In vitro* methylation of nuclear respiratory factor–1 binding site suppresses the promoter activity of mitochondrial transcription factor A. Biochem. Biophys. Res. Commun. 314, 118–122.

Clayton, D.A. (1998). Nuclear-mitochondrial intergenomic communication. Biofactors 7, 203–205.

Dalakas, M.C. (2001). Peripheral neuropathy and antiretroviral drugs. J. Periph. Nerv. Syst. 6, 14–20.

Doevendans, P.A., Kubalak, S.W., An, R.H., et al. (2000). Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes. J. Mol. Cell. Cardiol. 32, 839–851.

Ebert, K.M., Liem, H., and Hecht NB (1998). Miotchondrial DNA in the mouse preimplantation embryo. J. Reprod. Fertil. 82, 145–149.

Ekstrand, M.I., Falkenberg, M., Rantanen, A., et al. (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum. Mol. Genet. 13, 935–944.

Falkenberg, M., Gaspari, M., Rantanen, A., et al. (2002). Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat. Genet. 31, 289–294.

Fisher, R.P., and Clayton, D.A. (1988). Purification and characterization of human mitochondrial transcription factor 1. Mol. Cell. Biol. 8, 3496–3509.

Gahan, M.E., Miller, F., Lewin, S.R., et al. (2001). Quantification of mitochondrial DNA in peripheral blood mononuclear cells and subcutaneous fat using real-time polymerase chain reaction. J. Clin. Virol. 22, 241–247.

Gleyzer, N., Vercauteren, K., and Scarpulla, R.C. (2005). Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. Mol. Cell Biol. 25, 1354–1366.

Henderson, J.K., Draper, J.S., Baillie, H.S., et al. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20, 329–337.

Huo, L., and Scarpulla, R.C. (2001). Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. Mol. Cell. Biol. 21, 644–654.

Jansen, R.P., and de Boer, K. (1998). The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Mol. Cell. Endocrinol. 145, 81–88.

Kehat, I., Kenyagin-Karsenti, D., Snir. M., et al. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J. Clin. Invest. 108, 407–414.

King, M.P., and Attardi, G. (1996). Isolation of human cells lacking mitochondrial DNA. Meth. Enzym. 264, 304–313.

Larsson, N.G., Oldfors, A., Holme, E., et al. (1994). Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion. Biochem. Biophys. Res. Commun. 200, 1374–1381.

Larsson, N.G., Wang, J., Wilhelmsson, H., et al. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat. Genet. 18, 231–236.

Lavon, N., and Benvenisty, N. (2003). Differentiation and genetic manipulation of human embryonic stem cells and the analysis of the cardiovascular system. Trends Cardiovasc. Med. 13, 47–52.

Lehman, J.J., Barger, P.M., Kovacs, A., et al. (2000). Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J. Clin. Invest. 106, 847–856.

Li, H., Wang, J., Wilhelmsson, H., et al. (2000). Genetic modification of survival in tissue-specific knockout mice with mitochondrial cardiomyopathy. Proc. Natl. Acad. Sci. USA 97, 3467–3472.

Marchington, D.R., Hartshorne, G.M., Barlow, D., et al. (1997). Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. Proc. Natl. Acad. Sci. USA 60, 408–416.

Miller, F.J., Rosenfeldt, F.L., Zhang, C., et al. (2003). Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. Nucl. Acids Res. 31, e61.

Moyes, C.D., Battersby, B.J., and Leary, S.C. (1998). Regulation of muscle mitochondrial design. J. Exp. Biol. 201, 299–307.

Nelson, I., Hanna, M.G., Wood, N.W., et al. (1997). Depletion of mitochondrial DNA by ddC in untransformed human cell lines. Somat. Cell. Mol. Genet. 23, 287–290.

Paquin, J., Danalache, B.A., Jankowski, M., et al. (2002). Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc. Natl. Acad. Sci. USA 99, 9550–9555.

Piko, L. and Matsumoto, L. (1976). Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. Dev. Biol. 49, 1–10.

Piko, L., and Taylor, K.D. (1987). Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. Dev. Biol. 123, 364–374.

Poulton, J., Morten, K., Freeman-Emmerson, C., et al. (1994). Deficiency of the human mitochondrial transcription factor h-mtTFA in infantile mitochondrial myopathy is associated with mtDNA depletion. Hum. Mol. Genet. 3, 1763–1769.

Sathananthan, H., Pera, M., and Trounson, A. (2002). The fine structure of human embryonic stem cells. Reprod. Biomed. Online 4, 56–61.

Scarpula, R.C. (2002). Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. Gene 286, 81–89.

Seidel-Rogol, B.L., and Shadel, G.S. (2002). Modulation of mitochondrial transcription in response to mtDNA depletion and repletion in HeLa cells. Nucl. Acids Res. 30, 1929–1934.

Spitkovsky, D., Sasse, P., Kolossov, E., et al. (2004). Activity of complex III of the mitochondrial electron transport chain is essential for early heart muscle cell differentiation. FASEB J. 18, 1300–1302.

Talbot, N.C., Powell, A.M., and Garrett, W.M. (2002). Spontaneous differentiation of porcine and bovine embryonic stem cells (epiblast) into astrocytes or neurons. In Vitro Cell. Dev. Biol. Anim. 38, 191–197.

van den Bogert, C., De Vries, H., Holtrop, M., et al. (1993). Regulation of the expression of mitochondrial proteins: relationship between mtDNA copy number and cytochrome-c oxidase activity in human cells and tissues. Biochim. Biophys. Acta 1144, 177–183.

Virbasius, J.V., and Scarpulla, R.C. (1994). Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc. Natl. Acad. Sci. USA 91, 1309–1313.

Wallace, D.C. (1999). Mitochondrial diseases in man and mouse. Science 283, 1482–1488.

White, D.J., Mital, D., Taylor, S., et al. (2001). Sperm mitochondrial DNA deletions as a consequence of long term highly active antiretroviral therapy. AIDS 15, 1061–1062.

Wiesner, R.J., Ruegg, J.C., and Morano, I. (1992). Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues. Biochem. Biophys. Res. Commun. 183, 553–559.

Xia, Y., Buja, L.M., Scarpulla, R.C., et al. (1997). Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes governing mitochondrial proliferation and differentiation. Proc. Natl. Acad. Sci. USA 94, 11399–11404.

Zhang, H., Cooney, D.A., Sreenath, A., et al. (1994). Quantitation of mitochondrial DNA in human lymphoblasts by a competitive polymerase chain reaction method: application to the study of inhibitors of mitochondrial DNA content. Mol. Pharm. 46, 1063–1069.

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