Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells

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Abstract

Embryonic cells before implantation are exposed to a hypoxic condition and dependent on anaerobic metabolism. Human embryonic stem cells (HESCs) derived from pre-implantation blastocyst also grow well in hypoxic conditions. Expecting that the differentiating HESCs might mimic anaerobic-to-aerobic metabolic transition of the early human life, we examined the mitochondria-related changes in these cells. We observed that mitochondrial mass and mitochondrial DNA content were increased with differentiation, which was accompanied by the increase of the amount of ATP (4-fold) and its by-product reactive oxygen species (2.5-fold). The expression of various antioxidant enzymes including mitochondrial and cytoplasmic superoxide dismutases, catalase, and peroxiredoxins showed a dramatic change during the early differentiation. In conclusion, HESC differentiation was followed by dynamic changes in mitochondrial mass, ATP and ROS production, and antioxidant enzyme expressions. Therefore, the HESCs would serve as a good model to examine the mitochondrial biology during the early human differentiation.

Keywords: Embryonic stem cell; Mitochondria; Oxidative phosphorylation; Reactive oxygen species; Antioxidant

Human embryonic stem cells (HESCs) are derived from the inner cell mass of blastocyst, which is in pre-implantation stage [1]. Prior to implantation and vasceratization in vivo, embryonic cells are exposed to a hypoxic environment with an oxygen partial pressure below 40 mm Hg [2], in which anaerobic bacteria can easily colonize [3]. Since HESCs have metabolic characteristics of early intra-uterine life, they grow well in a hypoxic condition only with 1–5 % of oxygen [4]. In this hypoxic environment, HESCs are resistant to spontaneous differentiation and stably remain pluripotent [4].

Since cells in hypoxic condition cannot produce large amount of ATP via oxidative phosphorylation, which occurs in mitochondria, they must rely on anaerobic metabolism to produce ATP to meet their energy demand [5]. Considering the hypoxic condition in the uterine cavity, it is not surprising that HESCs have only few mitochondria with immature morphology [6,7]. With differentiation, it has been shown that the number of mitochondria increases and the morphology of mitochondria resembles that of mature cells [7]. Therefore, HESCs might recapitulate the early embryonic differentiation stages and could provide a good in vitro model to examine the dynamic changes in energy metabolism related to mitochondria of early life.

Once cells start to produce large amount of ATP through the mitochondrial electron transport chain...
(ETC), reactive oxygen species (ROS) will be produced as by-products of oxidative phosphorylation [8]. Because embryos are sensitive to oxidative damage [9,10], they should be equipped with effective antioxidant systems to detoxify ROS.

In this study, we examined the changes in mitochondrial biogenesis, ATP content, ROS production, and antioxidant enzymes during the spontaneous differentiation of HESCs in vitro.

Materials and methods

Culture of HESCs. We used an HESC line (SNUhES3) in this study. The derivation process and characteristics of SNUhES3 were previously described [6]. HESCs were maintained for up to 14 days without passaging of the cells. Medium consisted of DMEM (high glucose with L-glutamine) (Invitrogen). The culture medium was preincubated in a 37 °C shaking water bath. After thawing, these cells were seeded on 0.1% gelatin-coated culture dishes 1 day before the HESCs were plated.

Spontaneous differentiation of HESCs. Spontaneous differentiation was performed by removing feeder layer, bFGF, and serum replacement from the culture. These conditions for inducing spontaneous differentiation were maintained for up to 14 days without passaging of the cells. Medium change was performed every 2–3 days. For subsequent analyses, cells were rapidly harvested by mechanical dissociation and frozen at −80 °C for RNA and protein extraction.

Reverse transcriptase polymerase chain reaction (RT-PCR). To monitor the expression levels of differentiation markers, total RNA from the cells was prepared using the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was obtained using 1 μg RNA with random hexamers and avian myeloblastosis virus reverse transcriptase (Invitrogen). PCRs were carried out with 1 μl cDNA template, 10 μM of each primer, 8 μl of 2.5 mM dNTP mix, and 0.1 U of Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan) in a volume of 50 μl. The following primer sets were used: oct4 (forward: 5'-GAGGAATGAGGACCTCAGGA-3', reverse: 5'-TTCCGGGCCGTTTACAGAAACA-3'), nanog (forward: 5'-TGCCCTCACACGGGAGCTGTC-3', reverse: 5'-TGCTATTCCTTGCGCCACCTT-3'), keratin (forward: 5'-AGGAAATCTACTCAGGAGGAGGCGC-3', reverse: 5'-AAAGCCACAGATCTGGGAGGACTC-3'), z-fetoprotein (AFP) (forward: 5'-AGAACCCTGTCAAAAGCCTGTC-3', reverse: 5'-GAGACAGACGGGATGTTGTGTG-3'), myogenin (forward: 5'-GCAGAACGATTTCTCTCTCC-3', reverse: 5'-GAGGCAGCCGGTATGAAATTTAAG-3'), and GAPDH (forward: 5'-GGCTTCCACACGGGAGCTGTC-3', reverse: 5'-GTGATACCTGTCTTGAAAGGG-3'). The derivation process and characteristics of SNUhES3 were previously published [11].

Transmission electron microscopy (TEM). Cells were collected at different stages and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature. After being rinsed for 10 min in the same buffer, the cells were post-fixed with 1% OsO4 in 0.04 M phosphate buffer containing 0.14 M sucrose for 10 min at 4 °C. Following dehydration with standard ethanol series and infiltration with epoxy resin, cells were transferred to beam capsules for polymerization in the oven. The capsules were separated from the polymerized resin with a razor blade, and embedded cells in hardened blocks were viewed with an optical microscope so that the appropriate area was chosen for ultrathin sections. Consequently, ultrathin sections were obtained using an ultramicrotome (Sorval MT-6000; DuPont, Wilmington, DE) with a diamond knife. Heavy metal staining was done with 4% uranyl acetate and lead citrate, and the samples were examined through the electron microscope (H-7100; Hitachi, Tokyo, Japan) at 50 kV.

Quantiﬁcation of mitochondrial DNA using real-time PCR. The total DNA was extracted from cells using a commercial kit (Genta Systems, Minneapolis, MN), and its concentration was measured with a spectrophotometer. The mitochondrial DNA (mtDNA) copy numbers were measured by a real-time PCR method using an ABI Prism 7700 (Applied Biosystems, Lincoln, CA). The mtDNA quantity was corrected by simultaneous measurement of the nuclear DNA coding 28S rRNA. To determine the copy numbers, we cloned specific sequences from mitochondrial genome (2372–2672) and 28S rRNA gene (7358–7460) using pGEM-T easy vector (Promega, Madison, Wisconsin). The mtDNA- and 28S rRNA-specific ﬂuorescent probes were labeled internally using the ﬂuorescent dyes 5-carboxyfluorescein (FAM) on the 5′-end, and 6-carboxy-tetramethylrhodamine ( TAMRA) on the 3′-end. The primers for mtDNA and 28S rRNA were from the methods previously published [11]. All samples were run in triplicate in 25 μl reaction volume containing TaqMan Universal PCR Master Mix (Applied Biosystems). Standard curve was obtained using cloned sequences shown above and analyzed on an ABI Prism 7700 Sequence Detector (Applied Biosystems). Amplification was performed under the following conditions: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. During the PCR amplification, the amplified products were measured continuously by determination of the ﬂuorescence emission.

Measurement of intracellular ATP content. The cellular contents of ATP were measured using the luciferin-luciferase reaction with an ATP bioluminescence somatic cell assay kit (Sigma). The harvested cells were suspended with KRH buffer containing 0.1 mM glucose and 0.2% BSA. These cell suspensions were incubated in a 37 °C shaking water bath. After addition of an assay mixture containing luciferin and luciferase, luminescence was measured by 2,7′-dichlorofluorescein diacetate (DCFDA) (Invitrogen). Cells were plated on six-well plates and incubated with 1.5 μl/mL DCFDA at 37 °C for 30 min. ROS in the cells cause oxidation of DCFDA, yielding the fluorescent product 2,7′-dichlorofluorescein (DCF). The DCF fluorescence was measured in intact cells by fluorescence-activated cell sorting analysis using FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ).

Western blotting. Total cell lysates (20 μg protein) were subjected to 8–16% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with rabbit polyclonal antibodies against human glutathione peroxidase 1 (GPx1), Mn-superoxide dismutase (SOD), Cu/Zn-SOD, peroxiredoxins 1 and 2 (Prx1 and Prx2), and catalase (all at 1:1,000; LabFrontier Co., Seoul, Korea, http://bio.labfrontier.com), and with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) after three washings in PBS. Bound antibodies were visualized with an.
enhanced chemiluminescence system (Pierce, Rockford, IL). In the cases of the factors regulating mitochondrial biogenesis, rabbit polyclonal antibodies against human peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α, 1:1000; Santa Cruz), human nuclear respiratory factor 1 (NRF-1, 1:1000), or human mitochondrial transcription factor A (Tfam, 1:5000) were utilized as primary antibodies. The antisera against human NRF-1 or human Tfam were prepared in rabbits using the purified NRF-1 or Tfam proteins expressed in Escherichia coli. The antibody against GAPDH was utilized to confirm the equal loading of the sample.

Statistical analysis. Data were shown as means ± SD. Statistical significance was evaluated by $T$-test, and $P < 0.05$ was considered significant.

Results

Upon prolonged in vitro culture, without feeder cells, bFGF, and serum replacement, the HESCs spontaneously differentiated into cells with various morphologies (Fig. 1). With time, the morphologies of cells became more complicated (Fig. 1C and D). Oct4 and nanog gene expressions were only detected in undifferentiated HESCs. All markers indicative of ecto-, meso-, and endodermal derivatives were expressed in the cells of one- and two-week differentiation (Fig. 2).

MitoTracker Green staining in undifferentiated HESCs was faint. However, the peripheral portion of the undifferentiated HESC colony showed slightly stronger fluorescence compared to the central portion (Fig. 3A). In contrast, one- and two-week differentiated cells were strongly stained with MitoTracker Green (Fig. 3B and C).

TEM images showed that the undifferentiated HESCs had few mitochondria, which formed some clusters and had decreased number of cristae and electron-lucid matrix (Fig. 4A). They also had increased nuclear to cytoplasmic ratio and their cellular organelles were not so much distinct. In contrast, one-week differentiated cells had numer-

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**Fig. 1.** Phase contrast images of HESCs in various differentiation stages. (A) Undifferentiated HESCs on STO feeder layer, (B) three days differentiated cells (after removing feeder cells, bFGF, and serum replacement), (C) one-week differentiated cells, and (D) two-week differentiated cells (scale bars indicate 500 μm).

**Fig. 2.** RT-PCR for differentiation markers. Oct4 and nanog are stemness markers. Keratin, myogenin, and AFP represent ecto-, meso-, and endoderm differentiation, respectively. GAPDH was used as internal control for equal loading of cDNA. Abbreviations: undiff, undifferentiated HESCs; 1w, one-week differentiated cells; and 2w, two-week differentiated cells.
ous mature mitochondria dispersed in their cytoplasm, which had increased number of distinct cristae and dense matrix (Fig. 4B). Their cellular organelles looked much more evident than those of undifferentiated HESCs.

Real-time PCR showed that the mtDNA copies were increased more than 30% in one- and two-week differentiated cells compared to the undifferentiated HESCs (Fig. 5A). Unlike the mtDNA copy numbers and MitoTracker Green staining intensities, the protein expression of Tfam, PGC1α, and NRF-1, which are known as the regulators of mitochondrial biogenesis and mtDNA replication[12,13], was slightly increased in the undifferentiated HESCs compared to their differentiated counterparts (Fig. 5B).

Intracellular ATP content of one- and two-week differentiated cells increased about 4-fold compared to the undifferentiated HESCs (Fig. 6). Intracellular ROS content of one-week differentiated cells increased about 2.5-fold compared to the undifferentiated HESCs. With further differentiation, as shown in two-week differentiated cells, the intracellular ROS content showed a slightly decreasing tendency (Fig. 7A). In the undifferentiated HESCs, Mn-SOD and catalase were the dominant antioxidant enzymes. While the expression level of Mn-SOD was maintained with further differentiation, the expression level of catalase showed a marked reduction (Fig. 7B). The GPx1, Cu/Zn SOD, Prx1, and Prx2 were expressed at very low levels in the undifferentiated HESCs but their expressions were dramatically increased in one-week and two-week differentiated cells.

Discussion

Here, we report the dynamic changes in mitochondrial mass, ATP production, ROS production, and several antioxidant enzyme expressions in the HESCs of various differentiation stages. Since the HESCs are derived from the inner cell mass of blastocyst and have some metabolic characteristics of the pre-implantation stage[4], spontaneous in vitro differentiation of HESCs might provide a good model to examine the dynamic changes of anaerobic-to-aerobic metabolic transition, which might be facilitated by oxygenated blood from maternal-side through implantation and vascularization processes. As oxidative phosphorylation is extremely superior to glycolysis in terms of energy efficiency[5], there is no reason to persist in anaerobic metabolism in the condition with enough oxygen. Therefore, in differentiating or differentiated cells under aerobic condition, mitochondrial mass should be increased for efficient oxidative phosphorylation.

As was previously reported[7,14], we found that the mitochondrial mass clearly increased with differentiation. Undifferentiated HESC colony showed a faint mitochondrial staining except the peripheral portion. It was reported that the cells located in the peripheral portion of undifferentiated HESC colony show epitheloid appearance and
have specialized cell junctions [14]. Therefore, they could be regarded as cryptically differentiated cells and have more mitochondria than the cells in the central portion.

In accordance with increasing mitochondrial mass, the amount of mitochondrial genome was also increased, which was demonstrated by real-time PCR of mtDNA.

Mitochondrial biogenesis is controlled by PGC1α, NRF-1, and Tfam [12,13]. PGC1α interacts with NRF-1 and other transcription factors to control expression of genes of oxidative metabolism, including Tfam [12,15]. Tfam is known as the signal through which the nucleus regulates mtDNA transcription and replication [16]. However, we found a discrepancy between the regulatory factors of mitochondrial biogenesis and the actual mitochondrial mass. The latter has been clearly shown by MitoTracker Green staining and TEM. This paradox might be explained by the immature machinery for assembly or biogenesis of mitochondria, which could be supported by our observation that the proteins encoding mitochondrial respiratory complex I, III, and IV were similar regardless of differentiation stages (data not shown). Also, it was previously reported that gene expressions of complex I and IV in undifferentiated HESCs were similar.
to those of differentiated cells [7]. In addition, TEM images clearly showed that the cellular organelles were very immature in undifferentiated HESCs compared to the one-week differentiated cells.

Increased mitochondrial mass led to increased production of ATP. Since ROS is a by-product of oxidative phosphorylation, it is inevitable for the cells with oxidative metabolism to produce more ROS than the cells depending on anaerobic metabolism. Therefore, all aerobic cells should be equipped with effective antioxidant system. The incomplete reduction of oxygen to water during oxidative respiration results in the formation of superoxide anions, which are converted to hydrogen peroxide by SODs [17]. Hydrogen peroxide itself is not very reactive, but can be further reduced to the extremely deleterious hydroxyl radicals. Thus, hydrogen peroxide should be removed by various enzymes such as catalase, GPx, Prx, and others [17]. We found that the Mn-SOD and catalase are the major antioxidant enzymes in the undifferentiated HESCs. Other antioxidant enzymes such as Cu/Zn-SOD, Prx1, Prx2, and GPx1 have low level of expression in undifferentiated HESCs but increased dramatically in differentiated cells. Mn-SOD (SOD2) and Cu/Zn-SOD (SOD1) are encoded by distinct genes and exclusively located in the mitochondria and intracellular cytoplasmic spaces, respectively [18]. Although their biologic action is virtually same, the regulatory mechanisms of these two SODs are different [18].

From above observation, we might speculate that: (1) the ROS produced in the mitochondria of the undifferentiated HESCs can be effectively converted to hydrogen peroxide by Mn-SOD and then be catalyzed by catalase, (2) increased ATP production through mitochondrial ETC would be accompanied by increased production of ROS, which would be expected to leak into the cytoplasm, (3) cytosolic Cu/Zn SOD would be activated and convert leaked ROS into hydrogen peroxide, and (4) to deal with the increased production of hydrogen peroxide, other catalytic enzymes such as GPx and Prx would be activated.

In spite of increased antioxidant enzyme production, intracellular ROS amount was found to be increased with differentiation. It would be possible that the biological activity of antioxidant enzymes might be still low in this stage, which we did not measure in this study. Another possibility is the ROS produced in the differentiated cells might play an important role in cell signaling and differentiation [19, 20].

We showed dynamic changes in mitochondrial energy metabolism, ROS, and antioxidant enzymes in the HESCs of various differentiation stages. This in vitro model would be invaluable helpful to explore the mitochondrial biogenesis, energy metabolism, and maturation of antioxidant system during the early period of human embryogenesis.

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