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 (H₂O₂) 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 H₂O₂ as that in PAM, suggesting that H₂O₂ 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.

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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...
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 NHDFs 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 (H₂O₂), 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 hPSCs and NHDFs toward PAM. Different dilutions of PAM were added to hPSC and NHDF cultures, and viabilities of these cells were evaluated after 24 h. Undifferentiated hPSCs 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 hPSCs 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 investigated whether these parameters affected the sensitivity of hPSCs to PAM. The viability of iPSCs varied with cell density (Fig. 2A), with values of 113.9% ± 7.1% for 2.0 × 10⁴ cells/well, 81.9% ± 5.8% for 1.0 × 10⁴ cells/well, and 42.0% ± 2.8% for 5.0 × 10³ 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 hPSCs.

2.2. Selective elimination of undifferentiated cells by PAM

We next investigated whether PAM selectively eliminated undifferentiated hPSCs. We cocultured undifferentiated hPSCs and NHDFs. hPSCs 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 hPSCs that were treated with undiluted PAM detached from the surface and...
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 hiPSC:NHDF 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
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.

H2O2 is one of the main components of PAM [21]. Because expression levels of oxidative stress-related genes, including those encoding enzymes involved in H2O2 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 H2O2 as that in PAM. We measured H2O2 concentration in PAM (464 ± 59 μM) and prepared a medium containing 465 μM H2O2. Approximately 92.1% undifferentiated hiPSCs were killed after treatment with PAM, whereas only 57.4% undifferentiated hiPSCs were killed after treatment with the H2O2-containing medium (Fig. 6E). These results suggested that H2O2 as well as various reactive oxygen nitrogen species (RONS) present in PAM contributed to the selective elimination of undifferentiated hiPSCs.

**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.
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
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 H2O2-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 H2O2-containing medium [21]. These results suggested that mechanisms underlying PAM-induced death of undifferentiated hiPSCs are partially different from those underlying H2O2-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).
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 H2O2. In the present study, we used a commercially available clinical grade medium (Stemcell 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 hiPSCs. Therefore, this novel approach may serve as a promising tool in regenerative medicine for the treatment of regenerative therapy.

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 passaged using 0.05% trypsin/EDTA (3 mL) for 1 min at 37 °C in a CO2 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 × 10^6 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-cm² 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-cm² 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 CO2 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. 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. H2O2 concentration in PAM was measured using Amplitite Fluorometric 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 H2O2 was prepared using the concentration of H2O2 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/cm² laminin-511 E8 for 1 h at 37 °C in a CO2 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 CO2 incubator. On the following day, PAM, H2O2-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 H2O2-containing medium. The mixed PAM solution or H2O2-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 CO2 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 the 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

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/cm² laminin-511 E8 (Matrix-511, 381-07363; Wako, Japan)-coated 25-cm² 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-cm² flasks were coated with laminin-511 E8 for 1 h at 37 °C in a CO2 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 CO2 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 CO2 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 × 10^6 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.
seeded (1.0 \times 10^4 \text{ cells/well}) and treated with 60, 90, and 120 \mu M 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 \mu g/cm^2 laminin-511 E8 for 1 h at 37 ^\circ C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (5.45 \times 10^4 \text{ cells/well}) in the StemFit AK03 medium containing the rock inhibitor (final concentration, 10 \mu M) and were incubated for 24 h at 37 ^\circ C in a CO_2 incubator. On the following day, the medium was replaced with 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 \mu M CellTracker Green CFMFA 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 \times 10^5 \text{ 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; Invitrogen, USA) in DMEM lacking FBS and PS and the cells were incubated for 24 h at 37 ^\circ 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 \times 10^5 \text{ 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 kit (740955.50; MACHEREY-NAGEL, Germany) 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) following the manufacturer's instructions. Real-Time PCR was performed using Eco Real-Time PCR system (ILLUMINA, USA), 0.4 \mu g cDNA, and THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO). The conditions for real-time PCR were as follows: (1) 95 ^\circ C for 1 min, (2) 95 ^\circ C for 15 s, (3) 60 ^\circ C for 30 s, and (4) 45 cycles of (2) and (3). Melting curve was measured from 55 ^\circ C to 95 ^\circ C. Primers for PCR were purchased from FASMAC (Japan). Sequences of these primers are as follows: ATM forward: 5'-TTTACGAACTGACATGCTCTCATACT-3', ATM reverse: 5'-ACTCCCG-TAAGGCACTCTGAAAC-3', SOD forward: 5'-GAAAGTCGCTGGAAGCATATA-3', SOD reverse: 5'-CAATAGACACATCGGCCACA-3', CAT forward: 5'-GCCATGGCCACAAATTTACTT-3', CAT reverse: 5'-GAATCTCCGGACTTCCAG-3', GPX1 forward: 5'-CCAAGCTCATGATGTCAATGGTCTGGAA-3', GPX1 reverse: 5'-TCGATGTCAATGGTCTGGAA-3', GADDH forward: 5'-CTTGACGCTGCCGTCTAGAAA-3', GADDH reverse: 5'-CTTGACGCTGCCGTCTAGAAA-3'.

4.5. Quantification of the selective killing ability of PAM

For this, 12-well plates were coated with 0.5 \mu g/cm^2 laminin-511 E8 for 1 h at 37 ^\circ C in a CO_2 incubator, as described above. Varying numbers of 201B7 hiPSCs (5.0 \times 10^4, 2.5 \times 10^4, and 1.25 \times 10^4 cells) were seeded in these plates. After 48 h, NHDFs were fluorescently labeled with 5 \mu M CellTracker Green CFMFA Dye in DMEM lacking FBS and PS for 45 min at 37 ^\circ 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 \times 10^5 \text{ 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 \times TrypLE Select (Invitrogen, USA) for 4 min at 37 ^\circ C in a CO_2 incubator. The cells were then collected using a cell scraper and were centrifuged at 1500 \times 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 \mu g/cm^2 laminin-511 E8 (iMatrix-511) for 1 h at 37 ^\circ C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (1.2 \times 10^5 \text{ cells/compartment}) in the compartment with StemFit AK03 medium containing the rock inhibitor (final concentration, 10 \mu M) and were incubated for 24 h at 37 ^\circ C in a CO_2 incubator. On the following day, the medium was replaced with fresh StemFit AK03 medium containing 5 \mu M 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 \times 10^5 \text{ cells/compartment}) in other compartments. After 24 h, 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 \mu M calcein-AM and 3 \mu M PI solution in PBS were added to the dishes, and the cells were incubated for 15 min at 37 ^\circ 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 \mu g/cm^2 laminin-511 E8 for 1 h at 37 ^\circ C in a CO_2 incubator, as described above. Next, 201B7 hiPSCs were seeded (9.0 \times 10^5 \text{ cells/dish}) in a compartment with StemFit AK03 medium containing the rock inhibitor (final concentration, 10 \mu M) and were incubated for 24 h at 37 ^\circ 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 \times 10^5 \text{ 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 kit (740955.50; MACHEREY-NAGEL, Germany) 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) following the manufacturer’s instructions. Real-Time PCR was performed using Eco Real-Time PCR system (ILLUMINA, USA), 0.4 \mu g cDNA, and THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO). The conditions for real-time PCR were as follows: (1) 95 ^\circ C for 1 min, (2) 95 ^\circ C for 15 s, (3) 60 ^\circ C for 30 s, and (4) 45 cycles of (2) and (3). Melting curve was measured from 55 ^\circ C to 95 ^\circ C. Primers for PCR were purchased from FASMAC (Japan). Sequences of these primers are as follows: ATM forward: 5'-TTTACGAACTGACATGCTCTCATACT-3', ATM reverse: 5'-ACTCCCG-TAAGGCACTCTGAAAC-3', SOD forward: 5'-GAAAGTCGCTGGAAGCATATA-3', SOD reverse: 5'-CAATAGACACATCGGCCACA-3', CAT forward: 5'-GCCATGGCCACAAATTTACTT-3', CAT reverse: 5'-GAATCTCCGGACTTCCAG-3', GPX1 forward: 5'-CCAAGCTCATGATGTCAATGGTCTGGAA-3', GPX1 reverse: 5'-TCGATGTCAATGGTCTGGAA-3', GADDH forward: 5'-CTTGACGCTGCCGTCTAGAAA-3', GADDH reverse: 5'-TGCTTGACGCTGCCGTCTAGAAA-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.jreth.2016.07.001.
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