Maintenance of undifferentiated state of human induced pluripotent stem cells through cytoskeleton-driven force acting to secreted fibronectin on a dendrimer-immobilized surface

Mee-Hae Kim and Masahiro Kino-oka*

*Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka,
Suita, Osaka 565-0871, Japan*

*Correspondence: Masahiro Kino-oka, Ph.D.

Tel.: +81-(0)6-6879-7444

Fax: +81-(0)6-6879-4246

E-mail: kino-oka@bio.eng.osaka-u.ac.jp

Running title: Maintenance of hiPSCs on dendrimer surface

ABSTRACT

Understanding of the fundamental mechanisms that govern adhesive properties of human induced pluripotent stem cells (hiPSCs) to culture environments provides surface design strategies for maintaining their undifferentiated state during cell expansion. Polyamidoamine dendrimer surface with first-generation (G1) with dendron structure was used for co-cultures of hiPSCs and SNL feeder cells that formed tightly packed compact hiPSC colonies, similar to those on a conventional gelatin-coated surface. hiPSCs passaged up to 10 times on the G1 surface maintained their undifferentiated state. Immunostaining and RT-PCR analysis of fibronectin showed that the secreted fibronectin matrix from feeder cells on the G1 surface contributed to hiPSC attachment. Compared with cells on the gelatin-coated surface, F-actin and paxillin immunostaining revealed a well-organized network of actin stress fibers and focal adhesion formation at cell-substrate sites in hiPSC colonies on the G1 surface. E-cadherin expression levels on these surfaces were almost same, but paxillin and Rac1 expression levels on the G1 surface were significantly higher than those on the gelatin-coated surface. Zyxin showed prominent expression on the G1 surface at sites of focal adhesion and cell-cell contact in colonies, whereas zyxin expression on the gelatin-coated surface was not observed in regions of cell-cell contact. These findings indicate that transduction of mechanical stimuli through actin polymerization at sites of focal adhesion and cell-cell contact results in maintenance of undifferentiated hiPSC colonies on G1 surface. The G1 surface enables a substrate design based on the mechanical cues in the microenvironment from feeder cells to expand undifferentiated hiPSCs in long-term culture.

[**Keywords:** Human induced pluripotent stem cells; undifferentiation; mechanotransduction; cell-cell interaction; dendrimer surface]

INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) are genetically reprogrammed to an embryonic stem cell-like state by forced gene expression of factors that are important to maintain the defining properties of human embryonic stem cells (1-3). hiPSCs are characterized by their capacity to differentiate into cell types of all three germ layers (3). Because of their self-renewing and pluripotency, hiPSCs are a promising resource for many areas of research and medicine (1, 2).

Expansion of hiPSCs in an undifferentiated state remains challenging because spontaneous differentiation occurs during serial subcultures, resulting in the loss of their self-renewal and pluripotent qualities. Therefore, vigilant efforts to maintain the undifferentiated state are one of the major requirements. hiPSC culture requires the support of mouse or human fibroblasts for attachment and maintenance of pluripotency (4, 5). It is thought that feeder cells secrete various growth factors and extracellular matrices to support cell growth and maintenance of the undifferentiated state. Because signaling molecule responses are affected by interactions between the cells and the surrounding matrix, many studies have investigated the effects of the extracellular matrix secreted from feeder cells on the maintenance of undifferentiated hiPSCs during long-term culture (6). Recent reports have focused on the opportunity to tune the properties of synthetic substrates to better mimic the microenvironments of feeder cells for the long-term maintenance of undifferentiated hiPSCs *in vitro* (7-10). In this context, hiPSC culture has been advanced significantly by identification of essential growth factors and specific substrate coatings that allow for cellular growth, survival, and maintenance of the undifferentiated state (6, 8).

For the design of a synthetic culture surface to culture undifferentiated hiPSCs, many studies have been developed stem cell culture to mimic cell-cell and cell-matrix interactions and modulate stem cell self-renewal and differentiation characteristic of stem cell microenvironments (8-10). The strategies include influencing the cell-cell interactions and cellular interactions with the culture substrate through the physical properties of substrates, including topography, roughness, and elasticity (8). The transmission of mechanical forces from outside the cell through cell-substrate and

cell-cell interactions appears to control the maturation or disassembly of these adhesions and initiates intracellular signaling cascades that ultimately alter stem cell fate (6, 9). These studies have observed how stem cells respond to these cues and started to shed light on the pathways that dictate stem cell survival, self-renewal, and lineage commitment. The mechanisms by which mechanical cues from neighboring cells and the substrate activates the intracellular signaling pathways are widely studied in many cell types, but there have been few studies in hiPSCs.

In previous studies, we have investigated to design synthetic culture surfaces using a dendrimer substrate to create suitable microenvironments that regulate morphology and cellular function (11-14). Polyamidoamine dendrimer surfaces with dendron structure have been used to regulate the morphology and function of stem cells (11). In this study, we examined the morphological and functional responses of hiPSCs cultured on the dendrimer-immobilized surface. Moreover, fundamental mechanisms of cell and surface interaction are discussed in terms of cytoskeletal organization and focal adhesion formation to pluripotency maintenance of hiPSCs. The obtained results lead us to propose a synthetic culture surface to maintain the undifferentiated state of hiPSCs in long-term culture.

MATERIALS AND METHODS

Cells and culture conditions hiPSCs were provided from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (clone Tic, JCRB Number: JCRB1331). Routine hiPSC culture of were conducted in 0.1% gelatin-coated culture dish (surface area; 55-cm², Corning Costar, Cambridge, MA, USA) on a feeder layer of mitomycin C-treated SNL76/7 cells (European Collection of Cell Cultures, Salisbury, UK) using commercial medium (ReproStem, ReproCELL Inc., Tokyo) containing 5 ng/ml basic fibroblast growth factor. Cells were cultured at 37°C with 5% CO₂ atmosphere as described previously (15). Culture medium was replenished every day.

To subculture hiPSCs, the cells were washed once with phosphate-buffered saline (PBS, Sigma-Aldrich, MO, USA) and then incubated with CTK solution as described elsewhere (2).

1 hiPSC colonies in undifferentiated state were collected carefully by using cell scraper (Sumitomo
2 Bakelite Co., Ltd. Osaka). The suspension of collected undifferentiated colonies was pipetted gently
3 into small aggregates, and the suspension was dispersed into fresh culture vessel containing feeder
4 cells.

5 To prepare feeder layers, mitomycin C-treated SNL76/7 cells were cultured for 24 h in
6 Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 7% fetal bovine
7 serum (FBS, Invitrogen, Grand Island, NY, USA) with antibiotics (100 U/cm³ penicillin G, 0.1
8 mg/cm³ streptomycin and 0.25 mg/cm³ amphotericin B; all obtained from Invitrogen). Seeding
9 density was fixed at a viable cell concentration of 2.5×10^4 cells/cm².

10 **Cell culture on dendrimer surface** The first-generation dendrimer (G1) surface was
11 prepared using the conventional tissue culture polystyrene (PS) surface of a square 8-well plate
12 (surface area; 10.5 cm², Nunc, Roskilde, Denmark), as described previous report (10). Briefly,
13 hydroxyl groups were displayed on the PS surface by pouring potassium *tert*-butoxide into the wells.
14 Then, aqueous glutaraldehyde was introduced into the wells, with the wells were then treated with a
15 tris(2-aminoethyl) amine solution to produce a dendron structure. The wells were rinsed with sterile
16 water. To display glucose as a terminal ligand, D-glucose was applied.

17 For all experiments, at 1 day before plating hiPSCs, the SNL feeder cells were cultured on the
18 G1 surface, and then hiPSCs were plated on the conditioned G1 surface with feeder cells under
19 same conditions as those described above. Colonies deviated from undifferentiated state were not
20 removed before passaging the cells during serial passages.

21 **Immunofluorescence staining** The procedure used for immunofluorescence staining
22 was similar to a previously described method (12). Briefly, hiPSCs were fixed with 3.7%
23 paraformaldehyde (Wako Pure Chemical Industries, Osaka) for 10 min at room temperature and
24 rinsed with PBS, followed by soaking in PBS with 0.25% Triton X-100 for 4 min. After masking of
25 non-specific proteins by incubation in Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka)
26 for 1 h at ambient temperature, the cells were treated with a primary antibody at 4 °C overnight.

The cells were incubated with anti-Oct3/4 (Santa Cruz Biotechnology, CA, USA), anti-Nanog (Stemgent, San Diego, CA, USA), anti-SSEA-4 (Stemgent), anti-Tral-60 (Stemgent), anti-fibronectin (Santa Cruz Biotechnology), anti-paxillin (Millipore), and anti-zyxin (Abcam, Cambridge, MA, UK) 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 (Molecular Probes, Eugene, OR, USA) for 1 h. F-actin and cell nuclei were stained with rhodamine phalloidin (Molecular Probes) and DAPI (4',6-diamidino-2-phenylindole), respectively. Images were obtained using an image analyzer (In CELL Analyzer 2000; GE Healthcare, Buckinghamshire, UK) through 10× objective lens and confocal laser scanning microscope (Model FV-1000; Olympus, Tokyo) through 60× objective lens.

RNA isolation and reverse transcriptase PCR (RT-PCR) analysis RNA isolation, cDNA synthesis, and PCR analysis were conducted as described previously (16). Total RNA were isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and TRIzol (Invitrogen). One µg of total RNA was then reverse transcribed using SuperScript II Reverse Transcriptase kit (Invitrogen). The qPCR assays were conducted using SYBR Premix Ex Taq (Takara Shuzo, Shiga, Japan), and contained 1 µl of cDNA per 25 µl of reaction mixture. Assays were carried out on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative quantity of the target transcript was estimated by a standard curve, and the data were standardized based on the GAPDH expression. 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](#).

RESULTS

Long-term maintenance of undifferentiated hiPSCs Morphologies of hiPSCs and their colonies cultured on the G1 and gelatin-coated surfaces with feeder cells for 5 days were compared

as indicated in Fig. S1. Growth on gelatin was used as a reference condition to compare the growth and maintenance of hiPSCs on the G1 surface. Similar to undifferentiated hiPSCs cultured on the gelatin-coated surface, hiPSCs on the G1 surface grew as tight colonies with cells densely packed together. hiPSCs on the G1 surface maintained their characteristic morphology after 10 passages (50 days).

To confirm the phenotypes of hiPSCs cultured on the G1 and gelatin-coated surfaces in serial passages for long-term culture, we performed immunostainings of pluripotency markers, Oct3/4, Nanog, SSEA-4, and Tra1-60 of hiPSCs after 10 passages. As shown in Fig. 1A, the colonies passaged on the G1 or gelatin-coated surfaces were strongly positive, and the majority of cells in the colonies were stained for these markers.

Fig. 1

We also tested the hiPSCs from the G1 and gelatin-coated surfaces with feeder cells for the expression levels of pluripotency and early differentiation markers, by RT-PCR analyses after 1 passage and 10 passages. The gene expression of pluripotency markers (Oct3/4, Nanog, Sox2) in hiPSCs at 1 passage and 10 passages on the G1 surface was detected, similar to those in cells cultured on gelatin-coated surface (Fig. 1B1). In addition, the ectoderm (SOX1, NEUROD1), mesoderm (MESP1, T gene, EOMES), and endoderm (GATA4, GATA6, SOX17) differentiation markers were not detected in all culture conditions. Subsequent quantitative RT-PCR analyses demonstrated that three pluripotency markers did not show significant difference in mRNA levels (Fig. 1B2). These results indicate that the hiPSCs passaged up to 10 times on the G1 surface acquired the stable characteristics of undifferentiated cells, similar to those on gelatin-coated surface.

Fibronectin secretion from feeder cells on the dendrimer surface To verify the matrix organization of secreted fibronectin from feeder cells and their expression levels during hiPSC culture, cells cultured on the G1 and gelatin-coated surfaces with feeder cells for 5 days were analyzed by immunostainings and RT-PCR analysis. As shown in Fig. 2A, fibronectin secreted on the G1 surface had a diffuse distribution in distinct spots over the entire cells, similarly to those on

Fig. 2

gelatin-coated surface. Additionally, the comparison for the expression level of fibronectin did not show significant difference in fibronectin mRNA levels (Fig. 2B).

Cytoskeletal organization accompanied by focal adhesion formations of hiPSCs To assess the cytoskeletal organization accompanied by focal adhesion formation of hiPSCs, cells cultured on the G1 and gelatin-coated surfaces with SNL feeder cells for 5 days were immunostained for F-actin and paxillin, a focal adhesion protein. As shown in Fig. 3, the circumferential actin bundles were well recognized in the apical side of hiPSC colonies, and the staining patterns were similar on the two surfaces. However, many stress fibers appeared in the basal side of central and peripheral regions in hiPSC colonies on the G1 surface, and some stress fibers stretching across the cytoplasm were developed, compared with those on the gelatin-coated surface. Moreover, paxillin was diffusely distributed through the cytoplasm of cells in the basal side of colonies on the G1 surface, especially at the ends of stress fibers with intensive staining in the longitudinal direction in a scattered manner (Fig. 3D3). hiPSCs on the G1 surface showed increased paxillin staining of large adhesions, compared with those on the gelatin-coated surface. These observations indicate enhanced actin filament networks and focal adhesion formation of hiPSCs when they are grown on the G1 surface.

We also examined the expression levels of paxillin, Rac1, and E-cadherin in hiPSCs cultured on G1 and gelatin-coated surfaces with feeder cells for 5 days by western blot analysis. As shown in Fig. 4, the expression levels for paxillin and Rac1 in cells cultured on the G1 surface were significantly higher than those in cells cultured on the gelatin-coated surface. Furthermore, E-cadherin protein levels showed no significant difference in cells on the G1 and gelatin-coated surfaces.

To investigate the mechanical properties in hiPSCs, we examined the distribution of zyxin, a cytoskeletal protein, in hiPSCs cultured on the G1 and gelatin-coated surfaces with feeder cells. As shown in Fig. 5, zyxin expression on the G1 surface appeared to localize primarily at the sites of focal adhesion and cell-cell contacts in both the central and peripheral regions of colonies. However,

zyxin expression on the gelatin-coated surface was found only in the basal side with small scattered spots of staining. By comparing G1 and gelatin-coated surfaces, we found that zyxin expression was much stronger in cells cultured on the G1 surface.

DISCUSSION

Adhesion of hiPSCs to the dendrimer surface is facilitated by secretion of fibronectin matrix from feeder cells

It has been well documented that the *in vitro* expansion of hiPSCs relies on cell-substrate interaction that occurs between integrin and extracellular adhesion protein. Maintenance of the undifferentiated state and self-renewal of hiPSCs requires a co-culture environment in monolayer expansion, which is achieved by culturing on a layer of feeder cells (5). The secreted growth factors and extracellular matrices from feeder cells serve dual roles to support the attachment and maintenance of undifferentiated hiPSCs. Once feeder cells are attached to the culture surface, they begin to secrete and remodel extracellular matrix proteins, such as laminin, fibronectin, and vitronectin, to which integrin bind. One protein of particular interest to determine the relationship between cells and their substratum is fibronectin, because it is one of the major molecules that mediate cell attachment (16). Fibronectin might be affected by substrata that alter the distribution of cytoskeletal elements. This activity may provide fibronectin with the ability to mediate cell-substrate and cell-cell interactions. The cell-substrate and cell-cell interactions activate specific mechanotransduction pathways that control cell survival, self-renewal, and lineage commitment (17, 18).

In our previous study, we found that cell and colony morphology of hiPSCs associated with altered migration on the dendrimer surfaces were responsible for the coordinated regulation of balance between cell-cell and cell-substrate interactions, thereby switching their transition from self-renewal state to early endoderm differentiation in hiPSCs (14). hiPSCs cultured on the G1 surface for 5 days were maintained in an undifferentiated state, while those on the G5 surface exhibited the early commitment to differentiation toward endodermal fates. In this study, the G1

1 surface was used for co-cultures of hiPSCs and feeder cells during serial subcultures. hiPSCs on the
2 G1 surface were stably maintained for up to 10 passages without loss of their typical hiPSC
3 morphology, similar to those on a conventional gelatin-coated surface (Fig. 1). To determine the
4 effect of substratum surface topography on hiPSCs colony formation and changes in cell
5 morphology on the major extracellular adhesive proteins, we investigated the organization and
6 expression of fibronectin at protein and mRNA level. Fibronectin on G1 surface had a diffuse
7 distribution over the entire cells in the form of spots, similarity to those on gelatin-coated surface
8 (Fig. 2A). There are no significant differences in fibronectin mRNA levels (Fig. 2B). These findings
9 suggest that colony formation of hiPSCs on a G1 surface used as a synthetic substrate is regulated
10 by the actin cytoskeletal architecture and focal adhesion formation through feeder cells associated
11 with fibronectin.

12 **Cytoskeleton-driven force acting through focal adhesion sustains the undifferentiated**
13 **state of hiPSCs effectively during long-term culture**

Adhesion of stem cells to the
14 underlying culture surface modulates cell growth and differentiation through signaling
15 reorganization of the actin cytoskeleton (17, 18). The interaction between integrin and an intact
16 cytoskeleton as well as the assembly of actin stress fibers and cell-generated tension are all
17 thought to be important factors regulating cell fate decisions (19). Formation of the
18 integrin-mediated adhesions regulated focal adhesion-associated signaling molecules, paxillin and
19 zyxin, to coordinate spatiotemporal activation of a member of Rho family GTPases, Rac1 and their
20 associated effectors to regulate cell migration (19-21). Paxillin and zyxin have been suggested to
21 mediate an important subset of cellular responses to force during migration (22, 23). They localize
22 to the focal adhesions of adherent cells and localize to focal adhesion and cell-cell contacts to
23 regulate cytoskeletal organization and cell motility (23, 24). Transmission of forces from outside
24 the cell through cell-substrate and cell-cell contacts appears to control the maturation or
25 disassembly of these adhesions and initiates intracellular signaling cascades that ultimately alter
26 many cellular behaviors. In response to externally applied forces, cells actively rearrange the

organization and contractile activity of the cytoskeleton and redistribute their intracellular forces (23, 25). The cytoskeleton has been shown to bind to factors that control gene transcription (21-23). These studies have provided our current understanding of the focal adhesion mechanism to culture surfaces that functions as an adaptor protein to recruit diverse cytoskeleton and signaling proteins into a complex.

In this study, we showed that the secreted fibronectin matrix on the G1 surface was tightly coupled to events that play an important role not only in signaling at cell-substrate adhesions but also at the cell-cell contacts of hiPSCs (Fig. 2). In addition, we found that the cells cultured on the G1 surface had many stress fibers and focal adhesions with paxillin compared with those of cells on the gelatin-coated surface (Fig. 3). Western blot analyses of the expression levels of paxillin, Rac1, and E-cadherin revealed an increase of paxillin and Rac1 in the cells cultured on the G1 surface, but the E-cadherin protein level showed no significant difference in the cells on the G1 and gelatin-coated surfaces (Fig. 4). Our results suggest that the G1 surface induces increased turnover of focal adhesion to promote migration during hiPSC colony formation. Zyxin in cells on the G1 surface appeared at sites of cell-substrate and cell-cell contacts of hiPSCs in colonies on the G1 surface (Fig. 5C and 5D), whereas the zyxin on gelatin-coated surface could only be detected in these location at cell-substrate adhesion (Figs. 5A and 5B). It is noteworthy that zyxin shuttles between the sites of cell-substrate adhesion and cell-cell contacts of hiPSCs in colonies on the G1 surface. We propose that zyxin may regulate whether cell migration occurs independently in individual cells or is coordinated inside colonies that maintain balance between cell-substrate and cell-cell interactions. Zyxin is an alpha-actinin and stress-fiber-binding protein found in mature focal contacts (23, 24). Furthermore, zyxin has been shown to bind to factors that control gene transcription and they function as regulatory domains for controlling protein-protein interactions with components involved in cell-cell interaction. Our results suggest that maintenance of undifferentiated hiPSCs on the G1 surface is regulated by the well-organized actin cytoskeletal architecture through focal adhesion formation, which in turn elicit differential contractile forces and

1 adhesion to stimulate cell migration. An important consideration in hiPSC culture is that cell-cell
2 contractility within growing hiPSC colonies may change local region inside colonies, which may
3 affect maintenance of undifferentiated state. When the hiPSCs grown on the G1 and gelatin-coated
4 surfaces were compared, cells on the G1 surface are facilitated by mechanical forces acting through
5 zyxin with respect to attachments of the actin cytoskeleton to regions of cell-substrate and cell-cell
6 contacts. The effects of mechanical signaling through cytoskeleton in hiPSCs on the G1 surface
7 indicate that modulation of zyxin expression is sufficient to sustain their undifferentiated state. The
8 mechanisms by which mechanical cues from neighboring cells and the substrate facilitate the
9 spreading and self-renewal of hiPSCs are reported in other study. Jin et al. show that hiPSCs can be
10 maintained undifferentiated state on the synthetic peptide engineered surface (26). They showed
11 that the cytoskeleton characterization of hiPSCs grown on the synthetic peptide substrate revealed
12 formation of denser actin filaments and up-regulation of zyxin expression in the cell-cell contacts. It
13 is likely to interact with transcription factors to regulate gene expression associated with
14 maintenance of undifferentiated hiPSCs and its differentiation capacity (18, 27). While the
15 mechanism of these changes in cell cytoskeletal formation is unclear, it may still indicate a
16 reorganization of focal adhesions, which facilitates the spreading and self-renewal of hiPSCs on G1
17 surface.

18 In conclusion, the G1 surface can be used as an artificial substrate to support *in vitro* expansion
19 of hiPSCs while maintaining their undifferentiated state. hiPSCs cultured on the G1 surface formed
20 tight compact colonies that showed maintenance of undifferentiated characteristics similar to those
21 of cells cultured on gelatin-coated surface. We have shown that feeders-secreted fibronectin on G1
22 surface enhanced the availability of integrin-mediated adhesion of hiPSCs with enhancement of
23 actin polymerization at sites of focal adhesion and cell-cell contact. In addition, the cytoskeletal and
24 focal adhesion formation on this surface is responsible for the increased zyxin expression involved
25 in transducing mechanical stimuli, thereby facilitating maintenance of undifferentiated state of
26 hiPSCs in long-term culture. The applied culture surface may be a useful alternative to culture

hiPSCs in an undifferentiated state.

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Figure legends

FIG. 1. Maintenance of undifferentiated hiPSCs by passaging colony cells on the G1 and gelatin-coated surfaces. (A) Immunostainings of four of pluripotency markers, Oct3/4, Nanog, SSEA-4 and Tra1-60 in hiPSCs cultured on G1 and gelatin-coated surfaces after 10 passages. Scale bars represent 250 μm . (B) Conventional RT-PCR (B1) and quantitative RT-PCR (B2) analyses for three markers of undifferentiated stem cells (Oct3/4, Nanog, Sox2) and early ectoderm (SOX1, NEUROD1), mesoderm (MESP1, T gene, EOMES), and endoderm (GATA4, GATA6, SOX17) differentiation markers. In quantitative RT-PCR analysis, closed and open bars show G1 and gelatin-coated surfaces, respectively. The data were obtained from three independent experiments. Statistical significance was according to Student's *t*-test ($*p < 0.01$).

FIG. 2. Secreted fibronectin from feeder cells co-cultured with hiPSCs on G1 and gelatin-coated surfaces for 5 days. (A) Immunostaining of fibronectin (green, A1 and A3) derived from feeder cells in central region of hiPSC colonies. Cell nuclei were stained with DAPI (blue, A2 and A4). Scale bars show 50 μm . (B) Conventional RT-PCR (upper panel) and quantitative RT-PCR (lower panel) analyses of fibronectin expression of feeder cells co-cultured with hiPSCs on G1 and gelatin-coated surfaces. In quantitative RT-PCR analysis, the data were obtained from three independent experiments, and analyzed using the Student's *t*-test. There are no statistical significances among the data sets.

FIG. 3. Immunostaining of paxillin (green) in hiPSC colonies cultured on G1 and gelatin-coated surfaces for 5 days. F-actin and cell nuclei were stained with rhodamine phalloidin (red) and DAPI (blue). Scale bars show 50 μm . Panels A-D show the three-dimensional images in of hiPSCs. Panels A2-D2 show merged enlargements of demarcated box areas in A-D, respectively. Panels A3-D3 show the side (cross-section) views in A2-D2, respectively. Feeder cells are indicated by the asterisk.

FIG. 4. Western blot analysis of paxillin, Rac1, and E-cadherin of hiPSCs cultured on G1 and

gelatin-coated surfaces for 5 days.

FIG. 5. Immunostaining of zyxin (green) in hiPSC colonies cultured on G1 and gelatin-coated surfaces for 5 days. Cell nuclei were stained with DAPI (blue). Scale bars show 50 μm . Panels A and B show merged images of A1 and A2, and B1 and B2, respectively. Panels C and D show merged images of C1 and C2, and D1 and D2, respectively.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5