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Original Article

Plasma-activated medium selectively eliminates undifferentiated human induced pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells, including human induced pluripotent stem cells (hiPSCs), are promising materials for regenerative medicine and cell transplantation therapy. However, tumorigenic potential of residual undifferentiated stem cells hampers their use in these therapies. Therefore, it is important to develop methods that selectively eliminate undifferentiated stem cells from a population of differentiated cells before their transplantation. In the present study, we investigated whether plasma-activated medium (PAM) selectively eliminated undifferentiated hiPSCs by inducing external oxidative stress. PAM was prepared by irradiating cell culture medium with non-thermal atmospheric pressure plasma. We observed that PAM selectively and efficiently killed undifferentiated hiPSCs cocultured with normal human dermal fibroblasts (NHDFs), which were used as differentiated cells. We also observed that undifferentiated hiPSCs were more sensitive to PAM than hiPSC-derived differentiated cells. Gene expression analysis suggested that lower expression of oxidative stress-related genes, including those encoding enzymes involved in hydrogen peroxide (H_2O_2) degradation, in undifferentiated hiPSCs was one of the mechanisms underlying PAM-induced selective cell death. PAM killed undifferentiated hiPSCs more efficiently than a medium containing the same concentration of H_2O_2 as that in PAM, suggesting that H_2O_2 and various reactive oxygen/nitrogen species in PAM selectively eliminated undifferentiated hiPSCs. Thus, our results indicate that PAM has a great potential to eliminate tumorigenic hiPSCs from a population of differentiated cells and that it may be a very useful tool in regenerative medicine and cell transplantation therapy.

1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are potential sources of cells for use in regenerative medicine, drug screening, and cell transplantation therapy [1–4]. In 2014, the first human trial on hiPSC-derived retinal pigment epithelium [5] was performed by the Riken Center for Developmental Biology, Kobe, Japan. Thus, innovative therapy by using hiPSCs-derived differentiated cells is becoming an increasingly realistic prospect. However, hPSCs-based therapy is associated with some risks such as tumor and teratoma formation because of the residual tumorigenic potential of

Abbreviations: hPSCs, human pluripotent stem cells; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; PAM, plasma-activated medium; NHDFs, normal human dermal fibroblasts; SOD, superoxide dismutase; GPX1, glutathione peroxidase 1; CAT, catalase; ATM, ataxia telangiectasia mutated; ROS, reactive oxygen species; RONS, reactive oxygen/nitrogen species; PI, Propidium Iodide.

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undifferentiated stem cells that may be present in a population of differentiated cells [6]. Therefore, it is important to develop methods that selectively eliminate undifferentiated cells before transplanting differentiated cells to realize the safety of hPSC-based therapy.

Several methods have been developed to eliminate residual hPSCs from a population of differentiated cells, including induction of selective cell death by using cytotoxic antibodies [7,8], use of lectin-conjugated cytotoxic proteins [9], chemical inhibitors [10–12], use of conditionally replicating adenoviruses [13], selective separation by performing cell sorting with hPSC-specific antibodies [14], and metabolic properties [15]. However, these methods are associated with some limitations with respect to specificity, cost, efficacy, safety, and throughput. Therefore, alternative novel methods based on different mechanisms should be developed.

Plasma is an ionized gas containing positive and negative ions, radicals, electrons, uncharged (neutral) atoms and molecules, and UV photons [16]. In recent years, non-thermal atmospheric pressure plasma (NEAPP) has been used in various biological applications [17–19] and has emerged as a novel technology for medical applications such as cancer therapy. Moreover, both direct irradiation of cancer cells and indirect irradiation of medium or water with NEAPP affects cancer cells because of the presence of various reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, singlet oxygen, nitric oxide (NO), nitrate/nitrite (NOx), hydrogen peroxide (H_2O_2), and other uncertain species. Plasma-activated medium (PAM) or plasma-activated water exerts cytotoxic effects on various cancer cells compared with those on normal cells [20–22] but exerts a curative effect on age-related macular degeneration [23]. Moreover, recent studies have suggested that PAM or plasma-activated water is a new tool for treating various diseases and can be used in various biological researches.

Compared to differentiated cells, hPSCs respond differently to oxidative stress [24,25]. Prigione et al. [25] reported that (1) expression levels of some oxidative stress-related genes were lower in hPSCs than in differentiated cells and that (2) expression level of glutathione peroxidase-1 (GPX1), the most abundant glutathione peroxidase isozyme in mammalian cells [26], significantly decreased in hPSCs compared with that in differentiated cells. Therefore, we investigated whether hPSCs could be selectively eliminated by using PAM because it induces oxidative stress in cells. Results of the present study indicated that PAM could be a potential tool for eliminating residual hPSCs present in a population of differentiated cells.

2. Results

2.1. Differential sensitivities of undifferentiated and differentiated cells toward PAM

To determine whether PAM eliminated undifferentiated cells, we investigated the sensitivities of undifferentiated 201B7 hiPSCs and NHDFs toward PAM. Different dilutions of PAM were added to hiPSC and NHDF cultures, and viabilities of these cells were evaluated after 24 h. Undifferentiated hiPSCs were completely killed after treatment with 1- to 16-fold diluted PAM (Fig. 1). In contrast, NHDFs were not killed even after treatment with 8-fold diluted PAM (cell viability, 97.6% ± 10.4%). These results indicated that undifferentiated hiPSCs were more sensitive to PAM than NHDFs and suggested that PAM could selectively eliminate undifferentiated cells.

Since we previously reported that the effect of PAM varied with cell density or its volume, using a cancer cell line [21], we

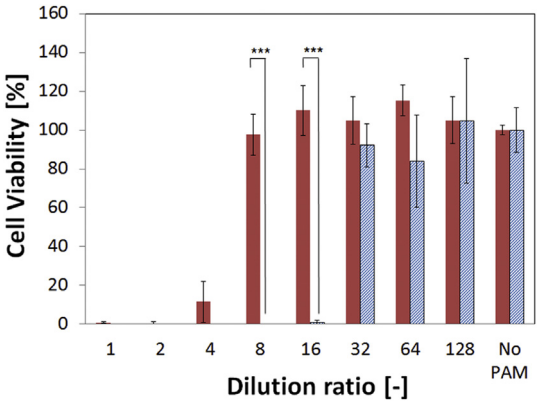

Fig. 1. Quantitative effects of PAM on undifferentiated 201B7 hiPSCs and differentiated NHDFs. PAM was diluted to an optimal concentration by using fresh Stemfit AK03 medium containing A, B, and C solutions and was added each wells (n = 3). After 24 h, cell viabilities (%) were evaluated using the Cell Counting Kit-8 by following the manufacturer's instructions. Red bars denote NHDFs, and blue diagonal bars denote 201B7 hiPSCs. The means ± SD of three experiments are shown; ***$p < 0.005$.

investigated whether these parameters affected the sensitivity of hiPSCs to PAM. The viability of iPSCs varied with cell density (Fig. 2A), with values of 113.9% ± 7.1% for 2.0×10^4 cells/well, 81.9% ± 5.8% for 1.0×10^4 cells/well, and 42.0% ± 2.8% for 5.0×10^3 cells/well. The viability of iPSCs also varied with PAM volume (Fig. 2B), with values of 96.7% ± 9.0% for 60 μL, 81.9% ± 5.8% for 90 μL, and 51.0% ± 12.3% for 120 μL. Considering these results, in subsequent experiments with different experimental conditions, the effective dilution ratio of PAM for each experiment was obtained and used to eliminate undifferentiated hiPSCs.

2.2. Selective elimination of undifferentiated cells by PAM

We next investigated whether PAM selectively eliminated undifferentiated hiPSCs. We cocultured undifferentiated hiPSCs and NHDFs. HiPSCs were first seeded in a multi-well plate and were cultured. After 48 h, fluorescently labeled NHDFs were seeded into the same plate and were cultured for 24 h. Next, the cells were treated with PAM for 24 h, stained with PI, and observed under a fluorescence microscope (Fig. 3). We observed that hiPSCs that were treated with undiluted PAM detached from the surface and

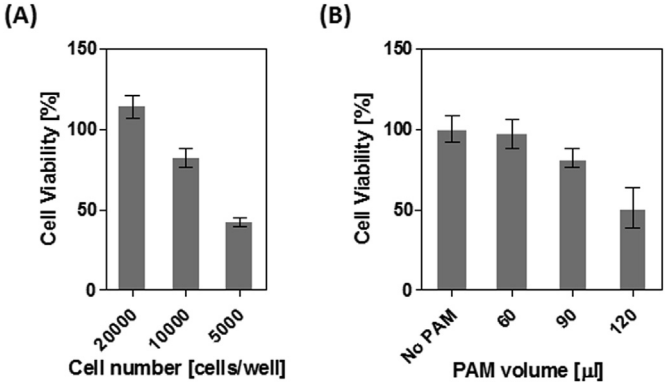

Fig. 2. Effects of cell density or PAM volume on the viability of undifferentiated 201B7 hiPSCs. (A) HiPSCs were seeded at densities of 2.0×10^4, 1.0×10^4, and 5.0×10^3 cells/well and were treated with 90 μL of 8-fold diluted PAM. (B) HiPSCs were seeded at a density of 1.0×10^4 cells/well and were treated with 60, 90, and 120 μL of 8-fold diluted PAM. After 24 h, cell viability (%) was evaluated using the Cell Counting Kit-8 in accordance with the manufacturer's instructions. The means ± SD of three experiments are shown.

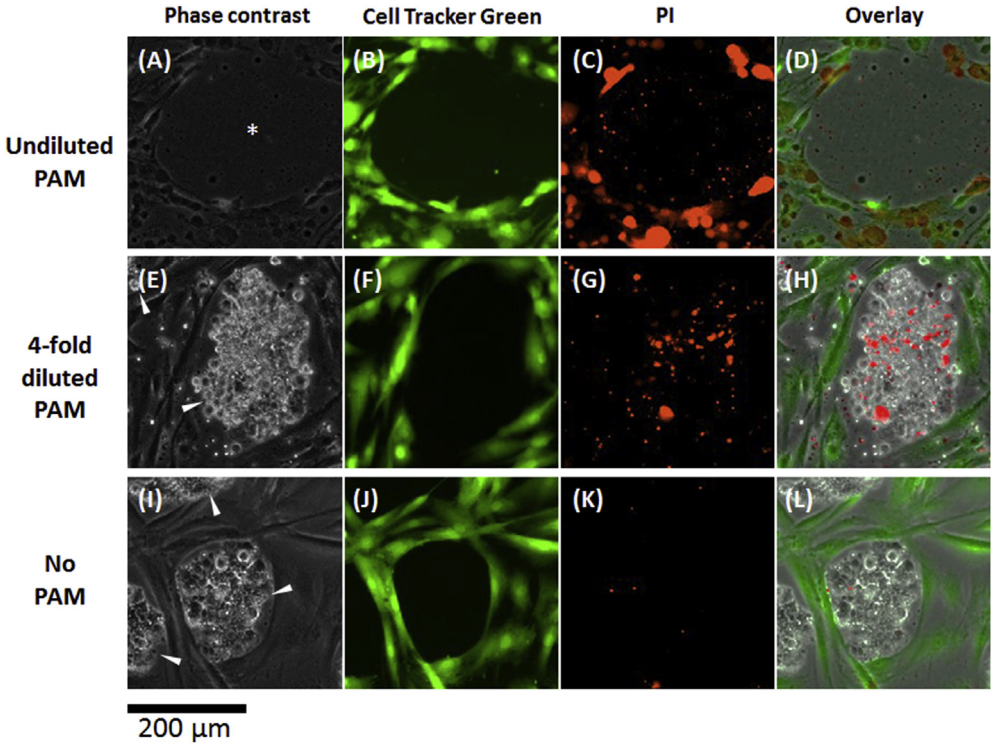

Fig. 3. Effects of PAM on undifferentiated 201B7 hiPSCs and differentiated cells NHDFs in a coculture. 201B7 hiPSCs were seeded in a 12-well plate. After 48 h, NHDFs stained with CellTracker Green were seeded in the 12-well plate containing hiPSCs. After 24 h, undiluted or 4-fold diluted PAM was added to the plate and the cells were incubated for 6 h. Next, PAM was removed, and the cells were stained with PI solution and were observed under a fluorescence microscope. (A ~ D) Cells treated with undiluted PAM, (E ~ H) cells treated with 4-fold diluted PAM, and (I ~ L) cells not treated with PAM. (A) The field of hiPSCs attachment before PAM treatment is indicated by an asterisk. (E and I) Arrow heads indicate hiPSCs.

were removed from the culture after a few washes (Fig. 3A–D). Most of the NHDFs treated with undiluted PAM appeared shrunken and were stained by PI (Fig. 3A–D). A proportion of hiPSCs that were treated with 4-fold diluted PAM were positively stained by PI and appeared shrunken (Fig. 3E–H). In contrast, NHDFs treated with 4-fold diluted PAM were not positive for PI (Fig. 3E–H), and proliferated normally to reach confluence (Fig. S1). HiPSCs and NHDFs without PAM treatment did not shrink or stain positively with PI (Fig. 3I–L). Considering these results, we concluded that undiluted PAM could kill both undifferentiated hiPSCs and NHDFs (Fig. 3A–D), whereas 4-fold diluted PAM selectively kills hiPSCs without exerting any apparent toxic effects on differentiated cells (Fig. 3E–H).

2.3. Evaluation of the elimination ability of PAM in a coculture by performing flow cytometry

Because PAM treatment of a coculture containing undifferentiated hiPSCs and NHDFs selectively eliminated the undifferentiated cells (Fig. 3), we evaluated the elimination ability of PAM in a coculture by performing flow cytometry. Undifferentiated hiPSCs and fluorescently labeled NHDFs were cocultured at different ratios (initial hiPSCs:NHDFs ratios were 1:2, 1:4, and 1:8) and were treated with 4-fold diluted PAM. After 48 h, the cells were collected and whole-cell populations containing different ratios of the 2 cell types were analyzed by performing flow cytometry. Results of flow cytometry showed that in control experiments, whole-cell populations containing initial hiPSC:NHDF ratios of 1:2, 1:4, and 1:8, contained 25.6%, 56.0%, and 83.2% NHDFs (Fig. 4, middle). Further, whole-cell populations containing initial hiPSC:NHDF ratios of 1:2, 1:4, and 1:8 contained 97.7%, 98.7%, and 98.6% NHDFs after treatment with 4-fold diluted PAM (Fig. 4, bottom). These results indicated that undifferentiated hiPSCs were almost completely eliminated from the whole-cell populations after treatment with 4-fold diluted PAM (Fig. 4, bottom). In addition, these results indicated that PAM was efficient in selectively eliminating undifferentiated hiPSCs from a coculture.

2.4. Differential sensitivity of undifferentiated hiPSCs and hiPSC-derived differentiated cells toward PAM

In the above experiments, NHDFs were used as differentiated cells (Figs. 1, 3 and 4). We also determined the effect of PAM on hiPSC-derived differentiated cells. HiPSC differentiation was induced by treatment with retinoic acid (RA) for 9 days [9,11]. The resulting differentiated cells and undifferentiated hiPSCs were cocultured and were treated with PAM (Fig. 5A). Cells treated with RA for 9 days were not stained with FITC–rBC2LCN, a fluorescent lectin probe, that was used to detect undifferentiated cells (data not shown). Most undifferentiated hiPSCs (not treated with RA) were killed after treatment with 16-fold diluted PAM (Fig. 5B [i–iv]). In contrast, most hiPSC-derived differentiated cells survived after treatment with the same dilution of PAM (Fig. 5B [v–viii]). These results indicated the potential of PAM for use in the actual differentiation process of the hiPSCs to other cell types such as cardiomyocytes and hepatocytes.

2.5. Mechanism underlying PAM-induced elimination of undifferentiated hiPSCs

To determine the sensitivity of undifferentiated hiPSCs to PAM compared with that of NHDFs, we evaluated the expression levels

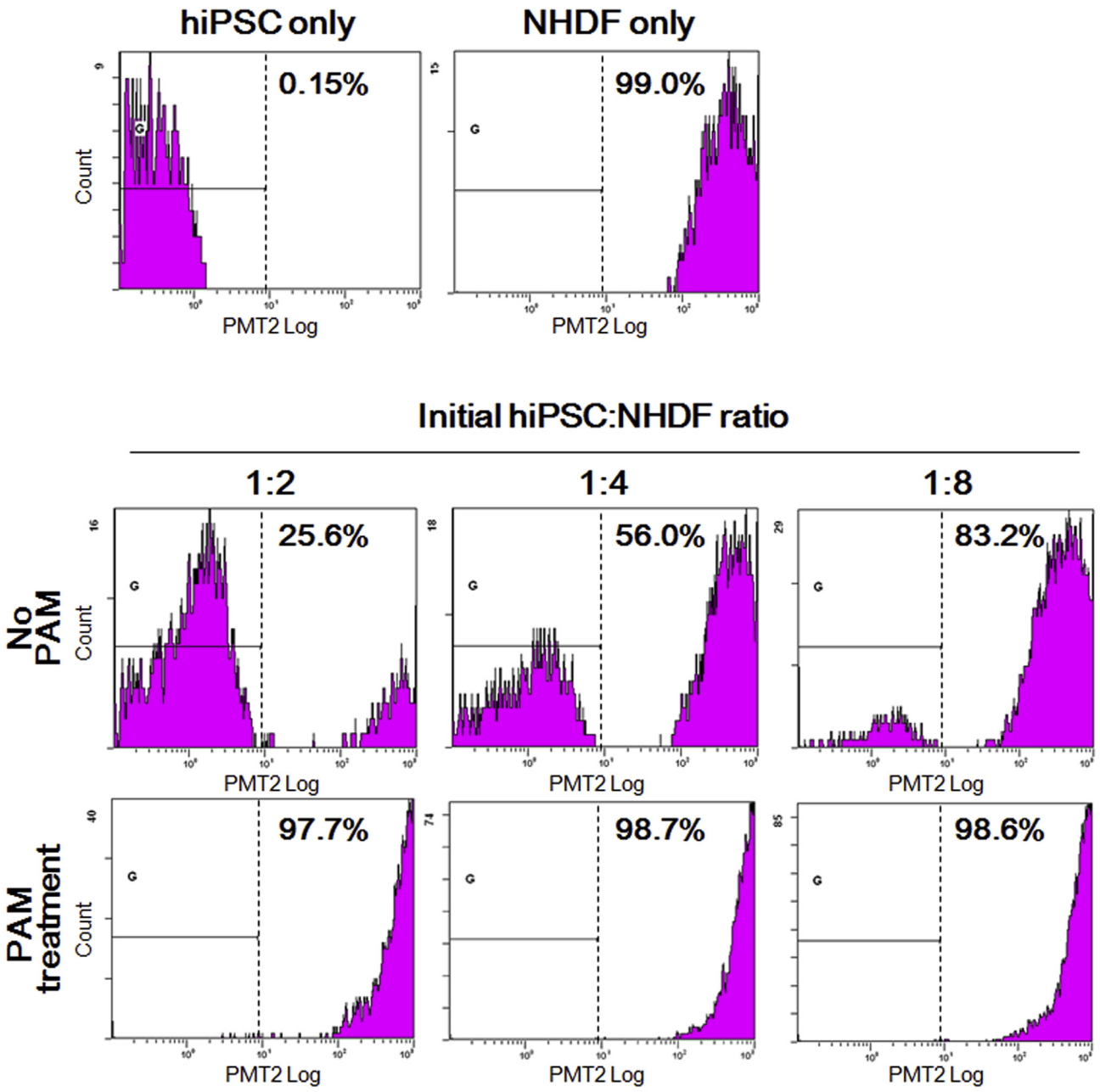

Fig. 4. Selective elimination of hiPSCs by PAM in a mixed-cell population. Varying numbers of 201B7 hiPSCs were cultured. After 48 h, NHDFs stained with CellTracker Green were seeded in a plate containing hiPSCs. After 24 h, 4-fold diluted PAM was added to the plate and the cells were incubated for 48 h. Next, PAM was removed, and the cells were washed with PBS. The cells were collected, and flow cytometry data were acquired.

of oxidative stress-related genes in undifferentiated hiPSCs and NHDFs. We selected 4 genes, namely, superoxide dismutase (*SOD*), *GPX1*, catalase (*CAT*), and ataxia telangiectasia mutated (*ATM*). SOD, GPX1, and CAT are involved in ROS generation and degradation. ROS is one of the main components of PAM [20–23]. ATM functions as DNA-repairing protein in human cells exposed to various stresses (e.g., ROS) [27]. Cells lacking ATM are hypersensitive to oxidative stress [28]. Expression level of *SOD* was similar in undifferentiated hiPSCs and NHDFs. However, expression levels of *GPX1*, *ATM*, and *CAT* were approximately 0.63, 0.46, and 0.09, respectively, in undifferentiated hiPSCs compared with those in NHDFs (Fig. 6A–D). These results suggested that low expression levels of oxidative stress-related genes in undifferentiated hiPSCs contributed to their selective elimination by PAM.

H_2O_2 is one of the main components of PAM [21]. Because expression levels of oxidative stress-related genes, including those encoding enzymes involved in H_2O_2 degradation, were low in undifferentiated hiPSCs (Fig. 6B–C), sensitivity of these cells to PAM was compared with their sensitivity to a medium containing the same concentration of H_2O_2 as that in PAM. We measured H_2O_2 concentration in PAM (464 ± 59 μM) and prepared a medium containing 465 μM H_2O_2. Approximately 92.1% undifferentiated hiPSCs were killed after treatment with PAM, whereas only 57.4% undifferentiated hiPSCs were killed after treatment with the H_2O_2-containing medium (Fig. 6E). These results suggested that H_2O_2 as well as various reactive oxygen nitrogen species (RONS) present in PAM contributed to the selective elimination of undifferentiated hiPSCs.

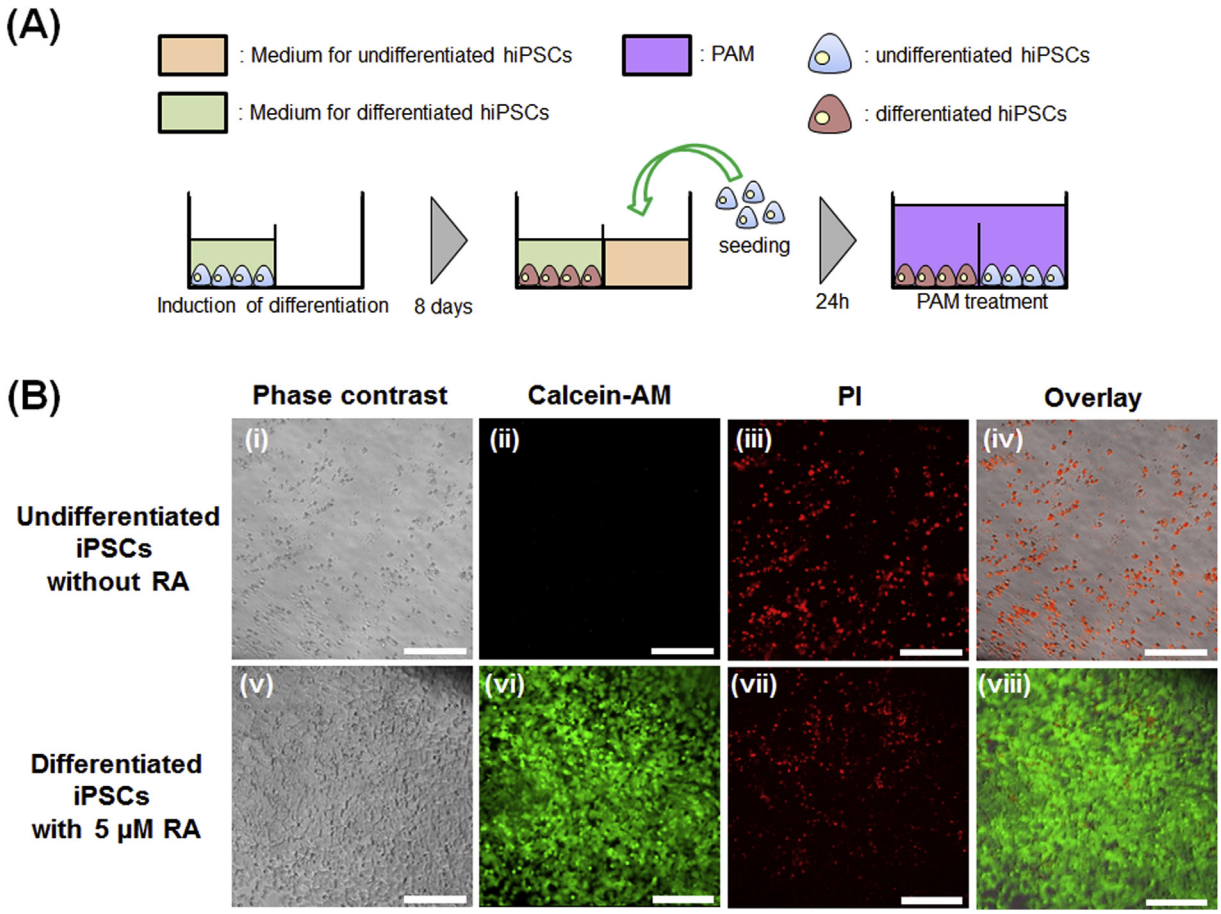

Fig. 5. Effect of PAM on the viability of undifferentiated 201B7 hiPSCs and RA-induced differentiated 201B7 hiPSCs. 201B7 hiPSCs were cultured in a medium containing 5 μM RA for 9 days. Undifferentiated 201B7 hiPSCs were seeded in a dish 24 h before PAM treatment. After 9 days of RA treatment, 16-fold diluted PAM was added to the dish and the cells were incubated for 24 h. Next, PAM was removed, and the cells were washed with PBS. The cells were then stained with calcein-AM and PI and were observed under a fluorescence microscope. (A) Schematic representation of the experimental procedure, and (B) experimental results. (i) Phase-contrast image of undifferentiated hiPSCs. (ii) Calcein-AM staining image of undifferentiated hiPSCs. (iii) PI staining image of undifferentiated hiPSCs. (iv) Overlay image of i–iii. (v) Phase-contrast image of differentiated hiPSCs. (vi) Calcein-AM staining image of differentiated hiPSCs. (vii) PI staining image of differentiated hiPSCs. (viii) Overlay image of v–vii. Scale bar, 200 μm.

3. Discussion

In this study, we showed that PAM containing RONS eliminated undifferentiated hiPSCs selectively and efficiently. We observed that 201B7 hiPSCs were more sensitive to lower concentration of PAM than NHDFs (Fig. 1). In addition, we observed that PAM selectively eliminated undifferentiated hiPSCs cocultured with NHDFs (Fig. 3). We performed flow cytometry to confirm that PAM had very high ability to eliminate undifferentiated hiPSCs (Fig. 4). In addition, we showed that the sensitivity of the differentiated cells and hiPSC-derived differentiated cells were apparently different, showing the potential of PAM for use in the actual process (Fig. 5). To the best of our knowledge, this is the first study to report the use of differential response of undifferentiated hiPSCs and differentiated cells to oxidative stress to selectively eliminate undifferentiated hiPSCs.

Expression levels of oxidative stress-related genes such as *GPX1*, *CAT*, and *ATM* were lower in undifferentiated hiPSCs than in NHDFs (Fig. 6B–D). Prigione et al. also reported that expression levels of oxidative stress-related genes were lower in hiPSCs than in iPSC-derived fibroblasts [24]. These results suggested that hiPSCs had lower ability to neutralize external oxidative stress than normal cells. This might be one of the reasons for the higher sensitivity of undifferentiated hiPSCs to external oxidative stress such as that induced by PAM than normal cells. PAM selectively eliminates cancer cells [20,21]. Activities of SOD and CAT are lower in cancer cells (e.g., colon and liver cancer cells) than in normal cells [29], which was similar to that observed in hiPSCs. Therefore, mechanisms underlying PAM-induced death of hiPSCs and cancer cells may be similar. Intracellular ROS concentration is higher in cancer cells than in normal cells because of the high mitochondrial activity in cancer cells [29]. However, undifferentiated cells such as hESCs do not show high mitochondrial activity [24]. Therefore, further studies should be performed to understand in detail the mechanisms underlying PAM-induced selective death of hiPSCs. An understanding of mechanisms underlying PAM-induced cancer cell death may provide some insights on mechanisms underlying PAM-induced death of hiPSCs.

PAM killed undifferentiated cells more efficiently than the medium containing the same concentration of H_2O_2 (Fig. 6E), which was similar to that observed with cancer cells [21]. PAM contains relatively short-lived superoxide anions, hydroxyl radicals, singlet oxygen, and NO; long-lived species such as H_2O_2 and NOx; and other uncertain species [20–23]. Therefore, the ability of PAM to effectively eliminate undifferentiated cells may be attributed to these components. Moreover, mechanisms underlying cell death induced by PAM are different from those underlying cell death induced by H_2O_2-containing medium. Adachi et al. reported that

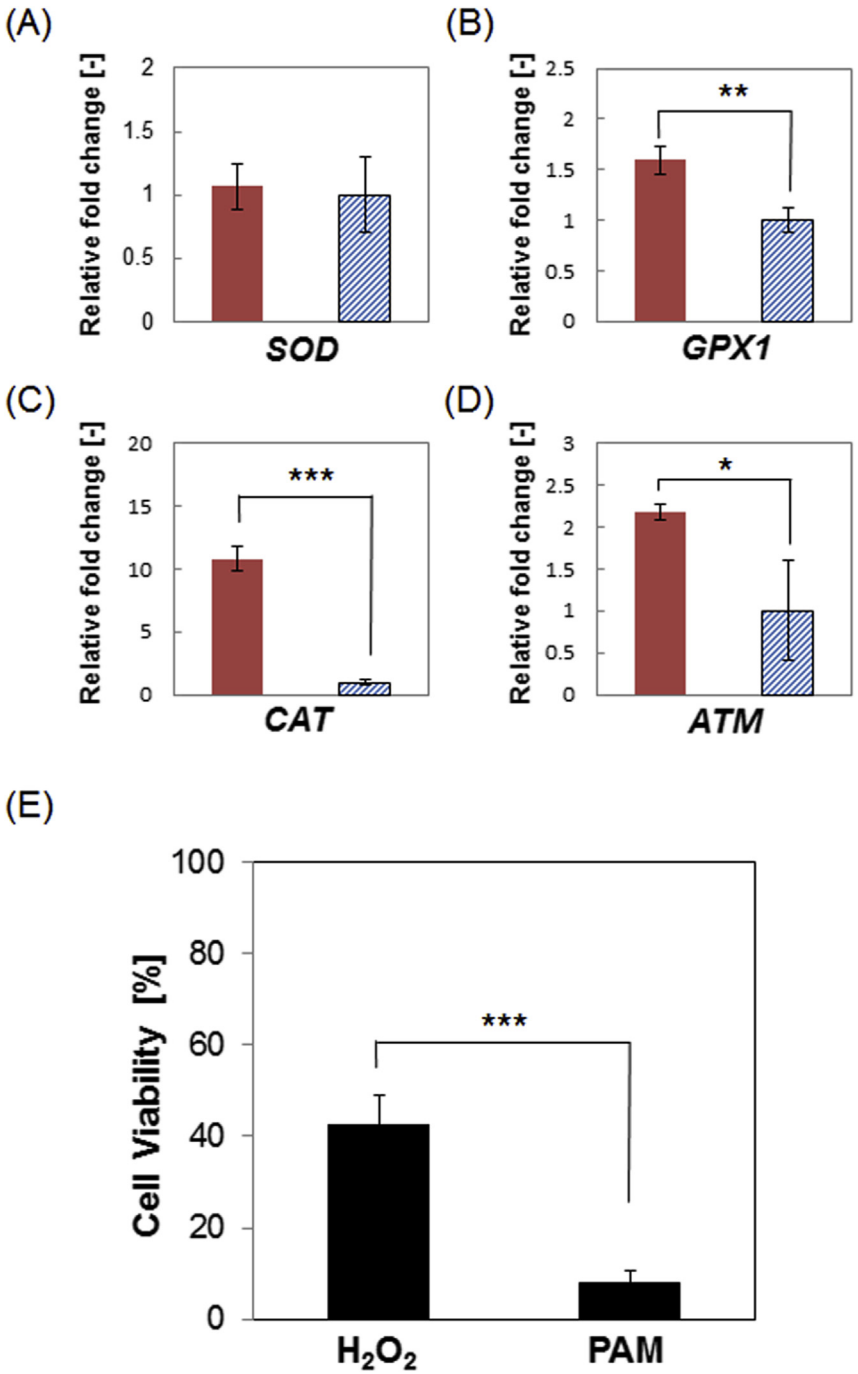

Fig. 6. Differences in the mRNA expression of oxidative stress-related genes between undifferentiated hiPSCs and differentiated NHDFs. Total RNAs of NHDFs (red bars) and hiPSCs (blue diagonal bars) were extracted from 4-day cultures, and mRNA levels were evaluated by performing real-time PCR. The mRNA levels of oxidative stress-related genes are expressed relative to those of GAPDH. Relative mRNA levels in hiPSCs are represented as "1". (A) *SOD*, (B) *GPX1*, (C) *CAT*, and (D) *ATM* ($p = 0.07$). (E) H_2O_2-containing medium was prepared by using the same concentration of H_2O_2 as that in PAM. H_2O_2 concentration in PAM was measured using Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit by following the manufacturer's instructions. PAM or H_2O_2-containing medium was added to each well containing 201B7 hiPSCs ($n = 3$). After 24 h, cell viabilities (%) were evaluated using the Cell Counting Kit-8 by following the manufacturer's instructions. Each bar represents mean ± SD ($n = 3$); $^{*}p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.005$.

the number of cancer cells labeled with annexin V–FITC, which is used to visualize plasma membrane injury, increased after treatment with PAM but not after treatment with H_2O_2-containing medium [21]. In addition, accumulation of YO-PRO-1 fluorescence, which is used to determine plasma membrane permeability due to apoptotic membrane injury, increased after treatment with PAM but not after treatment with H_2O_2-containing medium [21]. These results suggested that mechanisms underlying PAM-induced death of undifferentiated hiPSCs are partially different from those underlying H_2O_2-containing medium-induced cell death.

The effective PAM dilution ratios differed between experiments using different cell numbers (Figs. 1, 3 and 4). In monoculture experiments, 4-fold diluted PAM killed both hiPSCs and NHDFs, whereas 8- and 16-fold diluted PAM killed hiPSCs but not NHDFs (Fig. 1). In co-culture experiments, 4-fold diluted PAM was effective for selectively eliminating hiPSCs from NHDFs (Figs. 3

and 4). Previously, we reported that the effect of PAM varied with cell density or PAM volume using A549 cells (a human lung adenocarcinoma epithelial cell line) [21]. In the present study, we used undifferentiated hiPSCs and showed that cell viability differed with different cell densities or PAM volumes (Fig. 2). Considering these results, the optimization of PAM dilution and volume is necessary to eliminate residual hiPSCs during the differentiation process (to different cell types) for regenerative therapy.

PAM treatment is associated with some advantages. First, PAM is easy to use and has low manufacturing cost because it needs to be irradiated for only a few minutes. Second, PAM retains its elimination ability even when stored at less than −80 °C [21]. Third, PAM may induce no or minimal antigenicity because it contains small chemical compounds such as H_2O_2. In the present study, we used a commercially available clinical grade medium (Stemfit AK03) to prepare PAM [30] and successfully eliminated undifferentiated hiPSCs. Thus, our results suggest that PAM can be used clinically in the future to eliminate potentially tumorigenic undifferentiated hPSCs from a population of differentiated cells before transplantation.

However, for realizing clinical use of PAM for eliminating tumorigenic hiPSCs, influence of PAM treatment not only to the undifferentiated hiPSCs but also to the hiPSC-derived differentiated cells should be examined in detail. To date, several studies have examined the effects of PAM on differentiated normal cells and have shown that PAM exerts less harmful effects on these cells. Tanaka et al. [22] investigated the effects of PAM on glioblastoma cells and normal astrocytes and observed that PAM killed glioblastoma cells through PI3K/PTEN–AKT pathway without affecting normal astrocytes. Moreover, NEAPP did not affect the essential components in the medium used for culturing these cells [22]. Torii et al. [20] showed that WI-38 human fibroblasts are more resistant to PAM than gastric cancer cells. We also observed that NHDFs grew normally after treatment with PAM concentration that killed undifferentiated hiPSCs (Fig. S1). However, because ROS in PAM might induce DNA damage, including small or bulky modifications in bases as well as single- and double-stranded breaks [29], effect of PAM on the genomes of normal cells should be assessed very carefully.

In conclusion, we showed that PAM treatment selectively eliminated residual hiPSCs in a population of differentiated cells. Therefore, this novel approach may serve as a promising tool in regenerative medicine and cell transplantation therapy.

4. Materials & methods

4.1. Cell culture

HiPSC (201B7 [31]) was provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan. HiPSCs were cultivated in 0.5 μg/cm^2 laminin-511 E8 (iMatrix-511, 381-07363; Wako, Japan)-coated 25-cm^2 cell culture flask (430639; Corning, New York, USA) containing Stemfit AK03 (Ajinomoto, Japan). These cells were passaged after dissociation into single cells with TrypLE Select CTS (A12859-01; Life Technologies, California, USA) [30]. For passaging, 25-cm^2 flasks were coated with laminin-511 E8 for 1 h at 37 °C in a CO_2 incubator. Laminin-511 E8 solution was diluted with PBS before use. After coating, PBS solution was removed and 5 mL Stemfit AK03 medium containing rock inhibitor (Y-27632; final concentration, 10 μM; Wako) was added to the flask. The flask was incubated at 37 °C in a CO_2 incubator until the cells were seeded. HiPSCs were dissociated into single cells by using 0.5× TrypLE Select (1:1 dilution of 1× TrypLE Select and 0.5 mM EDTA/PBS) for 4 min at 37 °C in a CO_2 incubator. After trypsinization, trypsin solution was removed and the cells were washed with 4 mL PBS. PBS was then removed, and the cells were scraped using 2.5 mL Stemfit AK03 and were dissociated into single cells by pipetting them 10 times. The cells were counted using TC20™ Automated Cell Counter (Bio-Rad, California, USA) and were seeded (3.25 × 10^4 cells/flask). On the following day, the cultivation medium was replaced with a fresh medium (Stemfit AK03) to remove the rock inhibitor. The medium was changed every alternate day thereafter. The next passage was conducted when the cells reached 80%–90% confluency.

NHDFs (KF-4109; Kurabo, Japan) were cultivated in a 75-cm^2 cell culture flask (658170; Greiner Bio-one, Frickenhausen, Germany) containing DMEM (08458-16; Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% penicillin–streptomycin (PS; Life Technologies). For passaging, NHDFs cultured in 75-cm^2 flask were washed twice with 5 mL PBS and were dissociated using 0.05% trypsin/EDTA (3 mL) for 1 min at 37 °C in a CO_2 incubator. The cells were counted using TC20™ Automated Cell Counter and were seeded. The medium was changed every alternate day. The next passage was conducted when the cells reached 80%–90% confluency. Cells passaged for 6–10 times were used in subsequent experiments.

4.2. Preparation of PAM

NEAPP irradiation system used in this study included a power controller/gas flow regulator, argon (Ar) gas cylinder, and plasma source head [21]. Flow rate of Ar gas was set at 2 standard liters/min. PAM was prepared in 60-mm culture dishes (430166; Corning) by exposing plasma to 8 mL Stemfit AK03 medium lacking Stemfit B and C solutions. The distance (L) between the plasma source and the surface of the medium was fixed at 3 mm. The duration for PAM irradiation was 5 min. The prepared PAM was preserved in a refrigerator at −80 °C until further use. Stemfit B and C solutions were preserved in a refrigerator at −30 °C until further use. H_2O_2 concentration in PAM was measured using Amplite Fluorimetric Hydrogen Peroxide Assay Kit (11502; AAT Bioquest, Sunnyvale, USA) at near-infrared fluorescence by following the manufacturer's instructions. Stemfit AK03 medium containing equivalent concentration of H_2O_2 was prepared using the concentration of H_2O_2 in PAM and was preserved in a refrigerator at −30 °C until further use.

4.3. Cell cytotoxicity assay

Ninety-six-well plates (655180; Greiner Bio-one) were coated with 0.5 μg/cm^2 laminin-511 E8 for 1 h at 37 °C in a CO_2 incubator, as described above. HiPSCs or NHDFs were seeded (1.0 × 10^4 cells/well) in 200 μL Stemfit AK03 medium containing the rock inhibitor (final concentration, 10 μM) and were incubated for 24 h at 37 °C in a CO_2 incubator. On the following day, PAM, H_2O_2-containing medium, and Stemfit B stock solution were thawed in a water bath at 37 °C for 90 s. Stemfit C stock solution was thawed at room temperature. After thawing, 250 μL Stemfit B solution and 5 μL Stemfit C solution were mixed with 1 mL PAM or H_2O_2-containing medium. The mixed PAM solution or H_2O_2-containing medium was diluted to an optimal concentration. The cultivation medium in the 96-well plates was removed, and 150 μL mixed PAM solution was added to each well. The plates were then incubated for 24 h at 37 °C in a CO_2 incubator. After incubation, cell viability was determined using Cell Counting Kit-8 (347-07621; Dojindo, Japan) by following the manufacturer's instructions.

To investigate the effects of cell density on the viability of hiPSCs, cells were seeded (2.0 × 10^4, 1.0 × 10^4, and 5.0 × 10^3 cells/well) and treated with 90 μL of 8-fold diluted PAM. To investigate the effects of PAM volume on the viability of hiPSCs, cells were

seeded (1.0×10^4 cells/well) and treated with 60, 90, and 120 μL of 8-fold diluted PAM.

4.4. Selective killing of undifferentiated cells in a coculture

Twelve-well plates (665180; Greiner Bio-one) were coated with 0.5 μg/cm^2 laminin-511 E8 for 1 h at 37 °C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (5.45×10^4 cells/well) in the Stemfit AK03 medium containing the rock inhibitor (final concentration, 10 μM) and were incubated for 24 h at 37 °C in a CO_2 incubator. On the following day, the medium was replaced with a fresh medium to remove the rock inhibitor and the cells were incubated further for 24 h. On the third day, NHDFs were stained with 5 μM CellTracker Green CMFDA Dye (C2925; Thermo Scientific, USA) in DMEM lacking FBS and PS for 45 min. After staining, the medium was replaced with fresh DMEM containing 10% FBS and 1% PS and the cells were incubated for 30 min. Stained NHDFs were trypsinized, collected, and seeded in 12-well plates (1.09×10^5 cells/well) containing hiPSCs. After 24 h, the medium was removed and 1.63 mL 4-fold diluted mixed PAM was added to the wells. Mixed PAM was removed after 6 h, and the cells were washed twice with PBS. Next, PI solution (P378; final concentration, 15 μM; Dojindo) in PBS was added to the wells, and the cells were incubated for 15 min at 37 °C in a CO_2 incubator. The cells were then washed twice with PBS and were observed under a fluorescence microscope (IX81; Olympus, Japan).

4.5. Quantification of the selective killing ability of PAM

For this, 12-well plates were coated with 0.5 μg/cm^2 laminin-511 E8 for 1 h at 37 °C in a CO_2 incubator, as described above. Varying numbers of 201B7 hiPSCs (5.0×10^4, 2.5×10^4, and 1.25×10^4 cells) were seeded in these plates. After 48 h, NHDFs were fluorescently labeled with 5 μM CellTracker Green CMFDA Dye in DMEM lacking FBS and PS for 45 min at 37 °C in a CO_2 incubator. After staining, the medium was replaced with fresh DMEM and the cells were incubated for 30 min. Stained NHDFs were seeded (1.0×10^5 cells/well) and were cocultured with 201B7 hiPSCs for 24 h. On the following day, the medium was replaced with 1.5 mL fresh medium (Stemfit AK03) or 1.5 mL 4-fold diluted PAM. After 48 h, the cells were dissociated into single cells by treatment with 0.5× TrypLE Select for 4 min at 37 °C in a CO_2 incubator. The cells were then collected using a cell scraper and were centrifuged at 1500×g for 1 min. After removing supernatant, the cells were suspended in PBS containing 1% BSA. Finally, the cells were analyzed using a flow cytometer (Epics Altra; Beckman Coulter, USA).

4.6. Effect of PAM on hiPSC-derived differentiated cells

Schematic representation of this experiment is shown in Fig. 4A. Four compartments of 35-mm dishes (627975; Greiner Bio-one) were coated with 0.5 μg/cm^2 laminin-511 E8 (iMatrix-511) for 1 h at 37 °C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (1.2×10^4 cells/compartment) in the compartment with Stemfit AK03 medium containing the rock inhibitor (final concentration, 10 μM) and were incubated for 24 h at 37 °C in a CO_2 incubator. On the following day, the medium was replaced with fresh Stemfit AK03 medium containing 5 μM all-trans RA (182-01111; Wako) to induce the differentiation of 201B7 hiPSCs. The differentiation medium was replaced every alternate day. On the eighth day, undifferentiated 201B7 hiPSCs were seeded (1.2×10^5 cells/compartment) in other compartments. On the following day, the medium was removed and 16-fold diluted PAM was added to each compartments. After 24 h, PAM was removed and the cells were washed 3 times with PBS. Next, 5 μM calcein-AM and 3 μM PI solution in PBS were added to the dishes, and the cells were incubated for 15 min at 37 °C in a CO_2 incubator. After washing twice with PBS, the cells were observed under IX81 fluorescence microscope.

4.7. Real-time reverse transcription-PCR

For this, 35-mm dishes (3000-035; IWAKI, Japan) were coated with 0.5 μg/cm^2 laminin-511 E8 for 1 h at 37 °C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (9.0×10^4 cells/dish) in a compartment with the Stemfit AK03 medium containing the rock inhibitor (final concentration, 10 μM) and were incubated for 24 h at 37 °C in a CO_2 incubator. On the following day, the cultivation medium was replaced with fresh Stemfit AK03 medium to remove the rock inhibitor and the cells were incubated for 48 h. Next, NHDFs were seeded (9.0×10^4 cells/dish) in 35-mm dishes containing DMEM supplemented with 10% FBS and 1% PS and were cultivated for 72 h. After 72 h, total RNA of 201B7 hiPSCs and NHDFs was extracted using Nucleospin RNA (740955.50; MACHEREY-NAGEL, Germany) by following the manufacturer's instructions. Purity and concentration of the extracted RNA were determined using a spectrophotometer (V-730Bio; JASCO CORPORATION, Japan) at 260 and 280 nm, respectively. First-strand cDNA was prepared from the extracted RNA by using ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301; TOYOBO, Japan) by following the manufacturer's instructions. Real-Time PCR was performed using Eco Real-Time PCR system (Illumina, USA), 0.4 μg cDNA, and THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO). The conditions for real-time PCR were as follows: (1) 95 °C for 1 min, (2) 95 °C for 15 s, (3) 60 °C for 30 s, and (4) 45 cycles of (2) and (3). Melting curve was measured from 55 °C to 95 °C. Primers for PCR were purchased from FASMAC (Japan). Sequences of these primers are as follows: *ATM* forward: 5′-TTTACCTAACTGTGAGCTGTCTCCAT-3′, *ATM* reverse: 5′-ACTTCCG-TAAGGCATCGTAACAC-3′, *SOD* forward: 5′-GAAGGTGTGGGGAAG-CATTA-3′, *SOD* reverse: 5′-CAATAGACACATCGGCCACA-3′, *CAT* forward: 5′-GCCTGGGACCCAATTATCTT-3′, *CAT* reverse: 5′-GAATCTCCGCACTTCTCCAG-3′, *GPX1* forward: 5′-CCAAGCTCAT-CACCTGGTCT-3′, *GPX1* reverse: 5′-TCGATGTCAATGGTCTGGAA-3′, *GAPDH* forward: 5′-CCTGACCTGCCGTCTAGAAA-3′, and *GAPDH* reverse: 5′-TGCTGTAGCCAAATTCGTTG-3′.

4.8. Statistical analysis

Data are presented as mean values and standard deviation (SD), and a Student's t-test was used for evaluating statistical significance for comparison. A value less than 0.1, 0.05, 0.005 (*$P < 0.1$, **$P < 0.05$, ***$P < 0.005$) indicated statistical significance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.07.001.

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