Heliyon 8 (2022) e12009

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Non-thermal atmospheric-pressure plasma potentiates mesodermal differentiation of human induced pluripotent stem cells

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ARTICLE INFO

Keywords:
Atmospheric-pressure plasma
iPS cells
Cell differentiation
DNA damage
Mesoderm

ABSTRACT

Non-thermal atmospheric-pressure plasma has been used for biological applications, including sterilization and stimulation of cell growth and differentiation. Here, we demonstrate that plasma exposure influences the differentiation pattern of human induced pluripotent stem cells (hiPSCs). We treated hiPSCs with dielectric barrier-discharge air plasma and found an exposure dose that does not kill hiPSCs. Immunohistochemical staining for E-CADHERIN showed that the exposure affected cell-cell attachment and doubled the average size of the hiPSCs. Analysis of mRNAs in embryoid bodies (EBs) from plasma-treated hiPSCs revealed repression of ectoderm genes, including WNT1, and increased expression of mesoderm genes. Importantly, hiPSCs deficient in DNA repair only displayed minimal damage after plasma exposure. Collectively, our results suggest that plasma treatment can be another tool for directing the fate of pluripotent stem cells without disrupting their genomic integrity.

1. Introduction

Non-thermal atmospheric-pressure plasma consists of charged particles (electrons and ions) and neutral particles. Collisions with electrons turn oxygen and nitrogen into reactive species that can affect nearby biological specimens. Because this type of plasma provides reactive species at atmospheric pressure without thermal damage, it has been used for a variety of biological applications (Domonkos et al., 2021) such as antimicrobial therapy (Duarte and Panariello, 2020), gene transfer (Jinno et al., 2021), protein transfer (Yanagawa et al., 2017), and stimulation of plant germination (Dufour et al., 2021). Although active oxygen and nitrogen species have been implicated as the mediators of these effects, the light (especially in ultraviolet region) and electric field generated by the plasma may also contribute (Tan et al., 2020). Indeed, exposure to electric field itself has also been reported to have effects on biological events such as plant germination, growth and yield (Attri et al., 2022).

Plasma generation technology is continuously improving (Kumagai et al., 2016; Kobayashi et al., 2016) and several generators are now commercially available for wound treatment (Stratmann et al., 2020; Shimizu, 2021). Applications for biomedical research have been widely investigated (Ahmed et al., 2016; Tominami et al., 2017; von Woedtke et al., 2020). For instance, plasma treatment was reported to enhance the proliferation of various mesoderm-derived human adult stem cells (Park et al., 2019). Moreover, neural stem cells have been reported to differentiate predominantly into neuronal lineages upon plasma treatment (Xiong et al., 2014), and mouse embryonic stem (ES) cells to increase their expression of some ectodermal markers, although final differentiation was not examined (Miura et al., 2016; Nishihara et al., 2017). Most cell studies used a plasma jet. In such a setup, atmospheric-pressure plasma exits at high speed from a nozzle, and reacts with air to form reactive species such as ozone, NO, NO_2, N_2O, and N_2O_5. Different setups may generate a different mix of reactive species in the plasma, and affect cell activity differently (Tan et al., 2020). At the same time, the strong gas flow generated by plasma jets is not appropriate for delicate cells such as induced pluripotent stem cells (iPSCs).

Human iPSCs (hiPSCs) can proliferate indefinitely or differentiate into any types of cells *in vitro* (Takahashi et al., 2007), which makes them

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https://doi.org/10.1016/j.heliyon.2022.e12009
Received 11 April 2022; Received in revised form 8 September 2022; Accepted 23 November 2022

attractive systems for a number of applications, including regenerative medicine, drug discovery, and human developmental biology. In many cases, the cells need to be differentiated to a specified lineage. This differentiation is facilitated when the cells organize into intermediate structures called embryoid bodies (EBs) (Pettinato et al., 2014). EBs allow the initiation of spontaneous differentiation and the generation of all three germ layers (ectoderm, mesoderm, and endoderm). Cells in an EB further differentiate into more specific lineages, such as neural, cardiac, and hematopoietic cells. But to generate cells for specific purposes, better control over the differentiation process is essential (Fernandopulle et al., 2018; Valcourt et al., 2021). For example, if cardiac cells are required, EB cells should be directed toward a mesoderm lineage. Various methods for directional differentiation exist but they rely on expensive chemical reagents, biologics (Loh et al., 2014; Kimura et al., 2019) or tailored bioactive scaffolds (DeForest and Tirrell, 2015; Kobayashi et al., 2019). By contrast, non-thermal plasma exposure is a simple and inexpensive method that has already been shown to influence the differentiation of stem cells (Xiong et al., 2014; Nishihara et al., 2017). We reasoned that even if plasma treatment itself cannot induce full differentiation, combining it with other methods may help achieve preferential differentiation. A precedent for this hypothesis comes from the observation that combining physical stress with chemical stimuli can direct cell fate (Kaitsuka and Hakim, 2021).

In this study, we used a simple device to generate plasma rather than a plasma jet and analyzed the effects of plasma exposure on hiPSCs. Changes in cell lineage upon plasma exposure were assessed with a gene score card, an assay based on real-time quantitative PCR. Our results are important milestones for the use of plasma to control cell differentiation.

2. Materials and methods

2.1. Plasma generation

For plasma generation, we used a dielectric barrier discharge (DBD) source consisting of interdigitated electrodes covered with glass tubes under ambient conditions (Figure 1; Koga et al., 2016; Kobayashi et al., 2020). The Cu wires (φ0.5 mm, length 50 mm) were each covered with a glass tube (1.5 mm outer diameter and 1 mm inner diameter) and set parallel to each other. The distance between two wires was about 1.0 mm. A total of 28 Cu wires (14 per electrode) were used, making a plasma-generating area of about 4×4 cm^2 (Kobayashi et al., 2020). A 9 kV power supply (LHV-09K, Logy Electric Co. Ltd., Tokyo, Japan) was used with discharge frequency of 12.6 kHz. Discharge power calculated based on Q–V Lissajous characteristics was 0.3 W/cm^2. Distance between the cells and the plasma source was 10 mm. In contrast to other setups generating plasma jets, cells can be exposed to plasma without influence of gas flow.

2.2. Cell culture

We obtained the hiPSC line from Drs. B. Conklin and M. Mandegar (Mandegar et al., 2016). hiPSCs were maintained in StemFit (AK02N, Ajinomoto Co., Inc., Tokyo, Japan) medium at 37 °C with 5.0% CO_2. Dishes coated with 0.26 μg/cm^2 iMatrix-511 silk (Nippi, Incorporated, Tokyo, Japan) were used. Cell numbers were counted using dual--chamber slides (Cat. #1450011; Bio-Rad Laboratories, Hercules, CA, USA) on a TC20™ automated cell counter (Bio-Rad Laboratories). 10 μM Rho-associated kinase inhibitor (ROCK-i: Y-27632 dihydrochloride; Focus Biomolecules, Plymouth Meeting, PA, USA) was added when replating hiPSCs. Cell images were taken with a EVOS XL Core microscope (Life Technologies Corporation, Carlsbad, CA, USA).

For plasma exposure, 2.5×10^4 hiPSCs were plated in a 35-mm dish and cultured for 2 days. Just before plasma exposure, medium was removed and treatment was conducted for 10, 30 or 60 s. After 2 to 3 days, cell images were taken or cell numbers were counted. Cells appeared to be damaged with 30-s exposure, and many cells were dead with 60-s exposure (data not shown). For the rest of this study, 10-s plasma exposure was used as a standard protocol.

2.3. EB formation, differentiation and gene expression analysis

For embryoid body (EB) formation, 2.5×10^4 hiPSCs were seeded and cultured in a 35-mm dish. After being cultured for 2 days, medium was removed and 10-s plasma exposure was conducted.

2.4. Induction of excision repair deficiency in hiPSCs with doxycyline

Doxycycline (Dox)-inducible transcription-coupled nucleotide excision repair (TC-NER) deficient hiPSCs constructed according to published protocol (Mandegar et al., 2016) were used (Prell, 2022). For repression of *ERCC6* in the hiPSCs, the cells were cultured in the presence of 0.1 μg/mL Dox (Sigma-Aldrich).

2.5. UV irradiation

UV irradiation was conducted using a portable excimer light phototherapy device TheraBeam® UV308 mini (Ushio Inc., Tokyo, Japan). UV dose was calculated based on a dose-distance curve provided by the manufacturer.

2.6. Immunostaining

Cells were fixed with formaldehyde and stained with DAPI or immunostained using E-CADHERIN antibody (Cell Signaling Technology, Danvers, MA, USA). Images were taken with a Leica TCS SP8 (Leica Microsystems GmbH, Wetzlar, Germany) confocal microscope with a 63× objective lens. The size of 30 randomly selected cells in each image was measured using ImageJ (ImageJ, RRID: SCR_003070) software (Schneider et al., 2012). Statistical analysis was conducted by Student's *t*-test.

3. Results and discussion

3.1. Plasma exposure leads to loosening of cell packing

Plasma exposure has both positive and negative effects on biological organisms (Duarte and Panariello, 2020; Kobayashi et al., 2020). Importantly, it can affect cell differentiation (Xiong et al., 2014; Miura et al., 2016; Nishihara et al., 2017; Tan et al., 2020). To determine the best regimen of plasma exposure, we cultured hiPSCs (2.5×10^4 cells/35 mm-dish) for 2 days, exposed them to plasma for 10, 30 or 60 s, and counted the cells 3 days later (Figure 1). As exposure of 30 and 60 s led to significantly lower numbers of surviving cells at day 3 (data not shown), we used 10-s exposures for the rest of this study. We confirmed that plasma was indeed generated in our setup over this short time frame by measuring ozone, the main gas species generated by plasma (Figure S1) (Shimizu et al., 2012). Cell colonies appeared to grow larger after a 10-s plasma exposure (Figure 2). However, we rarely saw any increase in differentiated cells in the plasma-treated culture compared to untreated cells (Figure 2).

To clarify the cause of the increased colony size, we conducted immunostaining with an antibody against E-CADHERIN (Figure 3). E-CADHERIN plays an important role in cell-cell adhesion in hiPSCs (Chen et al., 2010), and in maintaining tissue architecture. We found that plasma treatment affected cell morphology and cell packing in colonies (Figure 3A–F). For instance, instead of a sharp E-CADHERIN signal in non-treated cells, indicative of tight cell-cell adhesion (Figure 3B), we observed a diffuse E-CADHERIN signal in plasma-treated cells, suggesting an altered E-CADHERIN distribution (Figure 3E). In addition, some areas between cells had no E-CADHERIN signal, suggesting an alteration in cell-cell adhesion. Furthermore, the average cell size, as measured on the microscopy images, was about twice greater for plasma-treated than for control cells (Figure 3G). Thus, the plasma treatment may impair

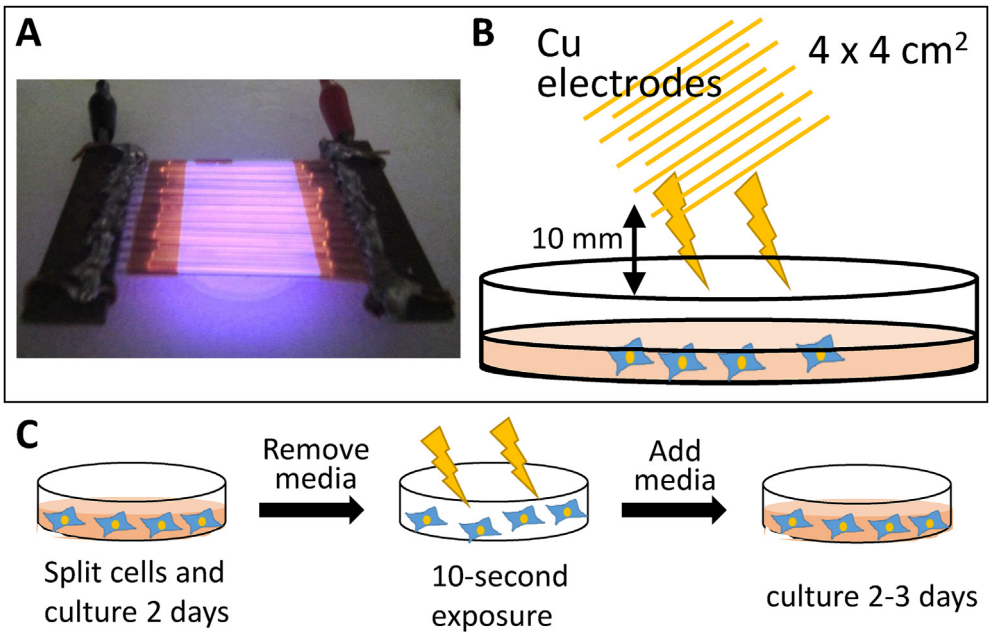

Figure 1. Photograph (A) and schematic (B) of hiPSCs exposure setup. Cells can be exposed to plasma without the influence of gas flow that is inevitable when plasma jet is used. (C) Plasma exposure protocol.

cell-cell adhesion of hiPSCs, resulting in looser colonies and enlarged cell and colony size, although it does not strongly induce differentiation. Why hiPSCs in a colony would both increase in size and loosen their tight junctions is unclear. As maintaining cell-cell contacts is energy-consuming, it is possible that to survive, the hiPSCs need to loosen their tight junctions, a trade-off that has been discussed in the evolution of yeast colonies (Jacobeen et al., 2018). In the future, it will be interesting to determine whether plasma treatment affects cell functions related to attachment, including cell migration.

3.2. Plasma exposure potentiates mesodermal differentiation of hiPSCs while repressing ectodermal gene expression in hiPSC-derived EBs

Pluripotent stem cells (PSCs) spontaneously differentiate into cells of the three germ layers *in vitro* when they are coaxed into three-dimensional aggregates known as embryoid bodies (EBs) (Hopfl et al., 2004). We therefore examined the fate of plasma-treated hiPSCs aggregated into EBs (Figure 4).

Right after plasma exposure (10 s, 10 mm distance between plasma source and culture), single-cell suspensions of the plasma-treated hiPSCs were made and then plated on a low attachment dish to induce EBs. EBs formed from the treated hiPSCs appeared similar to those from non-treated hiPSCs (Figure 5). In some cases, plasma-treated cells formed white transparent cysts (Figure 5B) reminiscent of those frequently observed in endothelial differentiation (K. Tomoda, personal communication). This observation suggested that the plasma treatment may push differentiation of hiPSCs toward the mesoderm lineage.

To analyze cell differentiation in the EBs, we collected their RNA one week after initiation and analyzed their gene expression profile by qPCR using TaqMan hPSC Scorecard™ Assay (Figure 6). A quick examination of the data revealed that pluripotency markers were expressed at low levels, while differentiation markers were highly expressed, suggesting that the hiPSCs had exited from the undifferentiated state and started differentiation, in EBs derived from both plasma-treated and non-treated hiPSCs (Figure S2).

A more detailed analysis uncovered 11 genes with statistically significant differences in expression in treated versus non-treated samples, among the 85 genes tested (Figure 6A, Figure S2). The expression of 7 ectodermal genes including *Pax 3* and *Wnt1*, and of 3 endoderm marker genes was suppressed while that of a mesoderm marker gene *PLVAP* (plasmalemma vesicle-associated protein) was upregulated (Figure 6A, Figure S2). At the same time, there was no statistically significant difference in the expression of other genes previously reported as germ layer markers, including *Pax6* (a neuroectoderm marker), *FGF4* and *T*

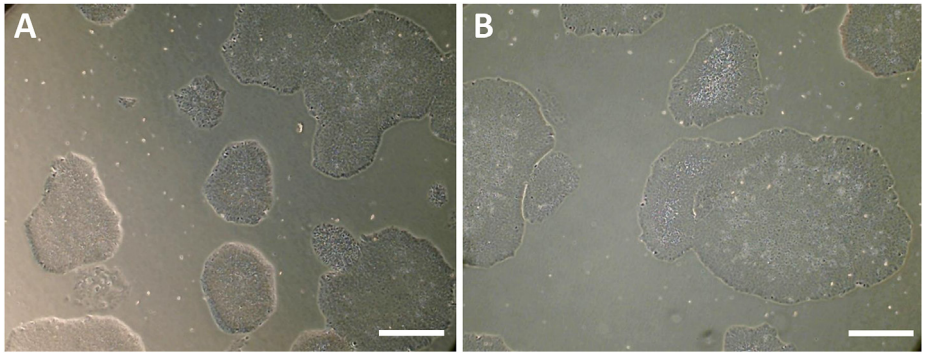

Figure 2. Plasma exposure affects cell morphology. Bright-field microscopy images of control (A) and plasma-exposed cells (B) 2 days after the treatment. Before plasma exposure, medium was removed and the treatment was conducted for 10 s. Medium was then added and the cells cultured for 2 days. Scale bars indicate 400 μm.

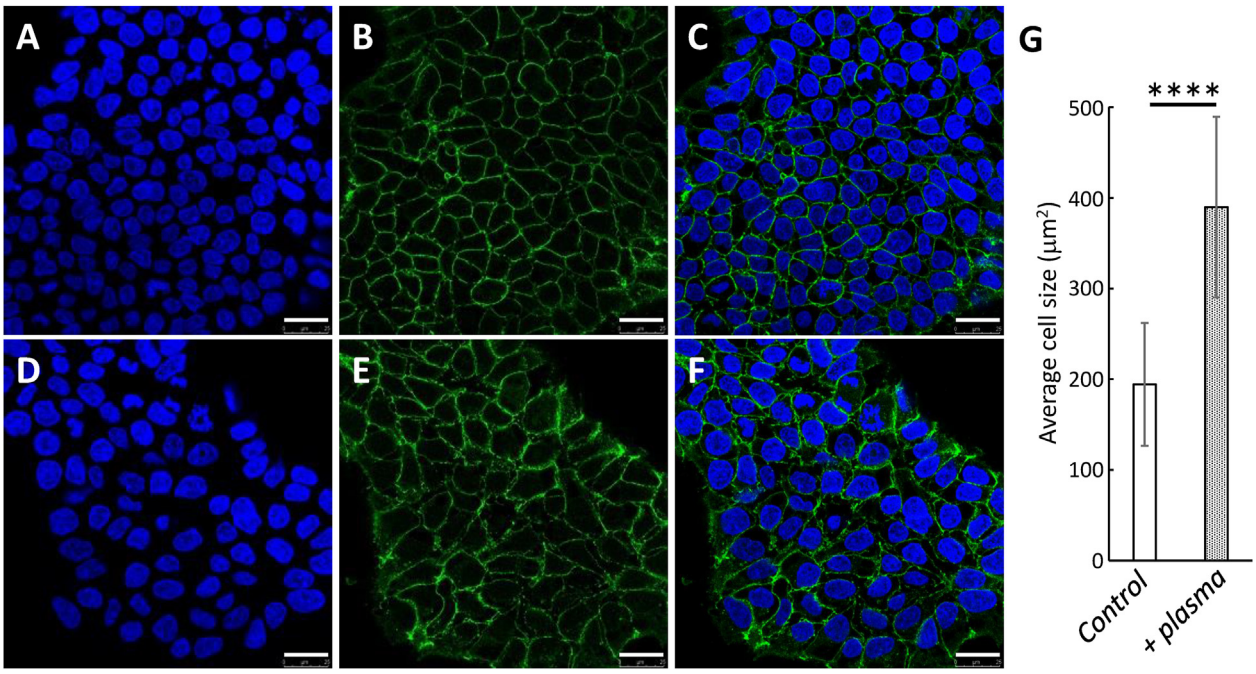

Figure 3. Plasma exposure leads to loosening of cell packing. Control cells (A, B, C) and plasma-treated cells (D, E, F) were stained with DAPI (A, D) or immunostained using E-CADHERIN antibody (B, E). C and F are merged images. Average cell size is shown in G. Before plasma exposure, medium was removed and the exposure was conducted for 10 s. Medium was added and cells were cultured for 3 days before they were fixed. Scale bars indicate 25 μm. Asterisks indicate statistical significance by Student's *t*-test (****, $P < 0.0001$).

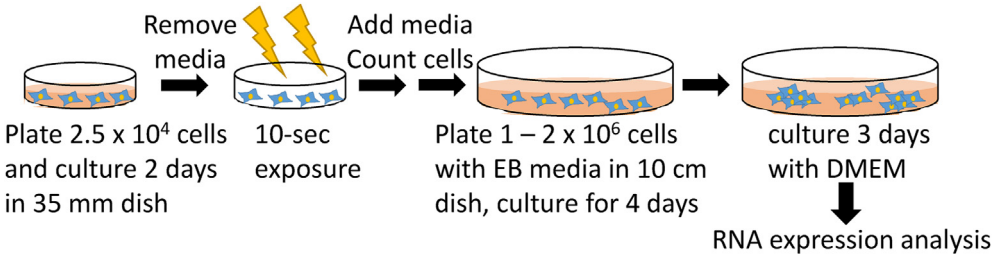

Figure 4. Schematic of embryoid body (EB) formation protocol.

(mesoderm markers) and *FoxA2* and *GATA6* (endoderm markers) (Figure 6A, Figure S2) (Nishihara et al., 2017). Importantly, the use of a scorecard algorithm confirmed a trend towards mesodermal differentiation, as only the mesoderm lineage score was significantly elevated (Figure 6B). By contrast, the ectoderm lineage score was generally down although this trend did not reach statistical difference, and the score for the endoderm lineage did not change significantly.

These findings contradict a previous study with mouse ES cells, which found that plasma exposure potentiated differentiation to the ectoderm lineage (Miura et al., 2016; Nishihara et al., 2017). However, these studies used plasma-treated medium, and different cell types (ES cells vs iPSCs), species (mouse vs. human), and plasma generation conditions compared to our study. In addition, it is known that the outcomes of hiPSC differentiation are highly dependent upon the quality of EBs

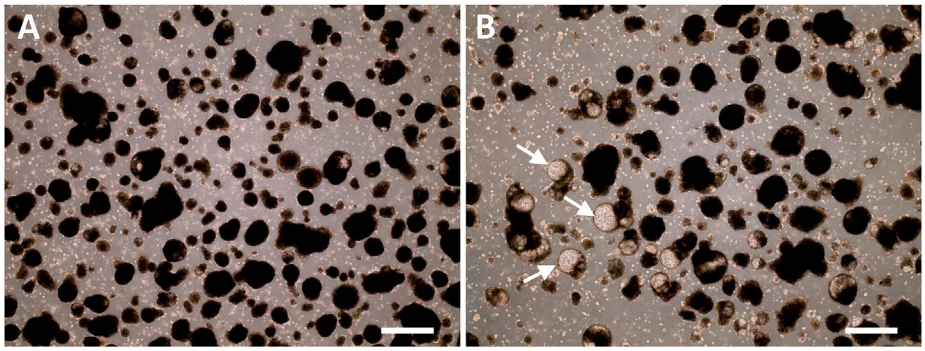

Figure 5. Plasma exposure does not inhibit EB formation. Bright-field microscopy images of control (A) and plasma-treated cells (B) taken on day 7. White arrows indicate transparent cysts, a potential indicator of mesoderm differentiation. Scale bars indicate 200 μm.

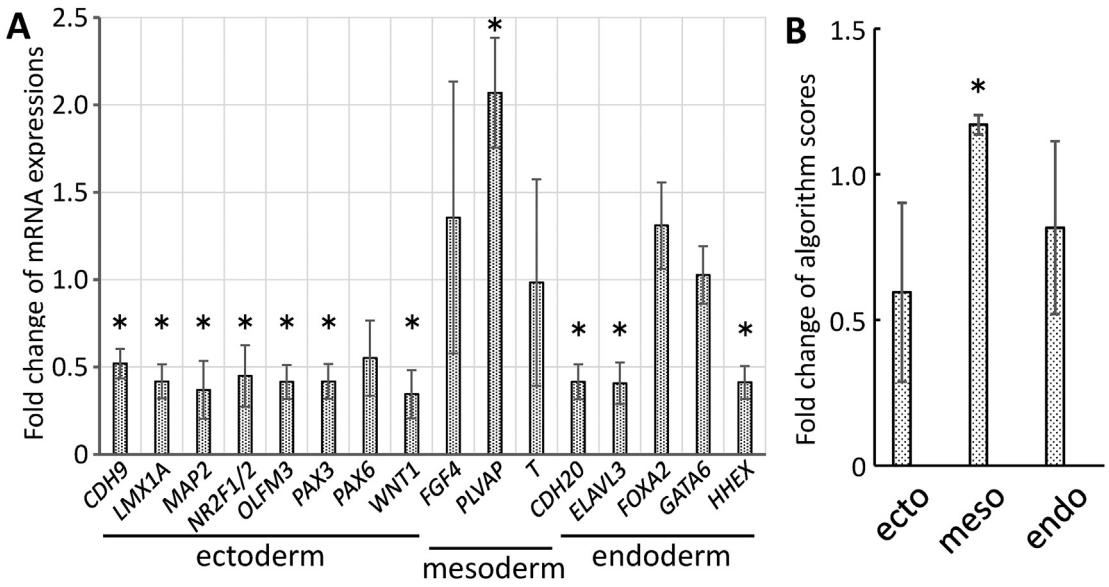

Figure 6. Plasma exposure affects differentiation preferences of embryoid bodies. (A) Fold expression change of genes representative of each primary cell layer. *CDH9*: cadherin 9, *LMX1A*: LIM homeobox transcription factor 1 alpha, *MAP2*: microtubule-associated protein 2, *NR2F1/2*: nuclear receptor subfamily 2 group F member 1/2, *OLFM3*: olfactomedin 3, *PAX3*: paired box gene 3, *PAX6*: paired box gene 6, *WNT1*: Wnt family member 1, *FGF4*: fibroblast growth factor 4, *PLVAP*: plasmalemma vesicle-associated protein, *T*: T-box transcription factor T, *CDH20*: cadherin 20, *ELAVL3*: ELAV like RNA binding protein 3, *FOXA2*: Forkhead Box A2, *GATA6*: GATA binding protein 6, *HHEX*: hematopoietically expressed homeobox. (B) Fold change of algorithm scores assessed by qPCR using hPSC Scorecard™ assay. Data were analyzed after three experimental replicates. Asterisks indicate statistical significance by Student's *t*-test (*, $P < 0.05$).

(Pettinato et al., 2014), which itself can vary between experimental conditions. Therefore, we do not think that our results challenge the validity of previous reports. Rather, we emphasize the importance of providing detailed experimental conditions when reporting research results. These details need to include the cell culture conditions as well as the plasma generation method, as different setups generate different profiles of active species. The mechanism of plasma effects on cultured cells has been discussed in relation to active oxygen and nitrogen species (Nishihara et al., 2017). However, active chemical species are probably not the only mediators of plasma effects. Moreover, whether these mediators differ between the commonly used plasma jets and the static plasma we used in our study is currently unknown.

How our plasma treatment biased hiPSCs towards mesodermal differentiation remains unknown. However, since it affected the E-CADHERIN expression pattern between cell-cell junctions in hiPSC colonies, it is conceivable that the alteration in cell-cell attachment caused a form of stress on the hiPSCs that helped promote their differentiation into mesoderm lineages.

3.3. Plasma exposure may not cause extensive DNA damage

Plasma exposure may damage the genomic DNA, hindering its applications to hiPSCs. To test this possibility, we first measured cell survival. Approximately 80% of the plasma-treated hiPSCs survived (Figure S3). Then, to measure damage to genomic DNA directly, we quantified the amount of 8-hydroxydeoxyguanosine (8-OHdG) in the genomic DNA of plasma-treated cells (Figure S4). 8-OHdG integration is considered as a biomarker of mutagenesis consequent to oxidative stress (Kasai 1997). There was no statistically significant difference in the amount of 8-OHdG in genomic DNA (approximately 200 pg per 1 μg) between plasma-treated and non-treated hiPSCs, although it trended slightly higher in plasma-treated hiPSCs (Figure S4).

To compare the effects of plasma on DNA to those of UV irradiation, we used a modified hiPSC line deficient in transcription-coupled nucleotide excision repair (TC-NER) (Prell, 2022). In this hiPSC line, the *excision repair cross-complementation group 6* (*ERCC6*) gene, encoding the NER protein ERCC6, can be knocked down with a six-day exposure to doxycycline (Dox) (Mandegar et al., 2016; Prell, 2022) (Figure S5). ERCC6 malfunction results in impaired recovery from DNA damage caused by ultraviolet (UV), leading to cell death (Mallery et al., 1998).

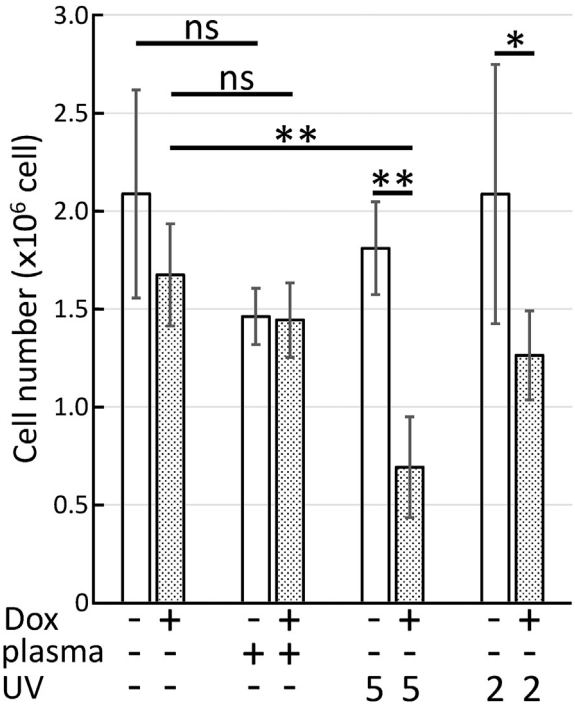

Figure 7. Cell DNA is not severely damaged by plasma exposure. Cells with a defect in DNA repair system (TC-NER) inducible by doxycycline (Dox) were used and exposed to either plasma or 308 nm UV irradiation at 5 or 2 mJ/cm^2. The addition of Dox did not alter cell survival rate compared with control cells. Data are from triplicated experiments and shown with mean ± SD. Asterisks indicate statistical significance by one-way ANOVA Turkey-Kramer (*, $P < 0.05$; **, $P < 0.01$; ns, not significant). Note that significant differences are not all indicated to avoid crowding the graph.

To test how the ERCC6-deficient hiPSCs would respond to plasma exposure, we treated them with Dox for six days to knock *ERCC6* down and then subjected them to the same plasma exposure dose (10 s, 10 mm) as used for the differentiation experiments. For comparison, we treated another batch of cells to UV irradiation at 2 mJ/cm^2 or 5 mJ/cm^2. Three days after the treatment, the cells were harvested and counted. We found similar numbers of cells in the cultures exposed to Dox and plasma and in the Dox-only cultures (Figure 7). The *ERCC6* knockdown itself reduced the cell numbers, but that reduction was the same in the plasma-treated and untreated *ERCC6*-deficient cultures. This observation is in stark contrast to the UV irradiation, which induced DNA damage and caused more cell death in the *ERCC6* knockdown cells (Figure 7). These results suggest that the plasma treatment, at the dose we used, may not severely damage the genomic DNA of the hiPSCs, and that the cells can be used for further applications.

4. Conclusions

In this study, we found a plasma exposure dose that affects the morphology of hiPSCs but does not appear to cause extensive DNA damage or cell death. At this dose, plasma exposure potentiated the mesodermal differentiation of hiPSCs.

Environmental stress is known to affect differentiation (Kaitsuka and Hakim, 2021). Plasma is cost-effective compared with chemicals or proteins commonly used to specify cell induction. It may not be possible for plasma treatment alone to induce specific cell lineages. However, pre-conditioning by plasma treatment may make it easier for other treatments to induce the differentiation into mesodermal cells such as cardiac cells.

4.1. Limitations of the study

Expression analysis of a panel of gene markers gives a good indication of the cells' characters, but it does not perfectly predict how the cells will differentiate into cells from specific lineage. Additionally, the genes analyzed are limited. An unbiased approach, such as RNA-seq, would give more detail on how the plasma treatment affects cell fate decisions in hiPSCs.

The mechanism by which plasma influences the differentiation of hiPSCs remains unknown and deserves future investigation.

Declarations

Author contribution statement

Mime Kobayashi, Ph.D; Kiichiro Tomoda, Ph.D: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hirofumi Morihara, Ph.D: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Michio Asahi, MD, Ph.D: Analyzed and interpreted the data.

Tetsuji Shimizu, Ph.D; Shinya Kumagai, Ph.D: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

Mime Kobayashi was supported by Plasma-bio consortium (01221908, 01222009, and 01223008), Interdisciplinary Project in Nara Institute of Science and Technology sponsored by the MEXT, Japan.

Shinya Kumagai was supported by Japan Society for the Promotion of Science KAKENHI (18K19942 and 19H04457), Uehara Memorial Foundation, Hibi Science Foundation, The Program for Forming Strategic Research Infrastructure (S1101028) sponsored by the MEXT, Japan.

Kiichiro Tomoda was supported by the on-site laboratory initiative launched by Kyoto University, Japan.

Michio Asahi was supported by OMC Internal Research Grant.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12009.

Acknowledgments

The authors thank M. Watanabe, S. Tsutsumi and Dr. M. Nakajima for technical assistance. We are grateful to Drs. B. Conklin and M. Mandegar for the iPSC line, and T. G. Prell for the Dox-inducible ERCC gene deficient cells. Critical reading of the manuscript by Dr. F. Chanut is also acknowledged.

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