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# Maintenance of an undifferentiated state of human-induced pluripotent stem cells through botulinum hemagglutinin-mediated regulation of cell behavior

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**Applications of human induced pluripotent stem cell (hiPSC) culture are impaired by problems with long term maintenance of pluripotency. In this study, we report that exposure to botulinum hemagglutinin (HA), an E-cadherin function-blocking agent, suppressed deviation from an undifferentiated state in hiPSC colonies. Time-lapse imaging of live cells revealed that cells in central regions of colonies moved slowly and underwent a morphological change to a cobblestone-like shape via interaction between contacting cells, forming dense, multiple layers. Staining and migration analysis showed that actin stress fibers and paxillin spots were diminished in colony central regions, and this was associated with alteration of cellular morphology and migratory behavior. However, in culture with HA exposure, cells in the central and peripheral regions of hiPSC colonies were migratory and arranged in loose monolayers, resulting in relatively uniform dispersion of cells in colonies. We also found that a well-organized network of actin stress fibers was of significance in the central and peripheral regions of a colony, resulting in activation of paxillin and E-cadherin expression in hiPSCs. After routine application of HA for serial passages, hiPSCs remained pluripotent and capable of differentiating into all three germ layers. These observations indicate that relaxation of cell–cell junctions by HA induced rearrangements of the cytoskeleton and cell adhesion in hiPSC colonies by promoting migratory behaviors. These results suggest that this simple and readily reproducible culture strategy is a potentially useful tool for improving the robust and scalable maintenance of undifferentiated hiPSC cultures.**



[**Key words:** Botulinum hemagglutinin; E-cadherin disruption; Human-induced pluripotent stem cells; Migration; Deviation from the undifferentiated state; Spatial heterogeneity]

Human pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells (hiPSCs) offer the opportunity to generate large amounts of human tissue for both clinical and industrial applications (1–4). The application of hiPSC-based technologies is hindered by a lack of robust, scalable technologies for culturing the quantities of cells anticipated to be required for widespread access. Culturing these cells, while maintaining their pluripotency in vitro, currently relies on feeder cells that secrete factors supporting proliferation in an undifferentiated state, or extracellular matrix (ECM) components such as gelatin, collagen and fibronectin (5,6). Although standard culture methods are sufficient to support attachment of hiPSCs, they are insufficient to maintain the growth and properties of these cells during long-term culture. Indeed, it has been shown that continued culture results in loss of self-renewal and differentiation potential. Typically, undifferentiated hiPSCs in monolayer culture form compact colonies of tightly associated cells, exhibiting a small, cobblestone-like morphology (7,8). Some hiPSCs undergo discernible morphological transformation by displaying elongated fibroblast-like morphology in colonies, suggesting loss of their undifferentiated state (9–11). These cell populations are able to proliferate in culture, gradually developing fibroblast-like morphology, and limiting the expansion of hiPSCs. Studies indicating that this morphological transformation in hiPSC colonies indicates deviation from the undifferentiated state of hiPSCs is accumulating. There is therefore a need to establish methodologies for sustaining the undifferentiated state of hiPSCs during the expansion process.

It has been well documented that the in vitro expansion of hiPSCs relies on dynamics of cell behavior that occur between cells, and between cells and the ECM (12,13). Many studies have provided clues as to how changes in cell behavior affect cell function, and how regulation of cell behavior by extracellular forces can coordinate self-renewal and differentiation of stem cells (14–18). In particular, colony morphology is the product of changes in the biomechanical and morphological properties of cells that are driven by interactions between the actin cytoskeleton, cell–cell adhesion, and cell–ECM adhesion (18–20). The integration of adhesion, biochemical signaling, and actin cytoskeletal network remodeling dynamically tunes the generation of intracellular force and regulates signal transduction cascades, as well as transcriptional events that control fundamental cell fate decisions (21,22). Recently, we have addressed possible mechanisms for controlling early cell fate decisions by influencing the balance between cell–cell and cell–substrate interactions in colonies of hiPSCs cultured with feeder cells (17,18,23,24). Colonies of hiPSCs expanded gradually and became larger and more tightly packed during culture. The cells underwent

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transition from small cells to large and flattened cells, with accompanying apoptosis. These behavioral observations can provide important information toward understanding the process of deviation from an undifferentiated state, which occurs at the center of hiPSC colonies. Growing evidence supports the idea that appropriate coordination of cell behaviors including cell–cell interaction, cell–substrate interaction, and cell migration in colonies is the key to maintaining hiPSCs in an undifferentiated state (12,13).

An increasing emphasis on design principles drawn from knowledge of the basic mechanisms of these cellular behaviors has now set the stage for their successful manipulation. hiPSC expansion strategies include methods to prevent or remove cells deviating from an undifferentiated state. We recently found that botulinum hemagglutinin (HA), an E-cadherin-blocking reagent, could selectively remove cells deviating from an undifferentiated state in hiPSC cultures (10,11). Its effect is achieved because of different E-cadherin expression levels, and E-cadherin re-establishment potentials, in undifferentiated and deviated regions of hiPSC colonies. It has become clear that HA possesses a potent ability to disrupt epithelial barrier function, and has distinct modes of action (25–28). HA showed temporary activity for disrupting E-cadherin-mediated cell–cell interactions to facilitate the selective removal of deviated cells in hiPSC colonies. The undifferentiated cells ability to regenerate E-cadherin can maintain hiPSCs in an undifferentiated state after disruption of E-cadherin binding through a Rap1-mediated positive-feedback mechanism (10). This correlates with the selective removal of deviated cells through E-cadherin disruption and the effects of this E-cadherin disruption will extend the prevention of the occurrence of deviated cells. These studies not only provide an important insight into the mechanisms of these epithelial barrier-disrupting activities, but also may lead to unique and powerful opportunities to develop a culture strategy to manipulate stem cell fate.

In this study, we report a simple and robust method to culture and propagate enriched hiPSCs using HA. Based on observed differences in cell behaviors between the center and periphery of single hiPSC colonies, we discuss the fundamental mechanisms of cell–cell and cell–substrate adhesion in relation to pluripotency maintenance of hiPSCs. The results we obtained led us to propose a culture tool to maintain the undifferentiated state of hiPSCs in long-term culture.

## MATERIALS AND METHODS

**Cells and culture conditions** hiPSCs were obtained from the Japanese Collection of Research Bioresources (clone Tic, JCRB Number: JCRB1331) and maintained in an undifferentiated state by coculture with mitomycin C-treated SNL76/7 feeder cells (European Collection of Cell Cultures, Salisbury, UK) in commercially available medium (ReproStem, ReproCELL Inc., Tokyo, Japan) containing 5 ng/ml basic fibroblast growth factor. Cells were passaged every five days, and media were changed daily.

For preparation of feeder layers, mitomycin C-treated SNL76/7 feeder cells were seeded at a density of $2.5 \times 10^4$ cells/cm$^2$ on the 0.1% gelatin-coated surfaces and cultivated for one day in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 7% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA).

For passages of hiPSCs, feeder cells were removed after a 1-min incubation with dissociation solution consisting of 0.1 mg/ml collagenase IV, 0.25% trypsin, and 20% KSR (all obtained from Thermo Fisher Scientific), and 0.1 mM $CaCl_2$ (Nacalai Tesque, Kyoto, Japan). The hiPSC colonies in the undifferentiated state were carefully collected using a cell scraper (Sumitomo Bakelite Co., Ltd., Osaka, Japan). The suspension of collected undifferentiated colonies was pipetted gently for dispersal into small aggregates, and then dispensed into a fresh culture vessel containing feeder cells. The medium was replaced by fresh medium every day.

**Exposure to HA complex** The procedure we used for reconstituting a functional HA complex was similar to a previously described method (28). HA concentrations were 5 and 50 nM. We cultured hiPSCs with SNL feeder cells for 48 h and then added HA to colonies. After HA exposure for 24 h, HA was removed during the course of routine medium changes, and then behavioral changes in undifferentiated cells in hiPSC colonies were examined.

**Time-lapse observations** To observe behavioral changes in hiPSC colonies, an image analyzer with a 4× objective lens (Biostudio; Nikon Engineering Co., Kanagawa, Japan) was used after seeding hiPSCs on a feeder layer.

**Fluorescence time-lapse observation** To track cell movement in hiPSC colonies, the cells were then stained, according to the manufacturer's instructions, with a cytoplasmic dye (CellTrackerTM Green; Thermo Fisher Scientific) used to distinguish cell behavior. Images were obtained every 45 min using a time-lapse confocal laser scanning microscope (FV-10i; Olympus, Tokyo, Japan) through a 60× objective lens under fluorescence excitation at 488 nm. The imaging processing of single cells within colonies was performed using image processing software (Imaris; Bitplane AG, Zurich, Switzerland).

**Quantitative analyses of migration rate and colony number** The procedure we used to analyze the migration rate of single cells in hiPSC colonies was similar to one described previously (11). Briefly, cell nuclei were stained with Hoechst33342 (Thermo Fisher Scientific) for 30 min in live cells. After incubation, cells were washed and fresh medium was added. Time-lapse images of cell nuclei were captured every 30 min over 6 h using an image analyzer with a 10× objective lens (IN Cell Analyzer 2000; GE Healthcare, Buckinghamshire, UK). The colonies were judged to be in an undifferentiated state at 120 h. For measurement of cell migration rate in the central and peripheral regions of colonies, an original image (6.9 mm × 6.9 mm, 16 bit, and $1.35 \times 10^6$ pixels/mm$^2$) was generated by tiling images. Data were obtained from the two regions of interest (ROIs; 150 μm × 150 μm) placed at the central and peripheral positions of the colony. The centroid position of the colony was set by the outer boundary of the colony in the bright-field image. For quantification of cell migration rate, the positional centroids ($x_i$, $y_i$) of each nucleus in the ROI were determined using image analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA). The migration rate, $V$, of single cells was estimated by measuring the centroid displacement of the nucleus in central and peripheral regions over 3 h. The average migration rate, $V_M$, and median migration rate, $V_m$, were calculated from migration rates, $V$, of 169–293 cells within ROIs of five randomly selected hiPSC colonies, and compared between the central and peripheral regions of single colonies before and after HA addition. The data on measured migration rate were grouped into bins defined by the [25th percentile −1.5 × (75th percentile −25th percentile)]–25th percentile range, 25th–75th percentile range, 75th–[75th percentile + 1.5 × (75th percentile − 25th percentile)] percentile range, and outliers of migration rates.

Colony number was measured according to a previously described method (11). Triplicate cultures were conducted in six-well plates (culture area in each well: 9.5 cm$^2$; Corning Costar, Cambridge, MA, USA) for 120 h. Bright-field images of hiPSC colonies were captured at 120 h. Original images (16 bits and $2.92 \times 10^5$ pixels/mm$^2$) were captured using an image analyzer with a 4× objective lens (IN Cell Analyzer 2000; GE Healthcare). Multi-position capturing provided tiling images for colonies in each whole well, which enabled detection of deviated hiPSC colonies by visual inspection of tiled images. The number of colonies in an undifferentiated state was evaluated to determine the ratio of undifferentiated colonies to total colonies.

**Immunofluorescence staining** The procedure used for immunofluorescence staining was similar to one described previously (29). Briefly, cells were washed with phosphate-buffered saline (PBS; Sigma–Aldrich), and then fixed with 4% paraformaldehyde (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for 10 min at room temperature. After washing with PBS, the cells were incubated with 0.5% Triton X-100 in PBS for 5 min and then washed twice more in PBS. For blocking attachment of nonspecific proteins, the cells were exposed with Block Ace (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) for 90 min at room temperature and then immersed at 4°C overnight with primary antibody: anti-laminA/C (Santa Cruz Biotechnology, Dallas, TX, USA), anti-E-cadherin (Cell Signaling Technology Inc., Beverly, MA, USA), anti-paxillin (Millipore, Billerica, MA, USA), anti-PAX6 (Sigma–Aldrich), anti-α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK), or anti-SOX17 (Abcam) in PBS with 10% Block Ace. For immunolabeling, the cells were rinsed with tris-buffered saline, and immersed for 1 h with secondary antibody: Alexa Fluor 488-conjugated goat anti-mouse or rabbit IgG (Thermo Fisher Scientific). F-actin and cell nuclei were stained with Alexa Fluor 594-conjugated phalloidin and 4',6-diamidino-2-phenylindole (both obtained from Thermo Fisher Scientific) in PBS, respectively. The cells were observed by an image analyzer with a 10× objective lens (IN Cell Analyzer 2000; GE Healthcare) or a confocal laser microscope (model FV-1000; Olympus) with a 60× objective lens under fluorescence excitation at 358, 488, and 594 nm.

**Induction of directed differentiation** For directed differentiation, the cells were dispersed and transferred into a new culture plate for seven-day culture using the STEMdiff trilineage differentiation kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions.

**RNA isolation and quantitative real-time RT-PCR** Isolation of RNA, cDNA synthesis, and PCR analysis were conducted as described previously (23). Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and TRIzol (Thermo Fisher Scientific). One mg of total RNA was then reverse-transcribed using a SuperScript II Reverse Transcriptase kit (Thermo Fisher Scientific). The quantitative real-time RT-PCR (qPCR) assays were conducted on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq

(Takara Bio Inc., Shiga, Japan), and contained 1 μl of cDNA per 25 μl reaction mixture. The relative quantity of the target transcript was estimated from a standard curve, and the data were standardized based on the expression level of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. The primer sequences are listed in Supplementary Table S1.

**Statistical analysis** The measured hiPSC colonies in each experimental condition were sampled from at least three different cultures. Statistical analysis was performed using either the parametric Student's *t*-test or the nonparametric Mann–Whitney *U*-test. The significance thresholds were $P < 0.01$ and $P < 0.05$.

## RESULTS

**Characteristics of HA-induced behavioral changes in hiPSC colonies** Similar to previously published reports on hiPSC culture (10,11,24), the deviation of undifferentiated hiPSCs in culture with SNL feeder cells spontaneously occurred in the central regions of colonies (Movie S1). Undifferentiated hiPSC colonies subsequently expanded gradually and became more tightly packed. Cells in the central regions of colonies underwent a transformation from small cobblestone-shaped cells to large flattened cells (Movie S1a). To understand the HA-induced behavioral changes in hiPSC colonies, we performed time-lapse imaging of fluorescently labeled colonies without and with exposure to HA. In control culture without HA exposure, the undifferentiated hiPSC colonies grew by a combination of cell division and migration (Movie S2a and b). The colonies gradually became larger and more tightly packed. In contrast to the control culture, cells in colonies exhibited a loose morphology initially after HA exposure (Movie S2c and d). In particular, cells in peripheral regions of cultures were migratory and arranged in loose monolayers, compared with those at the central region. Newly-divided cells in colonies continually migrated outward, thereby providing space for divided daughter cells.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.11.014

To clarify the effect of HA exposure on migration in an hiPSC colony in detail, we examined the migration rate of single cells in the central and peripheral regions of five colonies with or without HA exposure (Fig. 1A). For the comparative analysis, the data on measured migration rate, *V* in the central or peripheral regions for 3 h before and after HA addition are plotted in a box. In the control culture without HA exposure, the distribution of migration rates of single cells in the central region had a narrow range compared with those in the peripheral region of the colony. The ratio above the 75th percentile value (fast-moving populations) in both the central and peripheral regions has decreased over time from 25% to 17%. However, the ratio below the 25th percentile value (slow-moving populations) in the central region has drastically increased from 25% to 35% over the same time. There was statistically significant difference in distribution shift of median cell migrations between the comparing two groups ($^{\#}P < 0.01$ by Mann–Whitney *U* test).

In the hiPSC culture exposed to HA, the distributions of migration rate in the central and peripheral regions were significantly broader than those in non-HA treated cells before HA addition (in the central region, $^{\#\#}P < 0.05$; in the peripheral region, $P < 0.01$ by

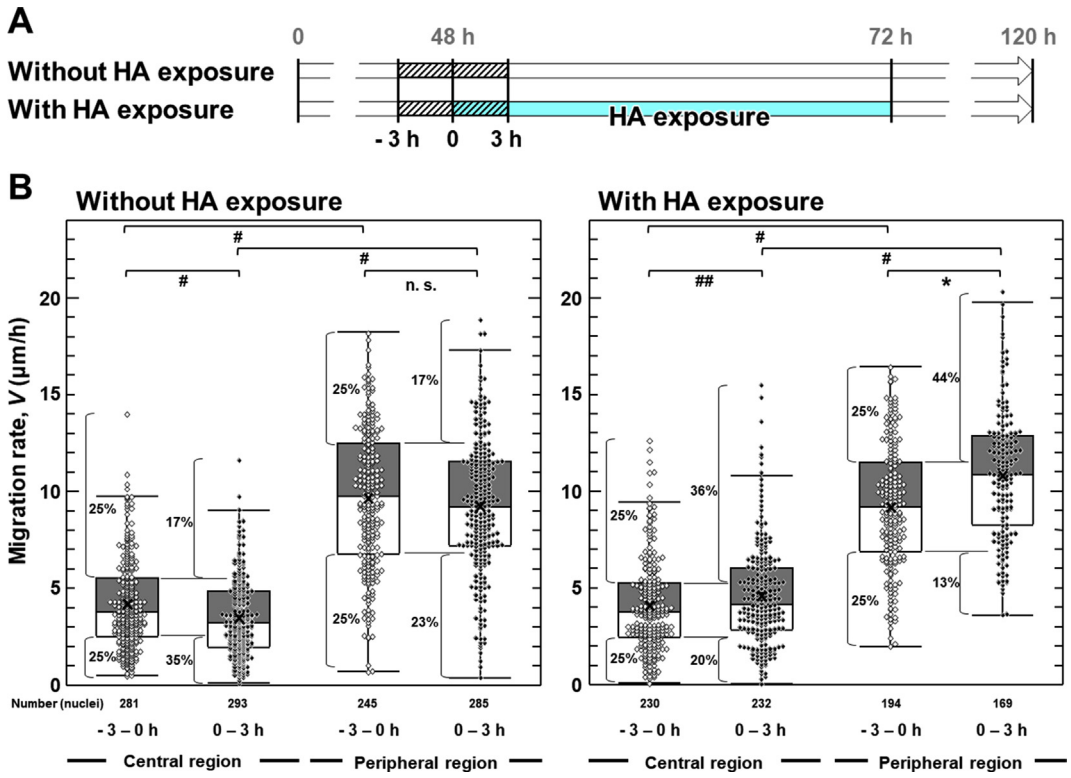


FIG. 1. HA-mediated changes of migration rate of single cells in central and peripheral regions of hiPSC colonies with or without HA exposure. (A) Timeline of experimental procedure. Cells were cultured on SNL feeder layers for 48 h and then replaced by medium supplemented with 50 nM HA. The migration rates of single cells in the central and peripheral regions of colonies were measured in two phases before and after HA exposure. (B) Distribution of migration rates of single cells at the central and peripheral regions of hiPSC colonies with or without HA exposure. The data were obtained from cells within ROIs of five randomly selected hiPSC colonies. Distributions of migration rates at the central regions of colonies without and with HA exposure followed a non-normal distribution. Open squares, −3 − 0 h; closed squares, 0–3 h. Cross marks indicate average values. Box-and-whiskers plots of migration rates of single cells at the central and peripheral regions of hiPSC colonies. For each box, the central bar is the median, and the edges are the 25th and 75th percentiles. The whiskers extend to the most extreme data points, not including outliers (n = 169–293 cells). Outliers are values outside of the range: [25th percentile −1.5 × (75th percentile − 25th percentile)] − [75th percentile + 1.5 × (75th percentile − 25th percentile)]. $^{*}P < 0.01$ (Student's *t*-test). $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ (Mann–Whitney *U* test).

Mann–Whitney *U* test). After HA exposure, the ratio above 75th percentile value in the central and peripheral regions has drastically increased over time from 25% to 36 % and from 25% to 44%, respectively. Specifically, the average migration rate in the peripheral regions was 1.2-fold higher than that before HA addition. There was a difference between statistical significance following HA treatment ($^*P < 0.05$ by Student's *t*-test).

**Formation of actin cytoskeleton and nucleoskeleton in hiPSC colonies** We tested whether HA exposure alters the structure of actin filaments associated with migratory behaviors in hiPSC colonies. Fig. 2 shows a series of confocal images representing horizontal sections through the apical–basal axis of the cells in single colonies in culture conditions with or without exposure to HA. From the spatial images of F-actin and nuclei, cytoskeleton-related F-actin assembled inside the undifferentiated cells in the peripheral or central regions of colonies. In peripheral regions, F-actin localized at cell–cell adhesions and appeared to connect intercellularly on the apical side; aligned actin fibers traversed the cell lengthwise on the basal side (Fig. 2Ba and Da). Notable differences between HA-treated and HA-untreated cells were seen for the basal-side actin fibers in the central regions of colonies (Fig. 2Aa and Ca). In HA-untreated colonies, cell nuclei in central regions exhibited a more restricted distribution with a fully disorganized overlapping layer (Fig. 2Ab). The cells exhibited complex F-actin structure and lost basal-side actin fibers in multiple layers where stratification showed that aggregation of nuclei had occurred (Fig. 2Aa). In contrast, in cultures exposed to HA for 24 h, cell nuclei exhibited a single-layered distribution of nuclei in the center of colonies (Fig. 2Cb). Actin bundle formation at the basal side was clearly observed and resembled cells in the peripheral region (Fig. 2Ca and Da). This is consistent with the expression pattern of the nucleoskeletal protein, lamin A/C in between HA-treated and HA-untreated colonies, as determined by immunofluorescence. Cells in peripheral regions of colonies showed a rim pattern of laminA/C expression around the nucleus (Fig. S1), and no remarkable differences in laminA/C expression pattern between HA-treated and HA-untreated colonies. However, while cells in central regions of HA-untreated colonies with multi-layered structure show a very strong rim pattern of nuclear staining, no staining was observed in central regions of HA-treated colonies (Fig. S1Aa and Ba).

**Localization of paxillin and E-cadherin in hiPSC colonies** To investigate the mechanisms of the effect of HA exposure on cell–substrate and cell–cell interactions, we performed immunostaining to determine the expression at 120 h of paxillin, an integrin-associated protein of focal adhesion, and E-cadherin, a cell–cell adhesion-associated protein. In HA-untreated colonies, F-actin had a linear morphology along the boundaries between adjacent cells, and a nebulous distribution of paxillin expression was observed for areas of cell–cell contact at the basal surface of cells in the central region (Fig. 3Aa). However, in cells in the peripheral region, distinct and straight stress fibers were observed, including at the leading edge with lamellipodia with intensive scattered staining (Fig. 3Ba). In contrast, in HA-treated colonies, similar pattern of actin and paxillin were found at the basal surface of cells between central and peripheral regions in hiPSC colonies (Fig. 3Ca and Da). In central regions, both paxillin recruitment to focal adhesions and actin stress fibers increased at the basal surface of cells in comparison with that in central region of non-HA-treated colonies. This staining pattern resembled that in peripheral regions (Fig. 3Ca and Da).

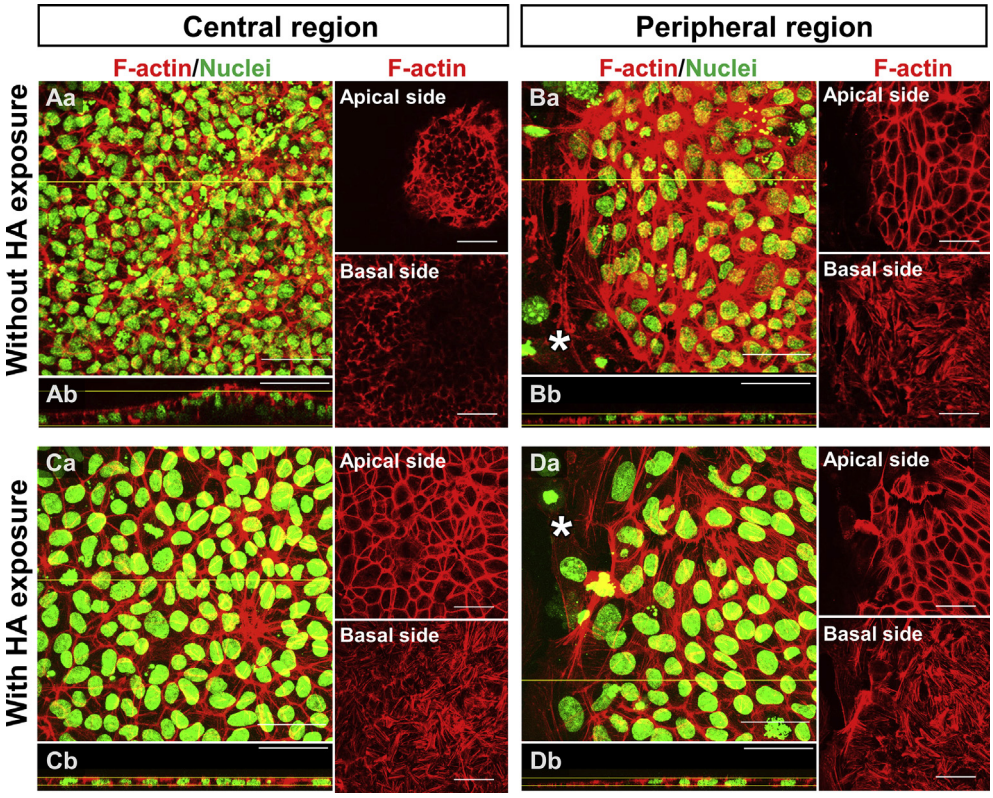


FIG. 2. Actin cytoskeletal formation related to nucleus distribution in hiPSC colonies without and with HA exposure. Confocal fluorescence images show organization of F-actin filaments (red) and nuclei (green). Panels Ab, Bb, Cb, and Db are the tomograms sectioned at *x*–*z* plane (yellow lines) in top-viewed panels Aa, Ba, Ca, and Da. The asterisks show feeder cells. Images were acquired after 120 h of culture. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

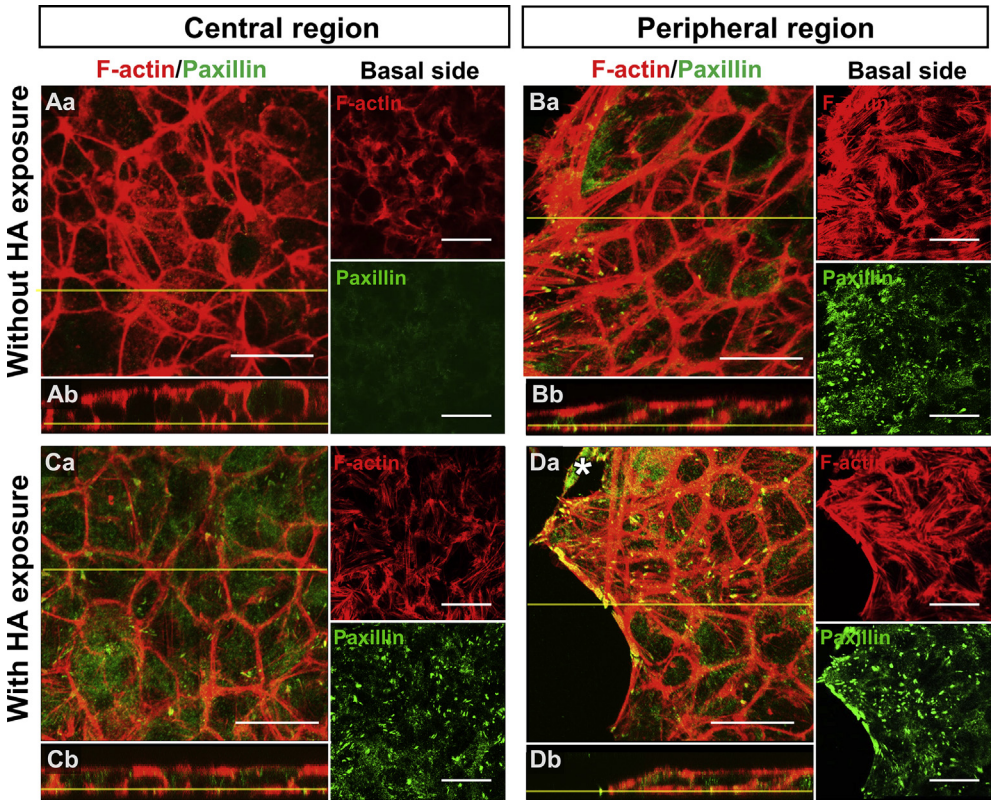


FIG. 3. Localization of paxillin associated with actin cytoskeleton in hiPSC colonies in cultures without and with HA exposure. Confocal fluorescence images show organization of F-actin filaments (red) and paxillin (green). Panels Ab, Bb, Cb, and Db are the tomograms sectioned at $x$–$z$ plane (yellow lines) in top-viewed panels Aa, Ba, Ca, and Da. The asterisks show feeder cells. Images were acquired after 120 h of culture. Scale bars: 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

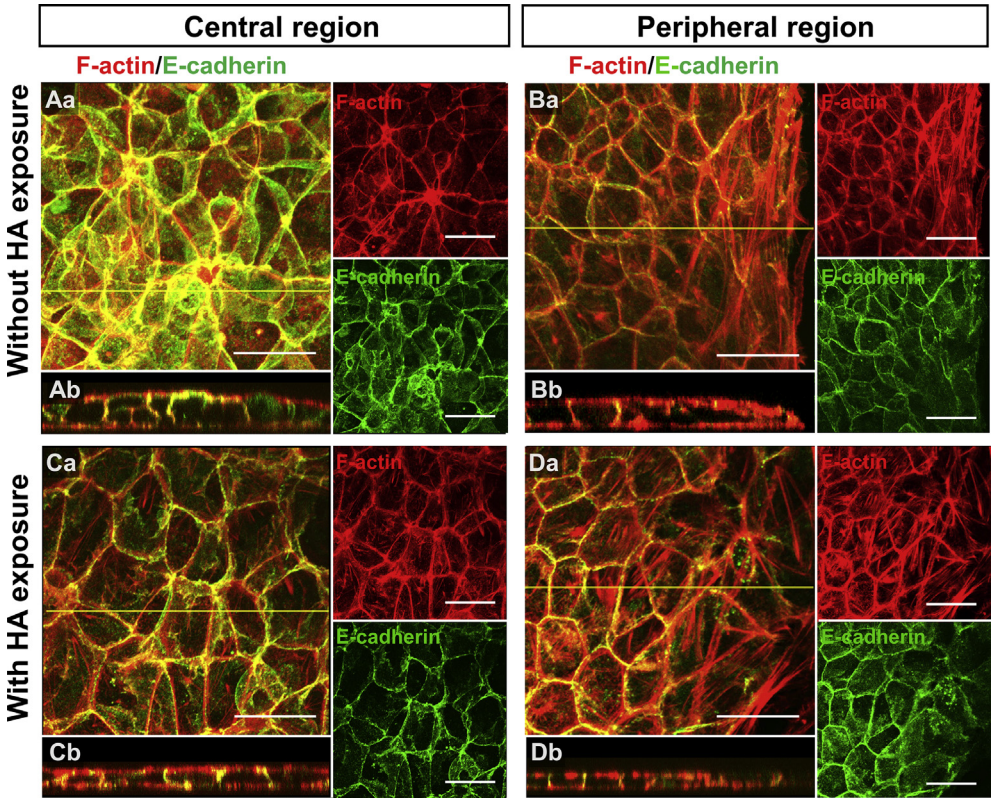


FIG. 4. Localization of E-cadherin associated with actin cytoskeleton in hiPSC colonies in cultures without and with HA exposure. Confocal fluorescence images show organization of F-actin filaments (red) and E-cadherin (green). Panels Ab, Bb, Cb, and Db are the tomograms sectioned at $x$–$z$ plane (yellow lines) in top-viewed panels Aa, Ba, Ca, and Da. Images were acquired after 120 h of culture. Scale bars: 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Subsequently, the results of immunostaining of E-cadherin and F-actin showed no notable location-dependent differences in their expression at 120 h. E-cadherin expression was very similar between central and peripheral regions under culture conditions with HA exposure (Fig. 4Ca and Da) or without HA exposure (Fig. 4Aa and Ba). Cells expressing E-cadherin exhibited a cuboidal morphology and were observed as a continuous line at the boundaries between neighboring cells (Fig. 4Aa, Ba, Ca, and Da). These findings indicate that the alteration of cell migration in hiPSC colonies following HA exposure has no effect on E-cadherin mediated cell–cell contacts.

**Long-term maintenance of undifferentiated hiPSCs** We next tested whether enriched preparations of undifferentiated hiPSCs could be obtained by performing several passages of cells in the presence of HA. During long-term passaging, the frequency of colonies with deviating cells decreased gradually following exposure to HA (Fig. 5). After 10 passages (50 days), the percentage of colonies with undifferentiated cells was significantly higher in HA-treated cells than in HA-untreated cells, supporting the effectiveness of our culture method for maintaining the undifferentiated phenotype of hiPSCs without use of manual elimination (Fig. 5B). We also tested hiPSCs cultured with or without HA exposure for the expression levels of pluripotency markers using q-PCR analyses after 10 passages. The expression levels of pluripotency markers (Oct4, Nanog, and Sox2) in HA-treated cells were detectably higher than in HA-untreated cells (Fig. S2). Finally, we determined whether propagated hiPSCs were capable of differentiation into the three major germ layers after 10 passages. They were positive for all markers associated with the three germ layers, namely nestin for ectoderm, α-smooth muscle actin for mesoderm, and SOX17, for endoderm (Fig. S3). These findings indicated that our HA-mediated culture method enabled highly efficient culturing of hiPSCs without loss of pluripotency.

## DISCUSSION

Key topics of research in the field of stem cell bioengineering include the search for triggers of deviation from the undifferentiated state, investigation of how cells self-renew and commit based on microenvironmental cues in culture, and design of culture strategies to elicit the desired cell fate (30–33). Particular normal cellular behaviors acting to regulate stem cell self-renewal and lineage fate, or maintain cellular diversity, have been described (12). As we observed here, changes in cellular behavior can be associated with reduced reproductive ability, creating a functionally heterogeneous cell population (Fig. 5). For example, the heterogeneous distribution of cells in culture is expected because cell growth and migration are generally promoted by colony formation. Previous studies have shown that spatial heterogeneity in colonies can affect deviation from the undifferentiated state, and have revealed the behavioral mechanisms for these effects (9,13,24). Within migrating colonies, cell–cell and cell–substrate interactions are integrated so that cells maintain interactions with neighboring cells and the underlying substratum. The coordinated organization of cadherin-based and integrin-based adhesion complexes is required for coordinated movement of large groups of cells, and spatiotemporal regulation of Rho family GTPases and actin dynamics is required for collective cell migration (9,13). In the case of hiPSC colonies with deviation, motility has been shown to decrease steadily with increasing population density at colony centers, and the cells overlapped, indicating that deviant cells in colony central regions partially detached from the substrate (24). These cells exhibited morphological changes and proliferated while undergoing transition from small to large, flattened cells. It was also demonstrated that the deviation from the undifferentiated state in hiPSCs occurs haphazardly, in consequence of anomalous cell migration. Deviation from the undifferentiated state in hiPSCs was attributed to the extents of the cell–cell and cell–substrate interactions derived from cell division and migration in the central regions of colonies (9,34). Cessation of cell migration apparently facilitated stable cell–cell interactions in the central regions of colonies, leading to imbalance between cell–cell and cell–substrate interactions. In cells in colony central regions, cytoskeletal forces result in an upward pull force away from the substrate. The cell body is lifted away from the surface and remains attached only at distinct adhesion points while the nucleus is not exposed to severe cytoskeletal forces. In cells located in these areas, cytoskeletal forces create a large push force toward the surface leading to compression and deformation of the nucleus. Deviation from the undifferentiated state occurs through the transition to heterochromatin via accumulation of laminA/C at the nuclear

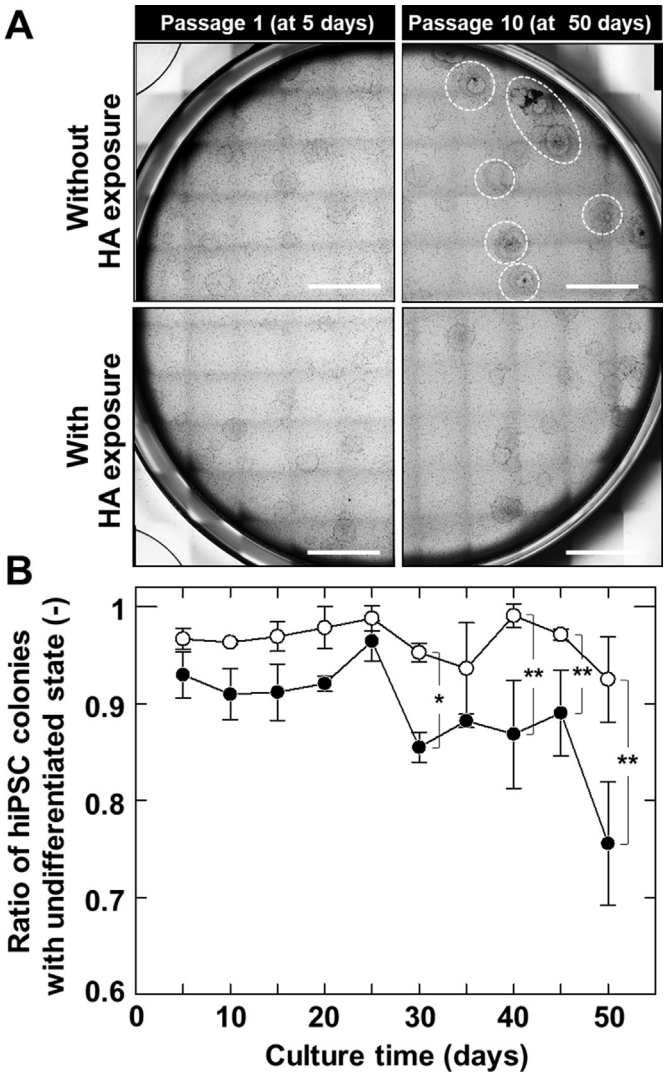


FIG. 5. Culture of hiPSCs in an undifferentiated state by several passages including HA exposure. hiPSCs were cultured with SNL feeder cells for 48 h and then added 5 nM HA to colonies. After HA exposure for 24 h, HA was removed during the course of routine medium changes. (A) Morphological properties of hiPSC colonies in dishes during serial-passage cultures without and with HA exposure. Dotted circles indicate hiPSC colonies with deviated cells from the undifferentiated state. Scale bars: 1 cm. (B) Ratio of hiPSC colonies with undifferentiated state in cultures with several passages. Closed circles, without HA exposure; open circles, with HA exposure. Data are given as mean and standard deviation ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.05$ (Student's $t$-test).

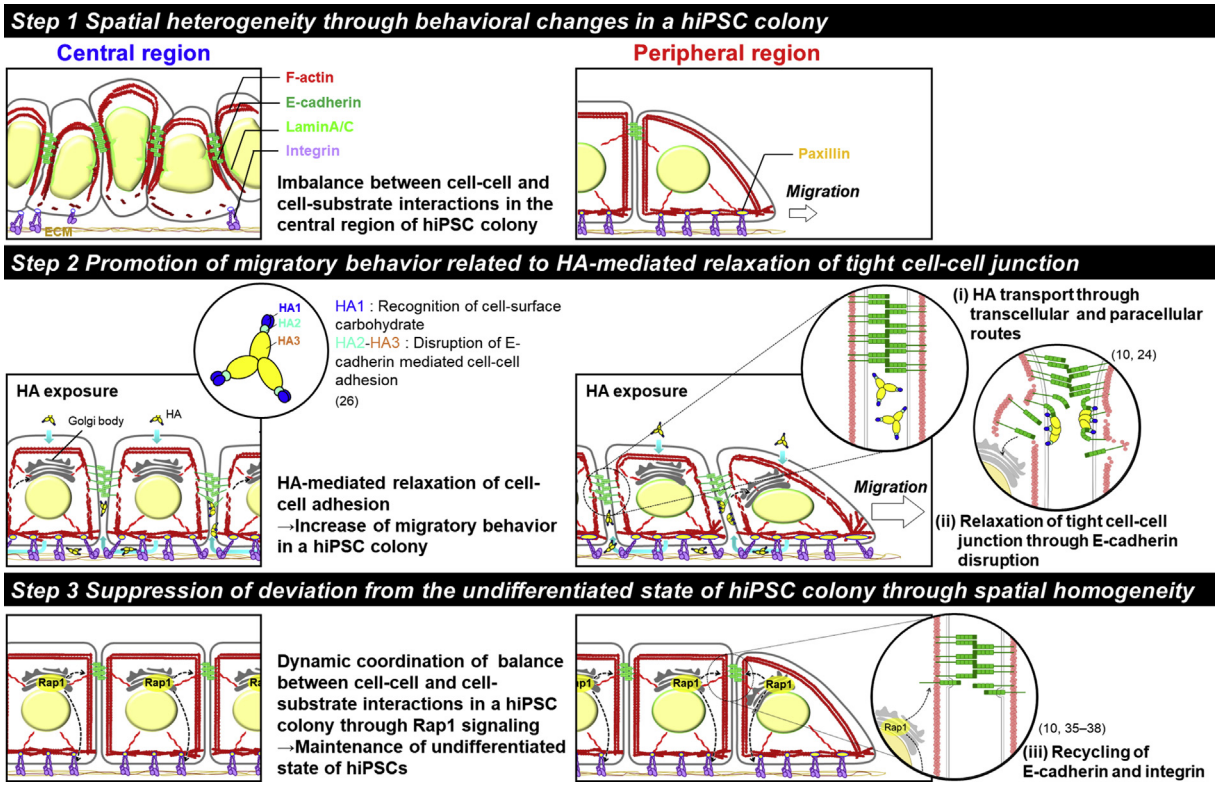


FIG. 6. Schematic drawings representing our hypothesis of the maintenance of an undifferentiated state of hiPSCs through HA-mediated cell-behavior regulation.

periphery through actin cytoskeleton-mediated cell–cell connections and cell–substrate adhesions in the region including the anomaly (24). These results provide important information toward understanding the processes of deviation from the undifferentiated state occurring at the center of hiPSC colonies, suggesting that the balance of interaction between adjacent cells and their substrate along with colony growth is closely related to the occurrence of deviated cells during cell expansion.

Here we presented evidence that HA can induce cell migration through relaxation of cell–cell junctions, leading to suppression of deviation from the undifferentiated state in hiPSC colonies. Fig. 6 shows a schematic illustration of our working hypothesis based on our observations and previous studies. The mechanism of disruption of E-cadherin adhesion by HA has been extensively studied and characterized (10,26,28). The main steps of this process are as follows: (i) HA can pass through cells (transcellular route) and between cells (paracellular route) when moving across cells. (ii) HA binds to E-cadherin and thereby disrupts E-cadherin-mediated cell–cell adhesion. (iii) Loss of E-cadherin adhesion rearranges cytoskeletal organization and focal adhesion by regulating the activity of Rap1, which functions in the interplay between cadherin and integrin. The effect of HA exposure on hiPSC culture may depend on the ability of E-cadherin re-expression after loss of E-cadherin adhesion. Herein, we observed that spatial distribution markedly differed between the central and peripheral regions of hiPSC colonies, and correlated with differences detected in expression of their E-cadherin and integrin. When grown with SNL feeder cells, hiPSCs formed a round morphology through cell division and migration, and cell density became greater at colony central regions than at the periphery (Movie S2). In culture with HA exposure, however, cells in the central and peripheral regions of hiPSC colonies were migratory and arranged in loose monolayers, resulting in uniform dispersion of cells in colonies. Cells in the central and peripheral regions of hiPSC colonies were clearly observed with mature actin stress fibers on the basal side (Fig. 2Ca and Da). The ability to regenerate E-cadherin can maintain hiPSCs in an undifferentiated state after disruption of E-cadherin binding (Fig. 4Ca and Da, Fig. 5). In particular, Rap1-mediated positive-feedback regulation could lead to E-cadherin and integrin-driven re-establishment of intercellular adhesion, and thereby enable undifferentiated cells to recover intercellular association and colony structure (35–38). Relaxation of E-cadherin-mediated cell–cell contacts reduces differences of cytoskeletal organization and focal adhesion between the central and peripheral regions of colonies by promoting migratory behaviors. These observations from immunostaining revealed that the balance between cell–cell and cell–substrate interactions of cultured hiPSCs is characterized by continuously changing cytoskeletal formation and focal adhesion concomitant with movement, leading to changes in cell distribution during culture. The coordinated regulation of balance between cell–cell and cell–substrate interactions through enhancement of cell migration leads to the prevention of nuclear laminA/C accumulation in the central region of hiPSC colonies. It is most likely that the nucleus can respond directly to control in migration in culture with HA exposure, with alterations in both gene regulation and nuclear mechanical properties that are independent of cytoplasmic biochemical and cytoskeletal responses. We thus consider that the change in cell behavior that occurred following HA treatment was responsible for the promotion of collective migration in hiPSC colonies during culture, thereby facilitating the maintenance of undifferentiated hiPSCs.

In conclusion, we developed a simple and robust method for culturing hiPSCs in an undifferentiated state using HA-mediated regulation of cell behavior. We found that spontaneous deviation from the undifferentiated state was suppressed by HA-mediated regulation of the balance between cell–cell interaction and cell–substrate interaction through cell migration in hiPSC colonies. This insight provides new avenues for suppressing the influence of

spatial heterogeneity on the behavior of hiPSC colonies during culture, and facilitates rational design of culture strategies for maintaining undifferentiated hiPSCs. In this regard, the principle of HA-mediated cell-behavior regulation in hiPSC-maintenance cultures will lead to novel approaches for expansion–culture processes designed to facilitate stable cultures, and enable operators to handle greater quantities of hiPSCs.

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