Mitochondrial pyruvate dehydrogenase phosphatase 1 regulates the early differentiation of cardiomyocytes from mouse embryonic stem cells

Hye Jin Heo1, Hyung Kyu Kim1, Jae Boum Youm1, Sung Woo Cho2, In-Sung Song1, Sun Young Lee1, Tae Hee Ko1, Nari Kim1, Kyung Soo Ko1, Byoung Doo Rhee1 and Jin Han1

Mitochondria are crucial for maintaining the properties of embryonic stem cells (ESCs) and for regulating their subsequent differentiation into diverse cell lineages, including cardiomyocytes. However, mitochondrial regulators that manage the rate of differentiation or cell fate have been rarely identified. This study aimed to determine the potential mitochondrial factor that controls the differentiation of ESCs into cardiac myocytes. We induced cardiomyocyte differentiation from mouse ESCs (mESCs) and performed microarray assays to assess messenger RNA (mRNA) expression changes at differentiation day 8 (D8) compared with undifferentiated mESCs (D0). Among the differentially expressed genes, Pdp1 expression was significantly decreased (27-fold) on D8 compared to D0, which was accompanied by suppressed mitochondrial indices, including ATP levels, membrane potential, ROS and mitochondrial Ca2+. Notably, Pdp1 overexpression significantly enhanced the mitochondrial indices and pyruvate dehydrogenase activity and reduced the expression of cardiac differentiation marker mRNA and the cardiac differentiation rate compared to a mock control. In confirmation of this, a knockdown of the Pdp1 gene promoted the expression of cardiac differentiation marker mRNA and the cardiac differentiation rate. In conclusion, our results suggest that mitochondrial PDP1 is a potential regulator that controls cardiac differentiation at an early differentiation stage in ESCs.

INTRODUCTION

Embryonic stem cells (ESCs) are self-renewing and pluripotent and differentiate into diverse cell lineages. Several molecular and conditional factors have been suggested to be major inducers of ESC-derived cardiomyogenesis, including cardiac transcription factors, reactive oxygen species (ROS) and hypoxic environments. Recent studies have suggested that several mitochondrial factors regulate cardiomyocyte differentiation, including mitochondrial DNA transcription factors, mitochondrial ROS levels, and mitochondrial permeability transition pore (mPTP) states. Recently, it was reported that the inhibition of mPTP promotes early cardiomyocyte differentiation from pluripotent stem cells and maturation in developing embryos. These observations suggest that mitochondria can determine cardiomyocyte differentiation capacity in both human and mouse ESCs (mESCs) via the modulation of energy metabolism. Energy metabolic turnover, from anaerobic glycolysis to oxidative phosphorylation, is predominantly regulated by mitochondria and is an essential step that triggers ESC differentiation into cardiomyocytes. Therefore, the robust regulation of mitochondrial energy metabolism shifts is essential to meet energy demands during cell differentiation.

The molecular mechanisms underlying mitochondrial energy metabolism during cardiomyocyte differentiation remain unclear. This study aimed to investigate possible mitochondrial regulators that are necessary for cardiomyocyte differentiation from mESCs. Among mitochondrial regulators, the pyruvate dehydrogenase (PDH) complex regulates the acetyl-CoA conversion rate from pyruvate. The acetyl-CoA conversion rate directly affects tricarboxylic acid (TCA) cycle activity, providing NADH to electron transfer chain complexes. Thus, the PDH complex has been hypothesized to be a mitochondrial molecular regulator of cardiomyocyte differentiation. In this study, we screened differentially expressed genes...

1National Research Laboratory for Mitochondrial Signaling, Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, Inje University, Busan, Korea and 2Laboratory of Vascular Biology and Stem Cell, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Korea

Correspondence: Professor J Han, National Research Laboratory for Mitochondrial Signaling, Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, Inje University, Busan 614-735, Korea.

E-mail: phyhanj@inje.ac.kr

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related to energy metabolism during embryonic body (EB) differentiation and found significantly modulated expression of PDH complex components.

Among these regulators, we report that the expression of the pyruvate dehydrogenase phosphatase catalytic subunit 1 (Pdp1) gene was significantly reduced during the early stages of mESC-derived EB differentiation. To examine whether Pdp1 affects cardiomyocyte differentiation, we established mESCs that transiently overexpressed or consistently inhibited Pdp1 in mESCs. In addition, we investigated PDH complex activity and mitochondrial function changes in these cells.

MATERIALS AND METHODS

Maintenance of undifferentiated mESCs
The mESC line R1 (ATCC) was routinely cultured and grown on mitotically arrested mouse embryonic fibroblasts (MEFs, 50 000 cells per cm²) in 60-mm culture plates. For MEF exclusion, EMG7 (mESCs), which have an α-myosin heavy chain (MHC) promoter controlling green fluorescent protein (GFP) expression, were maintained in 0.1% gelatin-coated 60-mm culture dishes without a feeder layer.

Induction of mESC-derived cardiomyocytes using EBs
The hanging-drop method was used to generate EBs from 750 cells per 20 μl differentiation media (differentiation day 0, D0). On day 4 of differentiation, EBs were seeded in 1% gelatin-coated culture dishes for each experiment. During differentiation, the culture media were changed daily. Beginning on day 6 of differentiation (2 days after plating), EB growth was observed daily for self-beating capacity. For each experiment. During differentiation, the culture media were changed daily. Beginning on day 6 of differentiation (2 days after plating), EB growth was observed daily for self-beating capacity. For each experiment.

Transient overexpression of Pdp1 in R1 mESCs
The Pdp1 gene was subcloned into the EcorRI/Xbal enzyme sites of the FUW expression vector. FUW vectors with the Pdp1 coding sequence or empty FUW vectors were transfected into HEK293FT cells to produce Pdp1-containing viruses. Two days after transfection, virus-containing media were collected to treat undifferentiated R1 cells. After treatment with virus-containing media, the hanging-drop method was employed to induce differentiation in mESC-derived cardiomyocytes.

Stable suppression of Pdp1 in EMG7 mESCs
For lentivirus-mediated Pdp1 gene transfer, the EMG7 mESC line was incubated for 15 min with 6 μg ml⁻¹ Polybrene. Cells were transfected with 10 μl of 1 × 10⁶ TU ml⁻¹ of viral particles (Sigma-Aldrich, St Louis, MO, USA). Twenty hours after the first transfection, virus-containing media were removed and three times the number of viral particles was added for the second virus transfection. To establish a stable cell line, we purified transfected cells using a final concentration of 3 μg ml⁻¹ of puromycin (Sigma-Aldrich) and used the hanging-drop method for EB generation.

Analysis of mRNA expression using quantitative real-time PCR
Total RNA was purified using the RNeasy mini kit (Qiagen, German-town, MD, USA). Complementary DNA (cDNA) was synthesized in a 50-μl volume using Super-Script III First-Strand Synthesis (Invitrogen, Waltham, MA, USA). cDNA was amplified using Taq DNA polymerase (Invitrogen) in the presence of 1 μM oligonucleotide primers. Quantitative real-time PCR was performed using the iQ5 real-time system and iQ SYBR Supermix (Bio-Rad, Hercules, CA, USA). Expression of target mRNAs relative to housekeeping gene expression (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA) was calculated using the threshold cycle (CT) method as \( \Delta \Delta CT \), where \( \Delta CT = CT_{\text{target}} - CT_{\text{GAPDH}} \) and \( \Delta(\Delta CT) = \Delta CT_{\text{D8}} - \Delta CT_{\text{D0}} \). The primer sequences are shown in Table 1 and Supplementary Table S1.

Western blot analyses
Cells were homogenized in protein lysis buffer and centrifuged at 10 000 g for 10 min at 4 °C. After centrifugation, protein samples (50 μg) were transferred onto nitrocellulose membranes, which were then incubated with primary antibodies against sarcomeric α-actinin (Sigma-Aldrich), cTnT (Abcam, Cambridge, UK), PDPI (Abcam), PDH-Elα (Abcam) and β-tubulin (Abcam). Immunoreactive protein bands were detected using the SuperSignal West Pico system and visualized using LAS-3000 PLUS (Fuji Photo Film Company, Tokyo, Japan).

Immunocytochemistry
Undifferentiated ESCs (D0) and differentiated EBs (D8) were cultured on gelatinized 12-mm glass coverslips, fixed for 30 min at room temperature in 4% paraformaldehyde, and permeabilized for 30 min at room temperature using 0.2% Triton X-100. After permeabilization, EBs were blocked for 10 min at room temperature with CAS-Block solution (Invitrogen) and incubated overnight at 4 °C with mouse monoclonal antibodies against Oct 3/4 (Abcam) and cardiac troponin T (cTnT, Abcam). In addition, goat anti-mouse IgG (Abcam) was applied to primary antibody-labeled EBs. Confocal images were obtained using a Carl Zeiss LSM 700 laser scanning microscope (Zeiss, Oberkochen, Germany).

Table 1 Quantitative real-time PCR primer sequences

| Primer   | Forward 5' | Reverse 3' |
|----------|------------|------------|
| α-actinin| 5'-AGCCAGGAAACAGATGAACAGA-3' | 5'-AAGCGATGAAAGGGCGTGG-3' |
| α-MHC    | 5'-GCCCACTTCCAGAAGCTG-3' | 5'-GCCTTAACATCTCCCTTCGG-3' |
| cTnl     | 5'-CCTGAGAGCGCGAGATGCAAC-3' | 5'-GTCCTCCTTCCACCTGCT-3' |
| cTnT     | 5'-CAAGAGGAAGCCAAGTACAGAGA-3' | 5'-CTCCATCGGGGATCTGGGT-3' |
| Cx43     | 5'-ACGGCAAGGTGAAAGTGAGA-3' | 5'-GAGAGACACAGGCCAGACCA-3' |
| Drp1     | 5'-GGTTGCTAGGAACCCCAAC-3' | 5'-TCAACTCTGCCGTTCTCG-3' |
| Mef2c    | 5'-ACCAGGAAAGAAATGAGGA-3' | 5'-GGCCGCTATTAGTGGTGAGG-3' |
| MLC2     | 5'-GAGCACAAGAAGAAGCGACA-3' | 5'-GGCCCTGACGTCTTGGTAT-3' |
| Mfn1     | 5'-GGGACCTGGACTCTCTGCT-3' | 5'-TACTTGTTGCTGCAGTGGT-3' |
| Mfn2     | 5'-TGAAACACCCAAGAAGA-3' | 5'-GCAAAGAATCGCCAGAC-3' |
| GAPDH    | 5'-CACATCTTCCAGGGACGGACAG-3' | 5'-CCTTCTCATGTTGAGTGAACAG-3' |
Flow cytometry analyses
Cells were stained with 0.1 nM tetramethylrhodamine, methyl ester (TMRM) and 5 μM MitoSox-Red (Invitrogen) for 30 min at 37 °C to detect mitochondrial membrane potential and mitochondrial ROS. To measure mitochondrial calcium levels, 5 μM Rhod2-AM (Invitrogen) was applied to dissociated EBs for 2 h on ice, followed by incubation for 30 min at 37 °C. After staining, cells were washed in phosphate-buffered saline (PBS) and analyzed on a dual-laser FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of oxygen consumption
After cell dissociation, the oxygen consumption rate (OCR) was measured using the NeoFox Phase Measurement system (Ocean Optics, Dunedin, FL, USA) and a 400-μl chamber at room temperature in air-saturated (220 nM per O2 per ml) culture media. After measurement, cells were collected for protein quantification. The OCR is expressed as nM O2 per min per mg of total protein.

ATP assays
ATP concentrations were analyzed using ATP bioluminescent assay kits (Sigma-Aldrich). Briefly, 100 μl of ATP assay mix working solution, 100 μl of ATP-releasing reagent and 50 μl of ultrapure water were mixed by vortexing in each assay vial. Cells were collected and 1×10⁵ cells were added to each vial. Luminescence was detected using SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA). ATP concentrations were calculated using a standard curve with known concentrations (0, 1.25, 2.5, 5 and 10 μM) of an ATP standard.

Target labeling and hybridization to microarray
For control (D0) and test RNAs (D8), cRNA probe synthesis and hybridization were performed using Agilent’s Low Input Quick Amp Labeling Kit (Agilent Technology, Santa Clara, CA, USA). Labeled target cRNA was quantified using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). After evaluating the labeling efficiency, 850 ng of each cyanine 3- and cyanine 5-labeled target cRNA was mixed and cRNA fragmentation was performed by adding 10× blocking agent and 25× fragmentation buffer, followed by incubation for 30 min at 60 °C. The fragmented cRNA was resuspended in 2× hybridization buffer and transferred to an assembled Agilent Mouse GE 4×44 K v2 Microarray. Arrays were hybridized at 65 °C for 17 h in an Agilent hybridization oven (Agilent Technology). The microarrays were washed according to the manufacturer’s washing protocol (Agilent Technology).

Measurement of PDH activity
Cells (D0 and D8) were prepared and adjusted to have the same cell number. After sample preparation, PDH activity assay kits (Abcam) were used to determine PDH complex activity.

Statistical analysis
The results are expressed as the mean±s.e.m. The data were analyzed using parametric unpaired Student’s t-tests. P-values <0.05 were considered statistically significant.
RESULTS

Differentiation of mESC-derived cardiomyocytes using EBs
To investigate whether EBs can differentiate into mature cardiomyocytes, R1 ESCs were cultured using the hanging-drop method. The beating EBs and beat-per-minute rates were measured in differentiation day 6 to day 12 cultures (Figure 1a–c). cTnT was strongly expressed and distinct sarcomere structures were observed in differentiating EBs (D8; Figure 1d).

Electrophysiological properties of mESC-derived cardiomyocytes in EBs
To evaluate the electrophysiological characteristics of beating cardiomyocytes from D8, three types of action potentials (APs) were recorded (Figure 1e–g). Depolarizing step pulses were performed to activate outward currents. We observed a biphasic time course with fast activation and slow inactivation (Supplementary Figure S1b). Outward currents were blocked in a reversible manner using 10 mM tetraethylammonium (TEA; Supplementary Figure S1b and S1c). However, there was no indication of an inward current carried through the inward rectifying K+ channel when hyperpolarizing test pulses between 100 and −70 mV were applied to cells from a holding potential of −60 mV (data not shown). The current–voltage (I–V) relationship of TEA-sensitive currents was determined by integrating the results from depolarizing and hyperpolarizing step pulses (Supplementary Figure S1d). The relationship was outward rectifying, suggesting that they were voltage-dependent K+ currents, which are primarily due to the KV family. Currents activated by depolarizing test pulses on D0 cells were linear and much smaller (<1/10) than those on D8 cells, indicating that D0 cells only exhibit background leak conductance (Supplementary Figure S1a).

To identify Ca2+-selective currents, a Cs+-rich pipette solution was employed and a Na+-rich solution with 10 mM Ba2+ was used as the bath solution. As shown in Supplementary Figure S2a, a series of depolarizing step pulses between −70 and +40 mV (from a holding potential of −80 mV) activated inward currents with fast activation and slow inactivation. The I–V relationship was constructed by plotting peaks of inward currents against each test potential and showed an inverted bell shape, where the maximum current density was between −40 and −30 mV (Supplementary Figure S2b). The inward currents were not sensitive up to 2 μM nifedipine, which is a specific L-type Ca2+ channel blocker. Mibefradil, which is specific for T-type Ca2+ channels, blocked inward current by ~50% at 1 μM (Supplementary Figure S2c). A dose–response relationship was obtained by plotting the degree of blocking against the concentration of mibefradil (Supplementary Figure S2d). The relationship fit the Hill equation, where the half-maximal effective concentration was 1.19 μM (n = 3). Considering that these currents are activated at potentials near rest, and they are sensitive to mibefradil, they are more likely to be Ca2+ currents belonging to T-type rather than L-type channels. Altogether, these data provide evidence of the electrophysiological characteristics of cardiomyocytes differentiated from EBs.

Mitochondrial function changes during differentiation
To determine whether mitochondrial functions were altered during mESC differentiation, we measured intracellular ATP levels and OCR at D0 and D8 (Figure 2a and b). Using flow cytometry analyses, we measured mitochondrial membrane potential (0.1 mM TMRM), mitochondrial ROS levels (5 μM MitoSox) and mitochondrial calcium levels (5 μM Rhod-2, AM; Figure 2c–e). All three parameters were significantly lower at D8 compared with D0, indicating that mitochondria functions were decreased in differentiating EBs compared with undifferentiated ESCs. OCR was not significantly different between D0 and D8 cultures.

Gene expression changes during differentiation
To examine alterations in gene expression, we performed microarray analyses of total mRNA in undifferentiated ESCs (D0) and differentiating EBs (D8). As shown in Figure 3c and Supplementary Table S2, many mitochondria-related genes were upregulated and downregulated at D8 compared with D0. A large proportion of the identified genes were associated with...
functional categories such as 'translation, ribosomal structure, biogenesis' and 'energy production and conversion' (Figure 3a). Moreover, pathway enrichment analysis revealed that the genes were related to several pathways especially in energy production (Figure 3b). In particular, the Pdp1 gene, which encodes a protein that converts pyruvate into acetyl-CoA in mitochondria, exhibited the lowest expression at D8 (Figure 3c and Supplementary Table S2). Quantitative PCR analyses confirmed that Pdp1 expression decreased during differentiation (Figure 3d).

**Pdp1 overexpression and mitochondrial function in mESC-derived EBs**

We transduced Pdp1 into mESCs to confirm the correlation of Pdp1 expression with mitochondrial function. We assessed ATP levels and OCR (Figure 4a and b) in control cells (mock) and Pdp1 -transduced cells (PDP1). We also evaluated the intensity of TMRE, MitoSox and Rhod-2 in mock and Pdp1 -overexpressing cells at D8 (Figure 4c-e). As shown in Figure 4, Pdp1 -overexpressing cells at D8 exhibited higher ATP levels, OCR, mitochondrial membrane potential and ROS levels compared with D8 mock cells. Moreover, Ca^{2+} levels were slightly increased in cells overexpressing Pdp1 at D8 compared with D8 mock cells.

**Pdp1 overexpression and changes in PDH complex activity in differentiating EBs**

We transiently transfected Pdp1 into undifferentiated mESCs, and expression was confirmed using quantitative real-time PCR and western blotting (Figure 5a and c). The Pdp1 group showed higher Pdp1 expression levels compared with mock at D8 (Figure 5a). Expression of PDP1 and the PDH complex protein PDH-E1α was also increased at D8 in the PDP1 group compared with control. However, the late stage of differentiation (D24) did not show significant differences between the two groups except PDH activity, which showed a difference (Figure 5b and c).

**Pdp1 overexpression and cardiomyogenesis in mESC-derived EBs**

To determine whether Pdp1 expression can regulate cardiac differentiation potential, we compared the expression levels of
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Mitochondrial PDP1 regulates cardiac differentiation from mESCs. Here we showed that mitochondrial PDP1 of mESCs could modulate the cardiac differentiation from mESCs.21 On the basis of these data, we hypothesized that there are mitochondrial modulators regulating cardiomyocyte differentiation from mESCs. Here we showed that mitochondrial PDP1 of mESCs could modulate the cardiac differentiation potential.

Using a standard ESC differentiation procedure, we successfully obtained differentiated EBs, including cardiomyocytes.18,22

DISCUSSION

Previous studies have demonstrated that mitochondrial function and biogenesis can determine cardiac differentiation potential from mESCs.15,20 Moreover, a mitochondrial functional index, such as the inner membrane potential, can be used as a practical tool for selecting differentiated cardiomyocytes.21 On the basis of these data, we hypothesized that there are mitochondrial modulators regulating cardiomyocyte differentiation from mESCs. Here we showed that mitochondrial PDP1 of mESCs could modulate the cardiac differentiation potential.
In the present study, differentiating EBs (D8) exhibited beating ability with a molecular identity similar to cardiomyocytes (Figure 1). Electrophysiological analyses confirmed that differentiating EBs, including cardiomyocytes, showed three types of APs, inward rectifying K+ channels (K_ir) and T-type Ca2+ channels, which are typical characteristics of cardiomyocytes. Generally, K_ir channels are crucial for stabilizing the resting membrane potential and action potentials.23,24 In addition, the T-type Ca2+ channel was recorded, likely indicating primarily cardiomyocyte characteristics and the necessary role for excitation-contraction and pacemaker activity in cardiomyocytes.25,26 However, undifferentiated mESCs maintaining pluripotency (D0) did not have electrophysiological activity with linear currents. These results suggest that our differentiated EBs were biologically and functionally similar to cardiomyocytes.

Next, we analyzed mitochondrial global gene expression profiles during differentiation. Compared with undifferentiated ESCs (D0), differentiating EBs (D8) displayed a lower expression of genes involved in regulating mitochondrial energy production and conversion. In particular, Pdp1 mRNA expression was decreased 27-fold in differentiating EBs (D8). It has been reported that the PDH complex acts as a crucial regulator of mitochondrial energy metabolism, linking the TCA cycle and subsequent oxidative phosphorylation with glycolysis.17 The PDH complex is controlled by PDP1, PDP2 and PDH kinase (PDK) 1-4 isoenzymes.27,28 PDH phosphatases comprise PDP1 and PDP2 and are located in the mitochondrial matrix space and regulate the PDH complex dephosphorylation and reactivation.27,28 Notably, the PDP1 and PDP2 isoforms are highly expressed in rat heart, kidney and skeletal muscle.29–31 Because PDPs
are key redox and biological energy sensors, PDP1 and PDP2 expression is acutely altered by starvation, diabetes and aerobic exercise, which subsequently modulates PDH complex activities.\textsuperscript{32}–\textsuperscript{34}

The phosphorylated PDH E1\(\alpha\) subunit causes PDH inactivation and decreases acetyl-CoA levels entering the TCA cycle.\textsuperscript{35}

The PDH E1\(\alpha\) subunit is highly phosphorylated in pluripotent stem cells compared with differentiated cells.\textsuperscript{17,27} Interestingly, PDP2 expression was decreased in both H7TF and IMR-90 differentiated cells compared with human pluripotent stem cells. In addition, hypoxia-inducible factor (Hif-1), which is overexpressed under hypoxic conditions, increases PDK1 activity, inhibiting the PDH complex from using pyruvate in the TCA cycle.\textsuperscript{17,36} Thus, lower mitochondrial oxygen consumption is observed and cell death is inhibited under hypoxic conditions. During mESC differentiation using EB formation, a hypoxic region is observed in the EB core in normoxic culture conditions.\textsuperscript{37} A recent study demonstrated that hypoxia increases cardiomyogenesis during human ESC differentiation.\textsuperscript{7} Consistent with previous studies, differentiating EBs (D8) exhibited lower energy production activity with decreased ATP levels and OCR compared with undifferentiated ESCs (D0) at an early differentiation stage. In addition, we showed decreased mitochondrial membrane potential, ROS levels and calcium levels in differentiating EBs (D8) (Figure 2).

To determine whether PDP1 is a potential mitochondrial modulator for cardiomyogenesis especially in the early differentiation stage, we investigated the effect of \textit{Pdp1} overexpression or suppression-mediated energy metabolism during mESC differentiation. \textit{Pdp1} overexpression resulted in increased mitochondrial function and PDH complex activity in D8, but the cardiac differentiation potential was significantly attenuated in differentiating EBs (D8; Figures 4 and 5). In contrast, \textit{Pdp1} suppression decreased mitochondrial function and PDH complex activity in D8, but the late differentiation (D24) did not represent a significant difference (Figures 6 and 7). Moreover, the shPDP1 group promoted cardiomyogenesis at both D8 and D24. These results clearly proved our hypothesis that PDP1 is a potential mitochondrial modulator for cardiomyogenesis at the early differentiation stage.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Changes in mitochondrial function after \textit{Pdp1} inhibition (shPDP1) in feeder-free mESCs. (a) The intracellular ATP content and (b) oxygen consumption rate in control and \textit{Pdp1} knockdown (shPDP1) EMG7-derived EBs were determined at D0 and D8. (c–e) Fluorescence-associated flow cytometry was performed to detect mitochondrial functions in Con and shPDP1 EMG7-derived EBs at D0 and D8. Cells were stained with TMRM (mitochondrial membrane potential) (c), MitoSox-Red (mitochondrial ROS) (d), and Rhod-2 AM (mitochondrial calcium) (e) and analyzed by flow cytometry. \(P<0.001\) vs Con. Con, control; mESC, mouse embryonic stem cells; EB, embryoid body.}
\end{figure}
In contrast to our findings, mitochondrial oxidative phosphorylation is generally increased during cardiomyocyte differentiation from pluripotent stem cells. These conflicting findings may depend on cell type, differentiation method, cell heterogeneity and culture environments, including hypoxic conditions. Indeed, consistent with our findings, the mitochondrial membrane potential was depolarized and ATP consumption and glycolysis-based energy metabolism were reduced in differentiated cells compared with human ESCs and iPSCs. Collectively, the previous and current data indicate that higher mitochondrial function may have stringent roles in pluripotent stem cells compared with differentiating EBs. However, our study is limited by the absence of PDK function data on cardiomyogenesis from pluripotent stem cells, and PDH complex activity was examined with the corresponding control group (Con). (a) Pdp1 expression was examined using quantitative real-time PCR. (b) PDH complex activity was measured in both groups at D0, D8, and D24. (c) Protein level of PDP1 and PDH-E1α were analyzed in both groups at D0, D8, and D24 by western blot. (d) Relative mRNA level of cardiac-specific markers were examined by quantitative real-time PCR in the control and shPDP1 groups. (e) The expression of cardiac-specific proteins (α-actinin and cTnT) was also evaluated by western blotting. (f) Immunohistochemistry results of αMHC-GFP and cTnT stained cardiomyocytes in Con and shPDP1. (g) Proposed role of PDP1 in cardiac differentiation. #P<0.01, †P<0.001 vs Con.

Moreover, we also need to prove that the exogenous donation of ATP may directly inhibit cardiac differentiation.

In conclusion, our data suggest that PDP1 in mESCs acts as a new mitochondrial regulator via the PDH complex and effectively increases cardiomyogenesis properties (Figure 7g), which is important for transplantation in cardiac stem cell therapy. Moreover, these results also provide new insights into cardiomyocyte differentiation metabolism in stem cell therapy and regenerative medicine.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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