Nanotopography Influences Adhesion, Spreading, and Self-Renewal of Human Embryonic Stem Cells

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cells that adhere to the extracellular matrix (ECM) can sense and respond to a wide variety of chemical and physical features of the adhesive surface, including the molecular nature of adhesive ligands and their local densities, as well as surface topography and rigidity.1–8 Topological sensing, the capacity to differentially respond to substrates with varying degrees of topology, is a general phenomenon affecting multiple cellular properties including morphology, migratory capacity, gene expression profile, and, eventually, cell differentiation and fate.9,10 Recent reports have shown that the nanoscale surface topography plays a critical role in regulating survival, proliferation, and differentiation of various types of adult multipotent stem cells.11–16 For example, human mesenchymal stem cells (hMSCs) cultured on nanoscale gratings on polydimethylsiloxane surfaces tend to align and elongate their actin cytoskeleton (CSK) and nuclei along the nanogratings.13 Likewise, gene profiling and immunostaining of these hMSCs demonstrate a significant upregulation of neuronal markers when cultured on the nanogratings, as compared to unpatterned flat controls.13 Recent published reports have also demonstrated that the nanoscale disorder in a nanopit array can stimulate osteogenesis of hMSCs in the absence of osteogenic supplements,12 while another study from the same group verifies that the perfectly ordered arrays of nanopits support long-term maintenance of hMSC phenotype and multipotency.15 Only in the past few years has experimental evidence begun to emerge showing that pluripotent stem cells such as embryonic stem cells (ESCs) are sensitive to the cell–ECM physical interactions,17–23 including their intrinsic sensitivity to the nanoscale surface topography.22,23 These early findings have promoted significant interest from the developing fields of functional tissue engineering and regenerative medicine, as ESCs are able to replicate themselves (self-renewal) while retaining their ability to give

ABSTRACT

Human embryonic stem cells (hESCs) have great potentials for future cell-based therapeutics. However, their mechanosensitivity to biophysical signals from the cellular microenvironment is not well characterized. Here we introduced an effective microfabrication strategy for accurate control and patterning of nanoroughness on glass surfaces. Our results demonstrated that nanotopography could provide a potent regulatory signal over different hESC behaviors, including cell morphology, adhesion, proliferation, clonal expansion, and self-renewal. Our results indicated that topological sensing of hESCs might include feedback regulation involving mechanosensory integrin-mediated cell–matrix adhesion, myosin II, and E-cadherin. Our results also demonstrated that cellular responses to nanotopography were cell-type specific, and as such, we could generate a spatially segregated coculture system for hESCs and NIH/3T3 fibroblasts using patterned nanorough glass surfaces.

KEYWORDS: human embryonic stem cell · nanotopography · microfabrication · mechanosensitivity · self-renewal

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rise to any type of specialized cell in the adult body (pluripotency), and as such, human ESCs (hESCs) are a promising cell source for disease modeling and future cell-based therapeutics to treat degenerative human diseases such as diabetes mellitus and trauma such as spinal cord injury.

To date, the existing work on regulation of ESC behaviors using nanotopography has focused on their neurogenic differentiation with regularly arranged nanoscale surface structures. For example, it has been shown that the electrospun fibrous scaffolds can not only enhance differentiation of mouse ESCs (mESCs) into specific neural lineages but also promote and guide the neurite outgrowth.

More recently, nanoscale ridge/groove pattern arrays generated on polyurethane acrylate surfaces are shown to induce morphological changes and differentiation of hESCs into a neuronal lineage without treatment with differentiation-inducing biochemical agents.

However, all the aforementioned work on nanotopography for stem cells has largely relied on complex and expensive nanofabrication techniques, such as electron beam and nanoimprint lithography, to generate nanoscale surface structures to regulate cell–ECM interactions. Further, it is still largely unknown to what extent such synthetic regular nanoscale structures can mimic the intrinsic random nanoscale topology associated with the in vivo cellular microenvironment. Herein, we developed an effective microfabrication method for precise control and spatial patterning of local nanoroughness on glass substrates using standard photolithography and reactive ion etching (RIE) techniques. Using our RIE-generated nanorough glass surfaces, we demonstrated that the nanoscale surface roughness is a potent physical signal in the cellular microenvironment to regulate a diverse array of hESC behaviors, including their morphology, cell adhesion, self-renewal, and pluripotency. Our experimental results further suggested the involvement of integrin-mediated focal adhesion (FA), myosin II activity, and E-cadherin-mediated cell–cell contacts in regulating topological sensing of hESCs.

RESULTS AND DISCUSSION

Fabrication Method. In this work, the surface nanoroughness on the silica-based glass wafers (Borofloat 33 glass) was generated with RIE, a well-established process used in semiconductor microfabrication (Figure 1). The etching of the silica-based glass wafer was consistent with a process of the ion-enhanced chemical reaction and physical sputtering as reported by others. Specifically, during the RIE process, bombardment by the reactive ion species generated using the SF$_6$ and C$_4$F$_8$ gases disrupted the unreactive glass substrate and caused damage such as dangling bonds and dislocations, resulting in the glass surface being reactive toward the etchant species. Interestingly, since small concentrations of impurities such as Al, K, and Na (about 6% w/w in total) existed in the silica glass, these impurities resulted in accumulations of less volatile species (such as AlF$_3$, KF, NaF) on the glass surface during the RIE process. Thus, when the reactive plasma species approached the glass surface, these less volatile compounds inevitably underwent numerous collisions with the plasma species. Some of these compounds were then backscattered onto the glass surface and formed randomly distributed small clusters that could shield the glass surface from bombardment and reaction with reactive ions. These compound clusters effectively generated the so-called “micromasking” effect that could randomly shadow the glass surface and thus result in nanoscale roughening of the glass surface during the RIE process. Therefore, under the same RIE process conditions, the surface nanoroughness of the glass wafer could be precisely controlled on the nanoscale by solely adjusting the RIE process duration.

The initial surface roughness of the unprocessed silica-based glass wafers, characterized by atomic force microscope (AFM) using the root-mean-square (rms) roughness $R_q$, was about 1 nm. The glass wafers were processed with RIE (LAM 9400, Lam Research, Fremont, CA, USA) for different periods of time to generate...
nanorough surfaces with $R_q$ ranging from 1 to 150 nm (Figure 1A), using $SF_6$, $C_4F_8$, He, and Ar gas mixtures. To spatially pattern nanoroughness on the glass wafers, the glass wafers were first spin-coated with photore sist, and the photore sist layer was then patterned with photolithography to physically expose different glass regions of various sizes and shapes for subsequent RIE etching. After the RIE process, photore sist was stripped using solvents, and the glass wafers were cleaned using distilled water and a Piranha solution (4:1 $H_2SO_4/H_2O_2$) to remove organic residues from the glass substrates. Thus, by precisely controlling photolithography and RIE, we could specify the location, shape, area, and nanoroughness level of different nanorough regions on glass substrates (Figure 1A–C).

Functional Responses of hESC. Using the nanorough glass substrates described above, we first examined functional responses of hESCs to different levels of nanoroughness, including their morphology, adhesion, proliferation, clonal expansion, and differentiation. Here, all glass substrates were precoated with vitronectin (5 μg/mL) by adsorption to support long-term self-renewal of hESCs as reported by others. Using AFM, we confirmed that the rms roughness $R_q$ of the smooth and nanorough glass surfaces did not significantly change before and after vitronectin coating (Supplemental Figure S1A,B). To further confirm that the density of the adsorbed vitronectin on the glass surfaces was independent of the nanoroughness $R_q$ of the glass surface, control assays were performed. Using fluorophore-labeled proteins, no apparent difference in fluorescence intensity was observed between glass surfaces of different nanoroughness $R_q$, indicating constant protein densities on the glass surfaces (Supplemental Figure S1C,D).

Scanning electron microscopy (SEM) images of single hESCs demonstrated that hESCs exhibited distinct morphological features on smooth and nanorough glass surfaces 24 h after cell seeding. A highly branched, filopodia-rich morphology of single hESCs was observed on the smooth glass surface ($R_q = 1$ nm; Figure 1E, top) as compared to the more compact cells with few, short cytoplasmic extensions on the nanorough surface ($R_q = 150$ nm; Figure 1E, bottom). Additionally, hESCs demonstrated significant adhesion selectivity between different levels of nanoroughness on the glass surfaces. For example, after 48 h of culture on a glass surface patterned with square-shaped, smooth islands surrounded by nanorough surfaces, hESCs selectively adhered to, and aggregated on, the smooth islands where $R_q = 1$ nm, but not on the nanorough areas where $R_q = 70$ nm (Figure 2A). During this selective adhesion and aggregation process, hESCs retained their stemness, as evidenced by their positive expression of Oct3/4 (Oct3/4+), a transcription factor and a hallmark of undifferentiated hESCs (Figure 2A). Interestingly, spontaneously differentiated hESCs, which would lose their expression of Oct3/4 (Oct3/4−), did not show any adhesion preference to either the smooth or nanorough surfaces, and Oct3/4− hESCs would randomly distribute on the patterned glass substrate (Figure 2B).

We quantified the cell adhesion rate, defined as the ratio of number of cells adhered to the glass surface to the total number of cells initially seeded, of single Oct3/4+ hESCs plated on glass substrates with different values of $R_q$. Our results showed that 24 h after cell seeding, Oct3/4+ hESCs at the single-cell level had a strong tendency to adhere to smooth glass surfaces with smaller $R_q$ (Figure 2C). In contrast, Oct3/4+ hESCs adhered less to surfaces with an increasing $R_q$. Both phenomena were consistent regardless of treatment with or without Y27632, a Rho-associated coil kinase (ROCK) inhibitor used to enhance survival of single fully dissociated hESCs (Figure 2C).

The effect of topological sensing of hESCs on their self-renewal and stemness maintenance was also investigated. In the experiments, Oct3/4− hESCs were seeded at a low density ($5 \times 10^3$ cells/cm²) on both smooth ($R_q = 1$ nm) and nanorough ($R_q = 150$ nm) glass surfaces and cultured for 7 days. Subsequently, cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) to identify nuclei and fluorescent-labeled antibodies for Oct3/4 (Figure 2F). Quantification of Oct3/4− hESCs as a function of $R_q$ showed a higher percentage of Oct3/4− cells (93.6%) on the smooth glass surface ($R_q = 1$ nm) as compared to cells seeded on nanorough surfaces (41% and 36.6% for $R_q = 70$ and 150 nm, respectively; Figure 2D). Thus, our results indicated that the smooth glass surface was conducive to self-renewal and maintenance of hESC stemness in long-term culture, while the nanorough glass surfaces resulted in a large portion of hESCs undergoing spontaneous differentiation, thus losing their pluripotency (Figure 2D,F).

To investigate the extent to which nanoscale topological cues could affect proliferation of hESCs, small clusters of undifferentiated hESCs were seeded on glass substrates with different values of $R_q$ (1, 70, and 150 nm) and observed for 7 days. Phase-contrast microscopic images of hESC colonies were taken 1, 2, 3, and 5 days after cell seeding for these different glass substrates. Owing to their self-renewal, colonies of hESCs on these glass substrates would continuously grow during this 7-day observation period. Colony sizes were analyzed using these colony images with the image analysis software ImageJ to calculate the cell population doubling time. hESCs proliferated more rapidly with a shortened cell population doubling time of 41 h on the smooth glass surface ($R_q = 1$ nm) as compared to cells on the RIE-generated nanorough surfaces, on which the cell population doubling time was 71 h for $R_q = 70$ nm and 77 h for $R_q = 150$ nm (Figure 2E,G). Together, these results confirmed that
the nanotopological cue on the RIE-generated glass surface to which hESCs adhered could provide a potent regulatory signal that affects a diverse array of hESC behaviors including cell morphology, adhesion, proliferation, and self-renewal.

**Topological Sensing by FAs, CSK Structure, and Cell–Cell Contacts.** The molecular mechanism for topological sensing by adherent cells remains largely undetermined, yet existing evidence from different cell types has suggested the involvement of integrin-mediated focal adhesion signaling. Integrins mediate cell adhesion to the ECM and contribute to cell–matrix signaling by activating intracellular tyrosine kinase and phosphatase signaling to elicit downstream biochemical signals important for regulation of gene expression and stem cell fate. Importantly, integrin-mediated FA signaling is closely related to its molecular arrangement and dynamic organization, which can be affected directly by local nanotopological cues.

To investigate the likely involvement of integrin-mediated FA formation in regulating topological sensing of hESCs, we examined FA formation of single hESCs plated on nanorough glass surfaces. After 48 h of culture, single hESCs exhibited distinct FA formations and organizations on the smooth and nanorough glass surfaces, as characterized by immunofluorescence staining of vinculin, a FA protein. On smooth glass surfaces, where $R_q = 1$ nm, vinculin-containing FAs formed primarily on the periphery of undifferentiated hESCs (Oct3/4$^+$) but distributed randomly throughout the whole cell spread area of differentiated cells (Oct3/4$^-$/Cd) (Figure 3A). However, on the nanorough surface where $R_q = 100$ nm, both Oct3/4$^+$ and Oct3/4$^-$/Cd hESCs exhibited randomly distributed, punctate FAs of small areas throughout the entire cell spread area (Figure 3A). Morphometric analysis of cell populations suggested that on the smooth glass surface Oct3/4$^+$ hESCs had a smaller mean cell spread area than Oct3/4$^+$ hESCs, and
the nanorough surface resulted in both Oct3/4+ and Oct3/4− hESCs having a small mean cell spread area comparable to Oct3/4− hESCs on the smooth surface. Furthermore, our results indicated that both Oct3/4+ and Oct3/4− hESCs on the nanorough surface formed FAs of smaller areas but with a greater density as compared with the cells on the smooth surface (Figure 3B). Collectively, our results indicated that the nanotopography significantly affected the molecular arrangement, formation, and distribution of FAs in hESCs (Figure 3).

Previous studies have suggested that nonmuscle myosin IIA (NMIIA)-dependent CSK contractility is a key mediator of the mechano-sensing and -transduction processes in different types of stem cells.18,40−42 For hESCs, recent studies show that the NMIIA-mediated CSK contractility plays a critical role in regulating hESC survival and cloning efficiency by controlling E-cadherin-mediated intercellular adhesion of hESCs.42−45 Thus, we hypothesized that a feedback regulation and mechanical–biochemical integration involving FA, NMIIA, and E-cadherin might be engaged in topological sensing of hESCs by their cross-regulation of intercellular adhesion and cell–ECM interactions (Supplemental Figure S2). To examine this possibility, we examined NMIIA (Figure 4A) and E-cadherin (Figure 4B) expressions in hESCs plated on both the smooth (Rq = 1 nm) and nanorough (Rq = 100 nm) glass substrates after culture of 48 h. The cells were costained for nuclei (DAPI; blue), Oct3/4 (red), and myosin IIA (A; green) or E-cadherin (B; green).
of the Oct3/4 expression levels, hESCs adhered to the nanorough surface demonstrated a random distribution of NMMIIA throughout the entire cells and a weak expression of E-cadherin, similar to Oct3/4+ hESCs adhered on the smooth surface, suggesting that topological sensing and functional regulation by hESCs might involve the functional interplay between the mechanosensory components of FA, NMMIIA, and E-cadherin.

We further performed Western blot analysis of full-length E-cadherin, vinculin, NMMIIA, focal adhesion kinase (FAK), Oct3/4, and β-actin in Oct3/4+ hESCs plated on smooth (Rq = 1 nm) and nanorough (Rq = 100 nm) glass substrates after 24 h (left) and 48 h (right) of culture.

Figure 5. Western blot analysis of full-length E-cadherin, vinculin, NMMIIA, focal adhesion kinase (FAK), Oct3/4, and β-actin in Oct3/4+ hESCs plated on smooth (Rq = 1 nm) and nanorough (Rq = 100 nm) glass substrates after 24 h (left) and 48 h (right) of culture.

However, expression of E-cadherin was clearly downregulated for hESCs plated on the nanorough surfaces as compared to smooth substrates, consistent with the immunostaining results shown in Figure 4. Combining the results in Figure 4 and Figure 5, it appeared that nanotopography might regulate hESC behaviors through its direct effect on the local molecular arrangement and formation of FAs that might in turn regulate the spatial organization of NMMIIA-mediated CSK contractility and the E-cadherin-mediated intercellular adhesion of hESCs, even though the total expression levels of FA proteins such as vinculin and NMMIIA might not be significantly affected by nanotopography. It was highly likely that E-cadherin-mediated cell–cell contacts of hESCs might serve as the downstream mediator to convert the nanotopographic signal through FAs and CSK contractility to regulate downstream gene expression and fate decisions of hESCs (Supplemental Figure S3).

Application to Cell Separation. To further explore the general phenomenon of topological sensing, experiments using the RIE-generated nanotopographical glass surfaces were performed with NIH/3T3 fibroblasts. Phase-contrast and immunofluorescence images of single NIH/3T3 fibroblasts taken 4 h after cell seeding on a glass surface patterned with different shaped nanorough islands (Rq = 70 nm, with the surrounding areas smooth) showed that NIH/3T3 fibroblasts attached...
preferentially to the patterned nanorough islands and spread to conform to the different geometries of the nanorough islands (Figure 6A–C). The adhesion selectivity of NIH/3T3 fibroblasts to the patterned nanorough islands was about 91% (Figure 6B), suggesting the patterned nanoroughness could serve as an effective means to control the adhesion location and cell shape or spread area of NIH/3T3 cells. In addition, when a high density of single NIH/3T3 fibroblasts were seeded on a glass surface patterned with large nanorough islands, the cells attached and aggregated to the patterned nanorough islands to form cell colonies that conformed to the different geometries of the nanorough islands (Figure 6D). Thus, the adhesion response of NIH/3T3 fibroblasts to nanoroughness was completely contrary to that of hESCs (Figure 2A), indicating cell-type specificities in cellular responses to nanotopography.

The cell-type-specific preference to adhere to either smooth or nanorough surfaces provided an opportunity to spatially separate different cell types for cell coculture applications. To explore this prospect, a mixture of hESCs and NIH/3T3 fibroblasts was cultured on a patterned nanorough glass substrate containing an array of nanorough strips separated by smooth regions. The cell adhesion and separation on the patterned surface after coculturing the cells for 48 h was examined, showing that hESCs and NIH/3T3 fibroblasts selectively attached and autonomously segregated to the smooth and nanorough regions, respectively (Figure 6E). Quantitative analysis suggested a superior cell segregation efficiency for hESCs and NIH/3T3 cells using the patterned nanorough surfaces, as 87% of the cells adhered on the smooth regions of the glass surface were hESCs, while on the nanorough regions 97% of cells were NIH/3T3 fibroblasts (Figure 6F).

CONCLUSION

In this work, we report an effective microfabrication strategy for precise control and patterning of the local nanoroughness on glass surfaces using photolithography and RIE. It was demonstrated that hESCs were intrinsically sensitive to the nanoscale topological cues on the substrate. Also it was demonstrated that smooth glass surfaces supported cell adhesion, rapid cell proliferation, and long-term self-renewal of hESCs, while the nanorough glass surfaces tended to induce hESCs into spontaneous differentiation. Cell shape is a potent regulator of stem cell fate both in vivo and in vitro, and conventional studies have used microcontact printing and other methods to control cell shape and geometry.\textsuperscript{40,46} In this work, we further generated nanorough patterns on glass surfaces and showed that such patterned nanoroughness could serve as an effective means for confining geometries or shapes of single hESCs or small hESC colonies. Our results further suggested that the cellular responses of hESCs to nanotopography might be functionally linked to their disrupted FA formation, NMMIIIA activity, and E-cadherin-regulated intercellular adhesion. A feedback regulation and mechanical—biochemical integration mechanism involving these intracellular mechanical-sensory components might play an important role regulating hESC behavior during long-term self-renewal. In addition, it was demonstrated that cellular responses to nanotopography were cell-type specific, as NIH/3T3 fibroblasts appeared to adhere preferentially on nanorough surfaces as compared to smooth ones. By taking advantage of such cell-type-specific adhesion preference, a spatially segregated coculture system for hESCs and NIH/3T3 using patterned nanorough glass surfaces was demonstrated. Together, these results provide important insights into the mechanosensitivity of hESCs to nanotopological cues in the cellular microenvironment and further highlight the important role of well-designed and controlled synthetic stem cell microenvironment to direct hESC behaviors that are desirable for functional tissue engineering and regenerative medicine.

METHODS

Fabrication and Surface Characterization of Nanorough Glass Samples. Glass wafers (Borofloat 33, Plan Optik, Elssof, Germany) were first spin-coated with photoresist. The photoresist layer was patterned using photolithography to physically expose different regions of the underlying glass wafer. The glass wafer was then processed with RIE (LAM 9400, Lam Research, Fremont, CA, USA) for different periods of time to generate the nanoscale surface roughness (ranging from 1 to 150 nm) on the open regions of the glass wafer, where the photoresist had previously been developed and dissolved. The corresponding RIE process conditions were selected: SF\(_6\) (8 sccm), C\(_4\)F\(_8\) (50 sccm), He (50 sccm), Ar (50 sccm), chamber pressure (1.33 Pa), bias voltage (100 V), and radio frequency power (500 W). The resulting glass etch rate by RIE was about 50 nm/min. After the RIE process, photoresist was stripped using solvents, and the glass wafer was cleaned using distilled water. For unpatterned nanorough glass samples, bare glass wafers were directly processed with RIE under the same RIE conditions as described above. All the processed glass wafers were cut into small pieces (1.5 cm × 1.5 cm) before assays with cells by using the ADT7100 dicing saw (Advanced Dicing Technologies Ltd., Yokneam, Israel).

Nanoroughness of the glass surfaces was measured at room temperature with the Veeco NanoMan Atomic Force Microscope (AFM, Digital Instruments Inc., Santa Barbara, CA, USA) using a noncontact, tapping mode and standard Si tapping mode AFM tips. The AFM scan image size for the glass surfaces was set as 10 \(\mu\)m × 10 \(\mu\)m with a scan rate of 1 Hz. The resulting map of the local surface height was represented using the AFM topographs. The nanoroughness of each glass sample was characterized by the root-mean-square roughness \(R_s\) of the local surface height over the scanned areas collected using the AFM topographs. The unprocessed bare glass wafer had an initial surface roughness \(R_s\) of 1 nm.

Cell Culture and Reagents. The glass substrates were functionalized with human vitronectin (R&D System, Minneapolis, MN, USA) by immersing the substrates in a vitronectin solution.
(20 μg mL⁻¹) in distilled water overnight. The glass substrates were rinsed twice with PBS before they were used for cell seeding. hESCs (H9; WiCell, Madison, WI, USA) were cultured on a feeder-free synthetic polymer coating (PMEDSAH) with the Human-Cell-Conditioned Medium (HCCM, GlobalStem, Rockville, MD, USA) supplemented with human recombinant basic fibroblast growth factor (bFGF; GlobalStem) at 37 °C in 5% CO₂. hESC colonies were observed every 24 h using a Leica stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL, USA). Differentiated cells were removed mechanically using a sterile pulled-glass pipet. The culture medium was replaced every 24 h. Cultures were passed every 4–7 days.

For the hESC colony-forming assay, large undifferentiated hESC colonies were selected and cut using the StemPro EZPassage disposable stem cell passaging tool (Invitrogen, Carlsbad, CA, USA) to achieve a homogeneous initial cell cluster size. hESC colonies were selected and cut using the StemPro EZPassage disposable stem cell passaging tool (Invitrogen, Carlsbad, CA, USA) to achieve a homogeneous initial cell cluster size. Clusters (20–50) of hESCs were transferred onto the vitronectin-coated glass surfaces and cultured using hCCM.

For the single-cell assay undifferentiated hESC colonies were mechanically cut into small aggregates using a sterile pulled-glass pipet and collected as described above, mixed in hCCM, seeded at a low density (3000 cells/cm²) in the growth medium containing the ROCK inhibitor Y27632 (10 μM; Tocris Bioscience, Ellisville, MO, USA) and 100 μM TPA, and passaged through a cell strainer with the PMEDSAH coating disposable stem cell passaging tool (Invitrogen, Carlsbad, CA, USA) to achieve a homogeneous initial cell cluster size. Clusters (20–50) of hESCs were transferred onto the vitronectin-coated glass surfaces and cultured using hCCM.

For immunostaining to visualize NMMIIA and E-Cadherin, hESCs were seeded on the glass substrates and cultivated in HCCM for the first 24 h. The cell media were then replaced with the bFGF-free hCCM for another 24 h to allow spontaneous differentiation of hESCs prior to the immunofluorescence staining experiments.

NIH/3T3 mouse embryonic fibroblasts (ATCC, Manassas, VA, USA) were maintained in a growth medium consisting of high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% bovine serum (Atlanta Biological, Atlanta, GA, USA), 100 μg mL⁻¹ L-glutamine, 100 units mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin. Fresh 0.25% trypsin-EDTA in PBS was used to resuspend NIH/3T3 cells. NIH/3T3 cells were seeded at a low density (3000 cells/cm²) in the growth medium onto the glass substrates and allowed to spread out overnight before other assays.

For coculture assays single hESCs and NIH/3T3 cells were collected as described above, mixed in hCCM, seeded at a density of 5000 cells/cm² for both cell types onto the patterned nanorough glass substrates, and cultured for 48 h prior to fixing and immunostaining.

**SEM Specimen Preparation.** Cell samples were washed three times with 50 mM Na-cacodylate buffer (pH 7.3; Sigma-Aldrich), fixed for 1 h with 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 50 mM Na-cacodylate buffer, and dehydrated in a graded series of ethanol concentrations through 100% over a period of 1.5 h. Deparaffinization in 100% ethanol was performed three times. After fixation, dehydrated substrates were dried with liquid CO₂ using a supercritical point dryer (Hitachi High Technologies America, Inc., Pleasanton, CA, USA).

**Immunofluorescence Staining.** In brief, cells were incubated in an ice-cold cytoskeleton buffer (50 mM NaCl, 150 mM sucrose, 3 mM MgCl₂, 1 μg mL⁻¹ aprotinin, 1 μg mL⁻¹ leupeptin, and 1 μg mL⁻¹ pepstatin A for 1 min and then permeabilized with 0.5% Triton X-100 (Roche Applied Science, Indianapolis, IN, USA) in the cytoskeleton buffer for 1 min. Detergent-extracted cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 30 min and washed three times with PBS. The fixed cells were then incubated with 10% goat serum (Invitrogen) for 1 h and then primary antibodies (produced in either mouse or rabbit) to Oct3/4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), vinculin (Sigma-Aldrich, St. Louis, MO, USA), nonmuscle myosin IIA (Abcam, Cambridge, MA, USA), and E-cadherin (Invitrogen) for 1 h. Alexa Fluor 488 and 555 conjugated goat anti-mouse (or anti-rabbit) IgG secondary antibodies (Invitrogen) were used as secondary antibodies. Alexa Fluor 555 conjugated phalloidin (Invitrogen) and DAPI (Invitrogen) were used for visualization of actin microfilaments and nucleus, respectively.

**Western Blot Analysis.** Whole cell lysates were prepared from cells, separated on 7.5% SDS-polyacrylamide gel, and transferred to PVDF membranes. The membranes were incubated with 5% milk in PBS for 1 h and then incubated with primary antibodies overnight at 4 °C. Primary antibodies used were as follows: mouse anti-NMMIIA (1:1000; Abcam), mouse anti-E-cadherin (1:1000; Invitrogen), mouse anti-vinculin (1:1000; Sigma-Aldrich), mouse anti-FAK (1:500; BD Biosciences), rabbit anti-Oct3/4 (1:2000; Santa Cruz), and rabbit anti-j-actin (1:1000; Cell Signaling, Danvers, MA, USA). Blots were incubated with peroxidase-coupled secondary antibodies (Promega, Madison, WI, USA) for 1 h, and protein expression was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA).

**Quantitative Analysis of Cell Spread Area and Focal Adhesions.** Cell spread area and focal adhesion formation were quantified as described previously. Briefly, immunofluorescence images of the actin cytoskeleton and vinculin were obtained using a Carl Zeiss Axio Observer Z1 microscope equipped with an AxioCam camera (Carl Zeiss Microimaging, Thornwood, NY, USA) and a 40× objective (1.3 NA, oil immersion; EC Plan APO) and processed using custom-developed MATLAB programs (Mathworks, Natick, MA, USA). To determine the spread area of each cell, the Canny edge detection method was used to binarize the actin fibers and FAs, and then image dilation, erosion, and fill operations were used to fill in the gaps between the white pixels. The resulting white pixels were summed to quantify cell area. To quantify FA number and area for each cell, the grayscale vinculin image was thresholded to produce a black and white FA image, from which the white pixels, representing FAs, were counted and summed.

**Quantification of Cell Population Doubling Time.** Cell population doubling time was calculated as the time required for the area of a cell colony to increase 2-fold as previously described. The cell colony area was calculated by using the surface area formula for an ellipse (πab), where a and b are the transverse and conjugate diameters, respectively. Phase-contrast microscopic images of hESC colonies (n = 20) were taken every 24 h after initial cell seeding. ImageJ software (http://rsb.info.nih.gov/ij) was used to process the images and measure the colony transverse and conjugate diameters. The colony areas were then calculated for different time points (24, 48, 72, and 120 h after cell seeding), and the cell population doubling time was estimated as an exponential function.

**Statistics.** p-Value was calculated using the Student t-test function in Excel (Microsoft, Seattle, WA, USA).

**Conflict of Interest:** The authors declare no competing financial interest.

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REFERENCES AND NOTES


