



Original Article

Effect of migratory behaviors on human induced pluripotent stem cell colony formation on different extracellular matrix proteins

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ABSTRACT

Introduction: Understanding how extracellular matrix (ECM) protein composition regulates the process of human induced pluripotent stem cell (hiPSC) colony formation may facilitate the design of optimal cell culture environments. In this study, we investigated the effect of migratory behaviors on hiPSC colony formation on various ECM-coated surfaces.

Methods: To quantify how different ECM proteins affect migratory behavior during the colony formation process, single cells were seeded onto surfaces coated with varying concentrations of different ECM proteins. Cell behavior was monitored by time-lapse observation, and quantitative analysis of migration rates in relation to colony formation patterns was performed. Actin cytoskeleton, focal adhesions, and cell–cell interactions were detected by fluorescence microscopy.

Results: Time-lapse observations revealed that different mechanisms of colony formation were dependent upon the migratory behavior of cells on different ECM surfaces. hiPSCs formed tight colonies on concentrated ECM substrates, while coating with dilute concentrations of ECM yielded more motile cells and colonies capable of splitting into single cells or small clusters. Enhanced migration caused a reduction of cell–cell contacts that enabled splitting or merging between cells and cell clusters, consequently reducing the efficiency of clonal colony formation. High cell-to-cell variability in migration responses to ECM surfaces elicited differential focal adhesion formation and E-cadherin expression within cells and colonies. This resulted in variability within focal adhesions and further loss of E-cadherin expression by hiPSCs.

Conclusions: Migration is an important factor affecting hiPSC colony-forming patterns. Regulation of migratory behavior can be an effective way to improve the expansion of hiPSCs while improving the process of clonal colony formation. We believe that this investigation provides a valuable method for understanding cell phenotypes and heterogeneity during colony formation in culture.

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1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic cells (hESCs) and human induced pluripotent cells (hiPSCs), harbor vast potential for use in application of tissue engineering

and regenerative medicine [1–3]. Under standard culture conditions, hESCs and hiPSCs grow as colonies; however, the commonly used methods of cell propagation result in colonies that are often characterized by mixed clonal origin. Additionally, extensive cell death after single cell dissociation upon cell passaging results in very low cloning efficiency [4–8]. In an effort to circumvent the problem of apoptosis in hPSC culture, use of Rho-associated kinase (ROCK) inhibitor has been shown to increase the development of hESC clones originating from more than one founder cell [9,10]. The importance of cell movement for cell–cell contact and cell survival, proliferation, and heterogeneity of hESCs has also been investigated in karyotypically normal and abnormal sublines of hESCs. This matter highlights the need for a deeper understanding of the

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processes by which individual hPSCs generate colonies. Moreover, to adapt hPSC differentiation protocols for large-scale assays and clinical trials, there is a great need for controlled and reproducible cell production strategies [11,12]. Thus, understanding the regulation of how hPSC colonies form from individual cells would be beneficial.

Because one goal of using hPSCs is for therapeutic purposes, more defined hPSC culture systems have been progressively developed. Many researchers have developed various serum-free culture media and extracellular matrix (ECM) to support the growth and pluripotency of hPSCs [13–20]. Recently, commercial xeno-free, defined culture media and ECM proteins have been developed for research and future clinical purposes. For example, recombinant laminin isoforms such as laminin-511 [16,17], laminin-521 [18], and laminin E8 fragments [19], as well as recombinant vitronectin [20], represent defined, xeno-free substrates that support hPSC undifferentiated growth and self-renewal. In addition, increasing evidence shows that specific laminin isoforms may play an important role in sustaining the long-term expansion of hPSCs. Intuitively, high single-cell plating efficiency of hPSCs using these ECM substrates could enable the propagation of cells from single cells. Although there have been several approaches to optimize hPSC cultivation, the modification of substrates used to coat culture surfaces has greatly contributed to improved viability after sub-culture and efficient expansion of hPSCs [4,7,8]. Despite significant advances, there remains a lack of robust methods for clonal culturing of hPSCs under chemically defined and xeno-free conditions. To date, mechanisms regulating the heterogeneous phenotype of hPSCs colonies and potential relationships to the functional properties of single cells are poorly studied. Furthermore, behaviors and fate of individual cells with respect to hPSC colony formation are not well understood; this hampers the ability to predict hPSC differentiation capacity.

In this study, we investigated the mechanism by which the ECM surface affects migratory behavior during hiPSC colony formation, as well as within the colony. In addition, we discuss the fundamental mechanisms of cell and ECM surface interactions with respect to the formation of actin cytoskeleton, focal adhesions, and cell–cell interactions.

2. Methods

2.1. Pre-coating of culture substrates

All ECM coating solutions were prepared as recommended by the manufacturers. Wells were coated with laminin511-E8 (LN511; Nippi, Tokyo, Japan), laminin-521 (LN521; BioLamina, Sundbyberg, Sweden), recombinant truncated human vitronectin (VTN; Thermo Fisher Scientific, Waltham, MA, USA), or Matrigel® (MG; Corning, New York, NY, USA) at concentrations of 0.25–1 µg/ml overnight at 4 °C prior to cell seeding. Matrigel was prepared by diluting the matrix solution 1:100 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12). The protein concentration of matrix solution was determined as 159 µg/ml by BCA assay, and was coated at concentration of 8.3–33.1 µg/cm² overnight at 4 °C prior to cell seeding. Volumes of coating solution were adjusted according to the growth area of the culture vessels used.

2.2. Cells and culture conditions

The hiPSC line 201B7 was purchased from iPS Academia Japan, Inc (Kyoto, Japan). Cells were transferred from a feeder-based to a feeder-free culture system according to standard guidelines established by the Center for iPS Cell Research and Application. In brief, cells were detached by enzymatic treatment with CTK solution (0.1%

collagenase IV, 0.25% trypsin, 20% KSR, and 1 mM CaCl₂ in PBS) for 30 s, and washed twice with PBS to release the feeder cells. Cells were then incubated for 4 min with 0.5 × TrypLE™ Select CTS™/0.25 mM ethylenediaminetetraacetic acid (EDTA) solution (Life Technologies). Next, StemFit®AK02N medium (Ajinomoto Co., Inc., Tokyo, Japan) containing 10 µM Y-27632 (Nacalai Tesque, Inc., 08945-84) was added to culture vessels and the cell suspensions were dissociated into single cells with gentle scraping and pipetting.

hiPSCs were maintained under feeder-free conditions on ECM protein-coated surfaces in StemFit®AK02N medium. For single cell passaging, cells were treated with a detachment solution consisting of 0.5 × TrypLE™ Select CTS™ (Thermo Fisher) containing 0.25 mM EDTA in PBS for 8 min at 37 °C. Medium including 10 µM Y-27632 was added to the vessel and single cell suspensions were created by pipetting several times before cell counting. Numbers of viable cells in culture vessels were determined using the trypan blue exclusion test measured by automated counting of detached cells from 50 images (Vi-CELL™ XR, Beckman Coulter, Brea, CA, USA). Cells were seeded at appropriate densities (1.0–4.5 × 10³ cells/cm²) on LN511, LN521, VTN, or MG surfaces in the presence of 10 µM Y-27632. For routine maintenance, cells were seeded at 1.5 × 10³ cells/cm² for LN511 and VTN, at 1.0 × 10³ cells/cm² for LN521, and 4.5 × 10³ cells/cm² for MG. Fresh medium was replaced on days 1, 2, 5, and 6, and cells were passaged on day 7.

2.3. Characterization of hiPSC growth and karyotype stability during long-term culture

During passaging, numbers of viable cells in culture vessels were determined using the trypan blue exclusion test measured by automated counting of detached cells (Vi-CELL™ XR, Beckman Coulter, Brea, CA, USA). Cumulative population doubling at each passage was calculated from the number of viable cells in each passage.

To analyze surface marker expression by flow cytometry, hiPSCs were harvested and processed according to the manufacturer's instructions. In brief, cells were detached from culture surfaces and fixed with Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA, USA) at 4 °C for 20 min, washed twice with BD Perm/Wash Buffer, then stained with Alexa Fluor® 647 mouse anti-SSEA-4 and Alexa Fluor 488 mouse anti-OCT3/4 (BD Biosciences) for 20 min in the dark. After two washes with BD Perm/Wash Buffer, stained cells were analyzed on a BD Accuri C6 flow cytometer.

For evaluation of karyotype stability, hiPSCs grown for 32 passages were assessed using a standard G-banding technique. Chromosomes were prepared using standard protocols, G-banded with trypsin and stained with Giemsa. For each culture, 20 metaphase spreads were examined.

2.4. Quantitative analyses of migratory behavior and colony formation

To investigate cell behaviors and colony formation, time-lapse observation of cells incubated on different ECM protein-coated surfaces was carried out for 72 h. To quantitate cell viability and colony-forming efficiency, three or more independent cultures under each condition were conducted for 72 h, and then cell numbers and colonies were counted from six captured images in each vessel. Cell viability was defined as the ratio of adherent cells at 3 h, determined from images, to that of seeded cells. The efficiency of overall colony formation was defined as the ratio of colonies at 72 h to the number of adherent cells at 3 h. The efficiency of clonal colony formation was defined as the formation of a colony from a single cell. The single cell origin of hiPSC colonies was

assessed by time-lapse imaging, with the origins of 230–500 colonies determined by backtracking.

All of the clonal colonies from 6 tiled images were traced in a time-lapse manner to track cells, analyze colony formation patterns, and record migration rates during the colony formation process. The schematic shown in Fig. 1 indicates the procedure for data analysis to assess migratory behavior. Images were captured to investigate cell behavior using an observation tool with a 4 × objective lens (BioStudio T, Nikon, Tokyo, Japan). Images were obtained from samples in 12-well plates, with 6 images (1280 × 1024 pixels/image; 0.77 pixels/μm²) captured at the center of the surface every hour for 72 h. Multi-position capturing provided tiling images for all cells within the center region of the culture vessel (six images tiled; 2552 × 3069 pixels). In this study, we classified two types of hiPSC colonies: clonal colonies (originating from a single cell) and non-clonal colonies. For measurement of cell migration for clonal hiPSC colonies, cells were assessed after 72 h of culture time to determine colony formation patterns; backtracking of 33–239 colonies for each coating condition was then conducted to evaluate cell behaviors. Positional centroids of each cell were determined

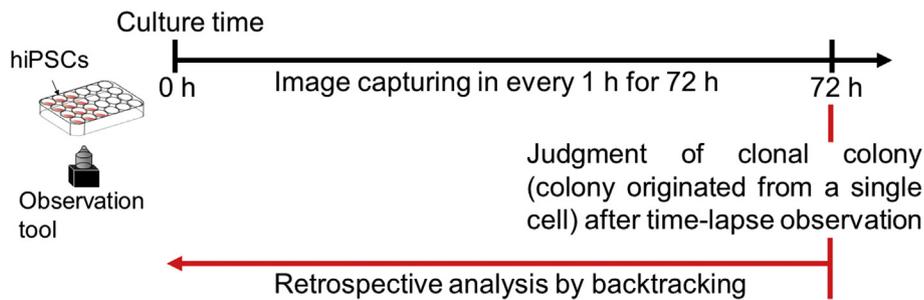
manually using image processing and analysis software (Image J; NIH, Bethesda, Maryland, USA). The migration rate of individual cells was determined from the average displacement of positional centroids over 72 h using the MTrackJ plugin for Image J.

To ascertain and quantify the association between migratory behavior and colony-forming pattern, frequencies of each biological event were estimated and related to colony forming patterns. Biological events occurring during colony formation were divided into three types: maintaining contact between neighbors (designated “no split”), splitting cell–cell contact at the two-cell stage (designated “cell split”), and splitting cell–cell contact at the multiple-cell stage with two or more cells (designated “colony split”). The frequency of each event was defined as the ratio of each event to the total number of events.

2.5. Immunofluorescence staining

hiPSCs were fixed with 3.7% paraformaldehyde (Wako) for 10 min at room temperature and then rinsed with PBS, followed by soaking in PBS with 0.25% Triton X-100 for 4 min. After masking of

A. Multi-position capturing of bright field images



B. Analyses of migration rate, efficiency of colony formation, and frequency of each event

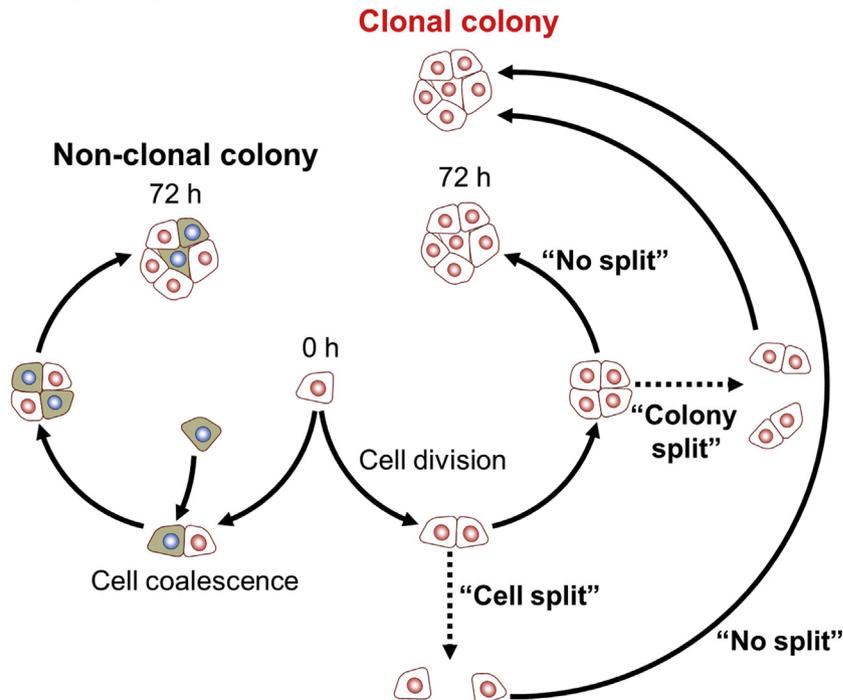


Fig. 1. Schematic drawing of data analysis procedures for time-lapse observation of hiPSCs.

non-specific proteins by incubation with Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) for 1 h at ambient temperature, cells were treated with primary antibody at 4 °C overnight. Specifically, cells were incubated with anti-Paxillin (clone 15D2, Zymed/Invitrogen) and anti-E-cadherin (Takara Biomedicals, Shiga, Japan) primary antibodies that were adequately diluted in PBS containing 10% Block Ace. Cells were washed with Tris-buffered saline followed by immunolabeling with Alexa Fluor 488-conjugated goat anti-rabbit or Alexa Fluor 594-conjugated goat anti-mouse IgG (Thermo Fisher) for 1 h. F-actin was stained with rhodamine phalloidin (Thermo Fisher). Images were obtained using a confocal laser-scanning microscope (FV-1000; Olympus, Tokyo) with a 60 × objective lens.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

RNA isolation, cDNA synthesis, and qRT-PCR assays were carried out as described previously (32). Total RNA was extracted from the cells (Maxwell 16 LEV simplyRNA Cells Kit, Promega Corporation, Wisconsin, WI, USA) according to manufacturer instructions. Reverse transcription was conducted using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan), and real-time PCR was performed using Fast SYBR Green Master Mix (Thermo Fisher) on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was normalized to 18S rRNA expression in order to obtain the ΔC_t value and calculated using the $2^{-\Delta\Delta C_t}$ method. All PCR products were evaluated according to melting curve analysis in order to exclude the possibility of multiple products or incorrect product size. The primer sequences are provided in Table S1.

2.7. Statistical analysis

To assess the significance of differences between cells grown on the four different ECM proteins, several statistical tests were used. Measurements for each experiment are reported as mean \pm standard deviation (SD). Differences between groups were analyzed for statistical significance with GraphPad Prism 7 software (GraphPad Software) using a Student's *t*-test or one-way ANOVA followed by Tukey's post hoc test. Pearson's correlation coefficient was determined to reveal bivariate correlation between two factors. Differences were considered significant when the *p* value was <0.05 .

3. Results

3.1. Characterization of hiPSCs grown on ECM protein-coated surfaces during long-term culture

To understand the effects of ECM surface for expansion of hiPSCs in combination with xeno-free culture media, various ECM proteins were tested and compared. As the ability of ECM surfaces to support long-term hiPSC expansion has previously been described in the literature [16–20], the model hiPSC line 201B7 was seeded onto different ECM surfaces in StemFit®AK02N as a xeno-free culture medium. We used four ECM proteins, LN511, LN521, VTN, and MG, as culture substrates because they are representative of protein-derivative culture substrates that support undifferentiated culture of hPSCs, and are commercially available. First, we evaluated these proteins for their ability to support hiPSC adhesion. Cumulative population doubling was calculated using the inoculation and final viable cell densities for each passage, as shown in Fig. S1. A similar cell growth curve was observed when culturing cells on all surfaces. Flow cytometry analysis of the cells indicated that cells

maintained high levels of markers both integral to and associated with PSCs. The percentage of OCT3/4/SSEA4-positive cells was $>95\%$ for all ECM surfaces. Finally, it was verified that hiPSCs in long-term culture displayed a normal karyotype (46 XX) after 32 passages on all ECM surfaces. Thus, the combination of ECM surface and xeno-free medium supports long-term culture of undifferentiated hiPSCs.

3.2. Cell behavior on ECM protein substrates

After demonstrating that ECM surfaces could support long-term culture of hiPSCs in xeno-free medium, ECM proteins were used to characterize cell behavior at the single cell level. To examine effects of substrate properties on cell behaviors, hiPSCs were cultivated on different ECM surfaces at concentrations ranging from 0.25 to 1.0 $\mu\text{g}/\text{cm}^2$ for LN511, LN521, VTN and 8.3–33.1 $\mu\text{g}/\text{cm}^2$ for MG. In these cultures, cell viability was similar among ECM proteins, hardly attaining 70% (Fig. 2A). There were no significant differences in cell viability among the ECM proteins tested. It was also confirmed that cells exhibited a normal growth rate without loss of cell viability.

To investigate the effects of ECM protein-coated surfaces on cell behaviors, we employed time-lapse analysis to study the behavior of hiPSCs during colony formation. In all cultures, after inoculation, most cells started to adhere to the surface and exhibited changes in their morphology within the first few hours (Movie S1). As culture time elapsed, cell division promoted clustering, leading to the development of cell colonies. Simultaneously, active migration was observed to cause coalescence between cells. Cells on LN521 and MG-coated surfaces appeared to actively migrate, while those seeded on LN511 and VTN-coated surfaces appeared to migrate more slowly. For migrating cells on LN521 and MG-coated surfaces, it was observed that some colonies originated from multiple cells or from colonies that merged to form a single colony. Thus, colonies were distinguished as either clonal colony or non-clonal colony. Fig. S2 shows time-lapse images of typically selected biological cell events during the course of cultivation on LN521. To illustrate the range of cell behaviors, we focused on four aspects: no split, cell split, colony split, and the merging of two or more cells (designated “coalescence”).

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.reth.2018.10.004>.

To compare rates of cell migration on different ECM surfaces of varying concentrations, migration rates were estimated using cell images captured over 72 h. Migration rates on LN521 and MG were relatively higher than those on LN511 and VTN surfaces (Fig. 2B). Despite wide heterogeneity in cell migration rates with respect to ECM type, as observed from the box plots, rates observed on LN521 and MG were above 10 $\mu\text{m}/\text{h}$ for dilute ECMs, with the highest migration rate observed on LN521 protein-coated surfaces at 0.25 $\mu\text{g}/\text{cm}^2$. The migration rate of cells increased noticeably with decreasing ECM concentration in coating solutions.

3.3. Correlation between migratory behavior and colony formation pattern on ECM protein substrates

To investigate the effect of migratory behavior on colony-forming patterns, we directly compared the migration rates and efficiency of colony formation on different ECM surfaces with varying concentrations of coating solution. The efficiencies of overall-colony formation originating from single cells on surfaces coated with LN521 and MG were higher than on LN511 and VTN, but considerably lower than the efficiencies of clonal colony formation (Fig. 3A).

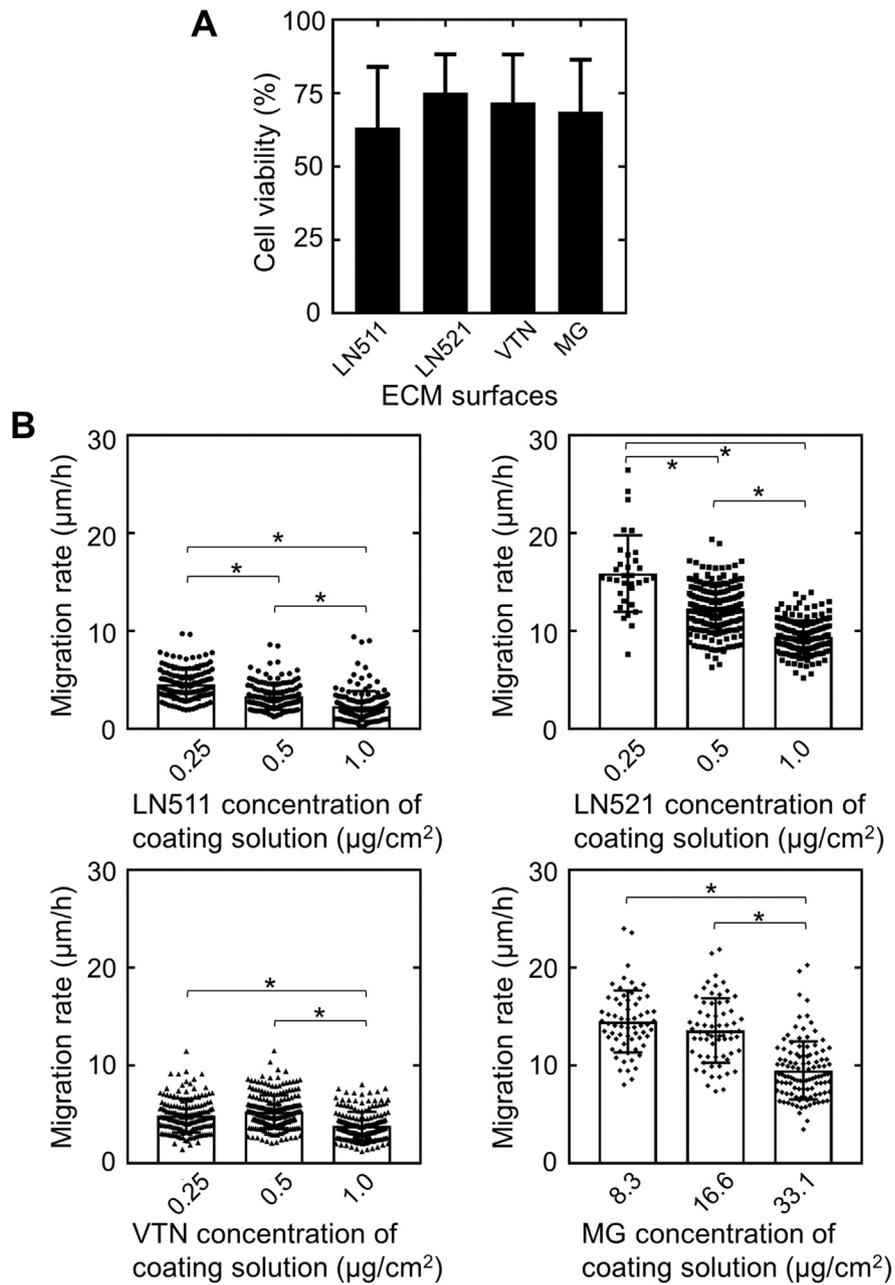


Fig. 2. (A) Cell viability of hiPSCs cultured on different ECM surfaces (0.25, 0.5, 1 µg/cm² for LN511, LN521, VTN or 8.3, 16.6, 33.1 µg/cm² for MG). Bars represent means ± SD from 23 to 36 individual wells (B) Cell migration rate of hiPSCs cultured on different ECM surfaces with varying concentrations of coating solution. Each data point represents the average migration rate of one trajectory. Bars represent the means ± SD from 33 to 239 cells in 5 or more independent wells (*p < 0.05 by one-way ANOVA followed by Tukey's post-hoc multiple comparisons test).

To clarify the correlation between migration rate and colony formation, the efficiencies of overall colony formation and clonal colony formation were plotted against migration rates (Fig. 3B). Although the efficiency of overall colony formation was not affected by migration rate, the efficiency of clonal colony formation decreased with increased cell migration. There was a positive correlation ($r = 0.6782$) between efficiency of overall colony formation and migration rate, while there was a negative correlation between efficiency of clonal-colony formation and migration rate ($r = -0.5337$). ECM substrates such as LN521 and MG allowed for high migration of hiPSCs, leading to more occurrences of coalescence and non-clonal colonies.

To determine whether there was a correlation between biological events associated with migration rate during clonal-colony formation, the frequencies of events were plotted against average migration rate for all culture conditions in Fig. 3. All trajectories of clonal colonies were tracked and the biological events were divided into three categories: (i) cell split; (ii) colony split; (iii) no split. Fast migrating cells tended to split after the first cell division (“cell split”) or even after colonies were formed (“colony split”). However, slowly migrating cells tended to form single colony without separating after cell division (“no split”). With increased cell migration on all culture conditions, regardless of ECM type and concentration, single hiPSCs exhibited more unpredictable colony formation

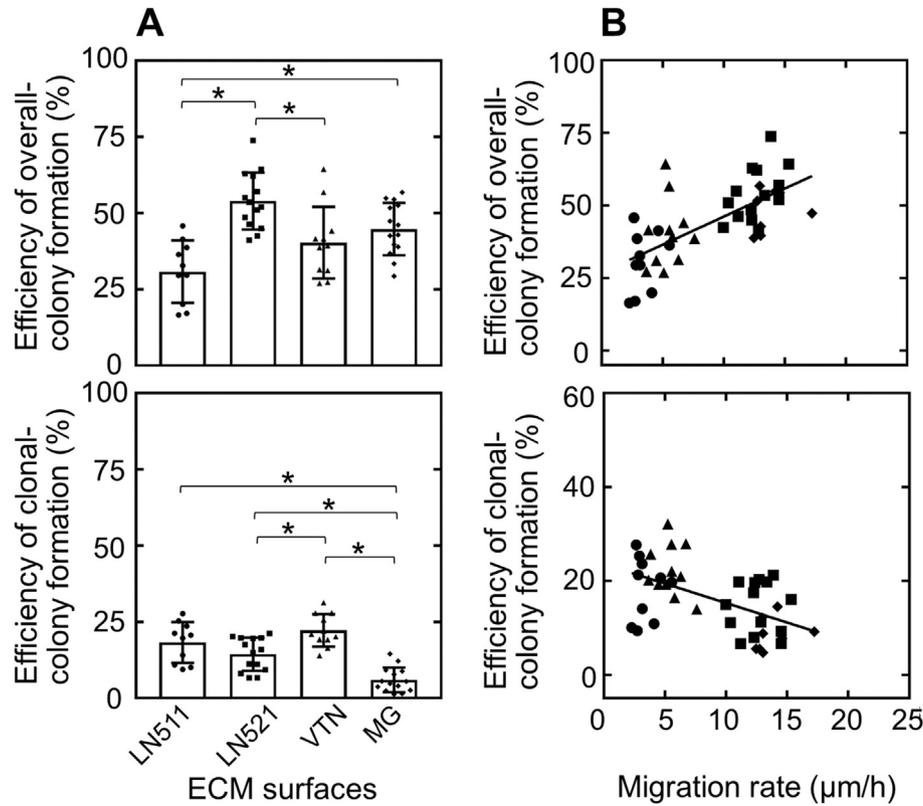


Fig. 3. (A) Migration rate and efficiencies of overall or clonal colony formation of hiPSCs cultured at 1000 cells/cm² on different ECM surfaces (0.5 μg/cm² for LN511, LN521, VTN or 16.6 μg/cm² for MG). Each point indicates the cloning efficiency from one well, and bars represent means ± SD from 10 to 14 wells (**p* < 0.05 by one-way ANOVA followed by Tukey's post-hoc multiple comparisons test) (B) Correlation between migration rates and efficiencies of overall or clonal colony formation. Migration rate for all ECM groups were plotted against the efficiencies of overall or clonal colony formation. Data points are representative of the average migration rate of all cells in a well (average migration rate from wells with 4 or more cell trajectories were included in the graph). Symbols: closed circle, LN511; closed square, LN521; closed triangle, VTN; closed diamond, MG. Correlation between efficiency of overall colony formation and migration rate, *r* = 0.6782; correlation between efficiency of clonal-colony formation and migration rate, *r* = -0.5337.

patterns, accompanied by more frequent incidences of cell split and colony split events (Fig. 4). Pearson correlation analysis was performed by comparing migration rates and the frequencies of each event for all culture conditions. For correlation analysis on culture conditions expect substrates coated with MG, strong and positive correlation was found between migration rates and frequencies of events (no split, cell split, and colony split; *r* = -0.975, 0.943, and 0.936, respectively). This is of great importance as the undefined nature of MG may lead to considerable fluctuations between migration rates and the colony formation pattern. These results indicate that the ECM surface regulated the migratory ability in such a way as to maintain higher clonal colony formation by hindering migration and the splitting of cells and colonies.

3.4. Cytoskeletal formation and E-cadherin expression

To examine cytoskeletal organization and focal adhesions occurring with each ECM protein-coated surface, cells were observed 72 h after seeding by fluorescence staining of F-actin and paxillin. As shown in Fig. 5A, cells at colony peripheries on LN521- and MG-coated surfaces show more lamellipodia and filopodia, compared with LN511 and VTN-coated surfaces. They were abundant in F-actin filaments of peripheral stress fibers and lamellipodia. In addition, many puncta positive for paxillin, a focal adhesion protein, appeared in both the cytoplasm and cell periphery. Cells cultured on LN521 and MG-coated surfaces exhibited a thin rim of F-actin at the cell periphery and numerous large puncta-like structures likely corresponding to focal adhesions. In contrast,

cells plated on LN511 and VTN-coated surfaces exhibited only a thin rim of F-actin staining and only a few small puncta positive for paxillin at the cell periphery.

Sequentially, we examined the effects of ECM surfaces on the localization of E-cadherin expression (Fig. 5B). Cells residing within the colony interiors on LN511- and VTN-coated surfaces exhibited a continuous line at boundaries between neighboring cells. However, cells cultured on LN521 and MG showed a discontinuous and fragmented E-cadherin staining pattern, compared with patterns observed on LN511- and VTN-coated surfaces. Cells residing at the colony periphery on MG-coated surfaces also showed loss of E-cadherin expression. Thus, cell-staining analyses revealed that the ECM surface affects the formation of F-actin filaments used to organize filopodia and stress fibers, as well as cell-cell contacts. In addition, LN521- and MG-coated surfaces enhanced the development of stress fibers with distinct paxillin puncta.

We further evaluated the temporal expression of gene for the cell-adhesion molecules (integrin β1 and paxillin) and cell-cell adhesion molecule (E-cadherin) using qRT-PCR analysis. At 24 h, gene expression levels of integrin β1 and paxillin as well as E-cadherin in cells grown on VTN- and MG-coated surfaces was relatively lower relative to cells grown on LN511-coated surface (Fig. S3). However, their expression exhibited expression changes in opposite patterns at the end of culture at 120 h. The expression differentiations in cells grown on VTN- and MG-coated surfaces between 24 h and 120 h were relatively high compared with LN511-coated surface. These findings support the hypothesis that alteration of cell migration within hiPSC colonies cultured on ECM

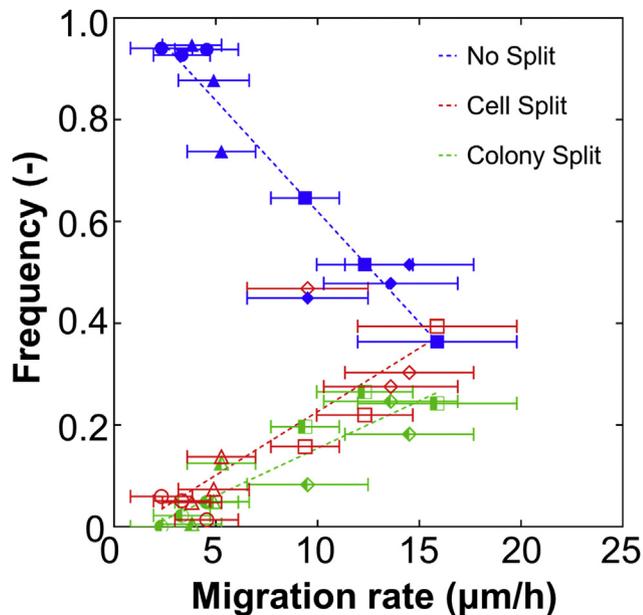


Fig. 4. Correlation between migration rate and frequencies of each event. Average migration rate for cells seeded at 1000 cells/cm² was plotted against the frequency of split events on different ECM surfaces. Each data point represents the average migration rate and frequencies of events. For average migration, each data point represents the average migration of 33–239 cell trajectories at a single ECM concentration from at least 5 independent wells. Corresponding frequency of split events (no split, cell split, colony split) represent the total rate of split events at each ECM concentration. Error bars represent the SD of the means of migration rate at each tested ECM concentration. Symbol colors: blue, no split; red, cell split; green, colony split. Symbols: circle, LN511; square, LN521; triangle, VTN; diamond, MG. Symbol filling: closed, no split; open, cell split; semi-closed; colony split. Symbol colors: blue, no split; red, cell split; green, colony split. Correlation between migration rate and frequencies of each event (excluding MG): no split, $r = -0.975$; cell split, $r = 0.943$; colony split, $r = 0.936$.

surfaces is associated with the temporal and spatial differences in integrin-mediated cell-substrates and E-cadherin mediated cell–cell contacts.

4. Discussion

4.1. ECM surface-driven migratory behavior leads to changes in hiPSC colony-forming patterns

Understanding how cell behaviors are controlled by extracellular cues is critical for better cell manipulation and for the translation of stem cell technologies to industrial application. The dynamic behavior of stem cells is highly related to the maintenance of undifferentiated hiPSCs, as this overall phenomenon involves cell adhesion, migration, and interactions between cells [6,11,12]. In this study, we focused on the behavior of single hiPSCs and their pairs as a first step towards understanding how a hiPSC colony is formed. This was achieved by performing behavioral analyses, including measurements of survival and migration rates during colony formation, as well as within hiPSC colonies. When hiPSCs were cultured on any ECM protein-coated surface, most cells adhered to the surface before they then began to move and divide (Movie S1 and Fig. S2). Some cell clusters began to coalesce or split into single cells or small clusters through active migration. We found that changes in cell behavior and hiPSC colony formation occurred through altered migration on the ECM surface, which varied with the concentration of the coating solution. In particular, cells with lower migration capacity on LN511 and VTN-coated surfaces

exhibited less loss of cell–cell contact between cells and colonies throughout the culture period, and a higher efficiency of clonal colony formation compared cells cultured on LN521 and MG-coated surfaces (Figs. 3 and 4). In addition, high proliferation rates with correspondingly low migration rates could cause clonal colony formation, indicating that colony derivation from a single cell facilitates cell–cell contact and promotes clonal proliferation.

The cell interaction with the ECM and with neighboring cells plays a central role in the regulation of cell behavior [21–26]. However, it is unknown how cellular mechanisms involved in individual cell migration are affected by ECM surfaces and whether integrin–substrate interaction is the only mechanism supporting cell migration during colony formation. Cell attachment appears to be accompanied by actin cytoskeleton organization with the formation of focal adhesions between the cell and its substrate [27–32]. This disrupts the actin cytoskeleton during migration, limiting the intracellular signaling pathways responsible for cell–cell adhesion formation [32]. For example, loss of E-cadherin expression in hPSCs, and other epithelial cells leads to abrogation of cell–cell interaction and increased motility, resulting in loss of cortical actin cytoskeleton arrangement and induction of cell polarization. In hPSCs that generate firm cell–cell interactions, aggregate formation is a competition between cell–cell versus cell–substrate interactions. Cells tend to form an aggregate when the cell–cell interaction is dominant over the cell–substrate interaction [32]. If the cell–cell interaction is stronger than the cell–substrate interaction, cells tend to remain aggregated when contractility increases. If contractility increases dramatically and cells to not detach from each other, retraction will occur. Because migration, cell–substrate interactions, and cell–cell interactions are important for the disassembly and assembly of cell aggregates, the ECM plays a key role in regulating scattering and clustering. The ECM determines cell migration rates, which can act to cause loss of cell–cell interactions. This splitting can proceed by altering the expression of the cell–cell adhesion molecule E-cadherin, or through signaling or cytoskeletal events, usually by altering the relative magnitudes of cell–cell or cell–substrate interactions [31,32]. In addition to these chemical inputs, mechanical inputs, such as the stiffness of the ECM substrate, can regulate traction force and determine whether cells split. In this study, we examined the characteristics of cytoskeletal formation and focal adhesions, as well as cell–cell contacts on different ECM surfaces. However, when we grouped cells according to migration rate and examined the characteristics of biological events during colony formation, we found that these characteristics differed between slow and fast moving cells. Slow cells exhibited a higher probability of maintaining colony formation, while fast cells had a higher probability of cell and colony splits occurring (Fig. S2 and Fig. 4). Additionally, cells with low migration rates exhibited many focal adhesions, while cells with high migration rates had few focal adhesions (Fig. 5). It was also found that the migratory behavior on ECM substrates affect cell–cell contact formation *via* E-cadherin. E-cadherin expression was seen for cells with low migration rates, resulting in continuous E-cadherin expression (Fig. 5), which contributed not only to the maintenance of strong cell–cell interaction but also to the blockage of nuclear β -catenin signaling [10]. This expression in cells with high migration rates was fragmented or lost from the membranes with only occasional weak intracellular staining. The binding of integrins to their respective ECM protein elicits intracellular signaling leading to the tyrosine phosphorylation of focal adhesion proteins and rearrangement of the actin cytoskeleton. The outside-in signaling triggered by this substrate–integrin interaction regulates cell–cell interaction or migration [28,29]. These results suggest that altered colony formation associated with migration on the ECM surface is responsible

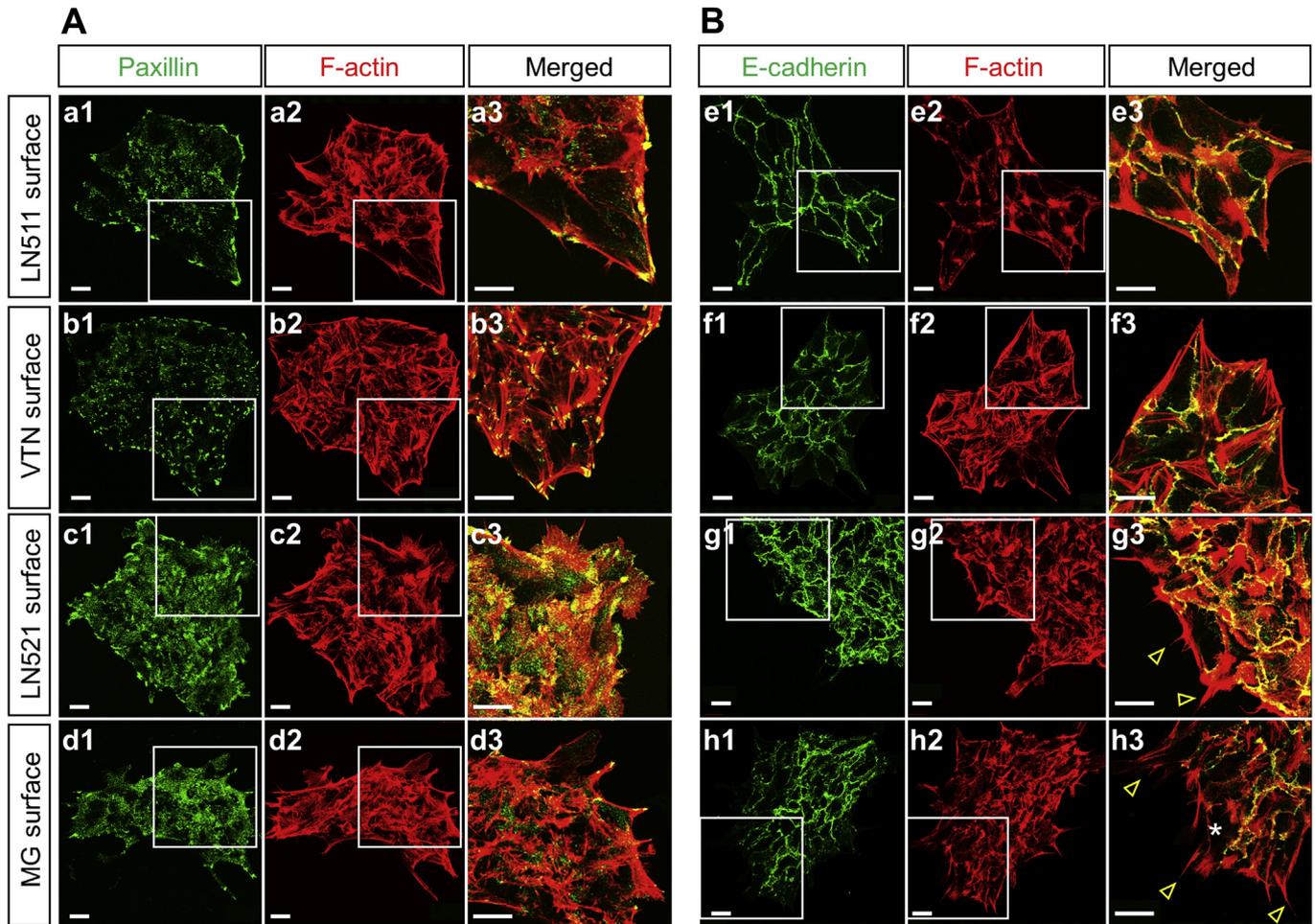


Fig. 5. (A) Immunostaining of paxillin (green) in hiPSC colonies cultured on different ECM surfaces at 72 h. F-actin was stained with rhodamine phalloidin (red). Panels a3-d3 show merged enlargements of demarcated box areas in panels a1-d1 and a2-d2, respectively. Scale bars show 20 μm (B) Immunostaining of E-cadherin (green) in hiPSC colonies cultured on different ECM surfaces at 24 h. F-actin was stained with rhodamine phalloidin (red). Panels e3-e3 show merged enlargements of demarcated box areas in panels e1-h1 and e2-h2, respectively. Arrowheads indicate the development of lamellipodia and filopodia at colony periphery. Asterisks indicate the loss of E-cadherin expression at colony periphery. Scale bars = 20 μm .

for coordinated regulation of the balance between cell–cell and cell–substrate interactions, thereby changing hiPSC colony formation patterns.

4.2. Cell-to-cell variability in ECM protein-derived migration influences heterogeneity within hiPSC cultures

By understanding how migration rates alters in response to ECM correlate with colony forming patterns, we can manipulate the migratory behaviors of cell through integrin and cadherin mediated interactions, providing a potential way to control migration in artificial matrices or during hiPSC culture. We observed considerable cell-to-cell variability with regard to the effect of ECM on cell migration rate and focal adhesion formation. Analyses of biological events associated with migration rate during clonal colony formation demonstrated stronger linear correlation upon exclusion of MG surfaces (Fig. 4). In addition, a significant increase in the variability of focal adhesions was observed on MG-coated surfaces; cells were intensely distributed at the colony periphery and fewer cell–cell interactions were observed (Fig. 5). It is noteworthy that LN511, LN521, and VTN are all defined, recombinant proteins, while MG is a complex protein mixture [13,14]. MG, which primarily consists of laminin, collagen IV, and enactin, is considered to be a reconstituted basement membrane preparation. In addition, MG

was previously reported to contain specific growth factors [13,14]. When a MG-coated surface was used, cell behavior was less predictable and harder to control, perhaps due to its complexity. These differences in architecture, biomechanical properties and composition of MG may induce fluctuations of cell behavior. Although we did not resolve such differences, it is possible that lower molecular weight ECM proteins allow for higher density of adhesion sites and, consequently, a higher density of integrin ligands on the substrate to permit cell adhesion. Importantly, such system properties can be revealed without the need for any perturbation by harnessing cell-to-cell variability; when enough single cells are quantified, correlations and causal interactions between properties can be inferred from the variability present within one cell population. Our study proposes a quantitative analytical method to evaluate temporal variations in the dynamic behavior of stem cells for assessing colony forming patterns during in vitro culture. This method allows for non-invasive characterization of hiPSC colonies, identification of cell fate history, and cell interaction with neighbors. This model provides a useful platform for testing the impact of culture media and culture substrate, and enables molecular studies of mutual cell interactions. Moreover, the developed methodology could be a useful tool for evaluating microenvironments for stem cell cultures in vitro. Such an analytical model may also provide the basis for understanding the relationship between cell behavior and kinetics,

which in turn, may pave the way toward more quantitative views of cell culture. These results can be extended to investigating cell behaviors emerging from hPSC differentiation, thus enabling wider applications for hPSC phenotyping during cell culture expansion, and differentiation.

5. Conclusion

In this study, we assessed migration rates during the hiPSC colony formation process by behavioral analysis of time-lapse images of individual cells on different ECM surfaces. We found that hiPSCs formed tight colonies on concentrated ECM substrates, while cells were more motile on ECM surfaces at dilute coating concentrations, with cells and colonies splitting into single cells or small clusters. Cells with higher migration rates on ECM surfaces experienced loss of cell–cell contacts and colony re-aggregation, resulting in lower efficiency of clonal colony formation. These results suggest that ECM proteins supporting low motility facilitate cell–cell contact and promote clonal proliferation. Thus, the results of our approach lead to a new understanding of the establishment of a supportive niche in hiPSC cultures, and the dynamic interplay between cell subpopulations in defined culture conditions.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2018.10.004>.

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