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A Strong Contractile Actin Fence and Large Adhesions Direct Human Pluripotent Colony Morphology and Adhesion

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SUMMARY

Cell-type-specific functions and identity are tightly regulated by interactions between the cell cytoskeleton and the extracellular matrix (ECM). Human pluripotent stem cells (hPSCs) have ultimate differentiation capacity and exceptionally low-strength ECM contact, yet the organization and function of adhesion sites and associated actin cytoskeleton remain poorly defined. We imaged hPSCs at the cell-ECM interface with total internal reflection fluorescence microscopy and discovered that adhesions at the colony edge were exceptionally large and connected by thick ventral stress fibers. The actin fence encircling the colony was found to exert extensive Rho-ROCK-myosin-dependent mechanical stress to enforce colony morphology, compaction, and pluripotency and to define mitotic spindle orientation. Remarkably, differentiation altered adhesion organization and signaling characterized by a switch from ventral to dorsal stress fibers, reduced mechanical stress, and increased integrin activity and cell-ECM adhesion strength. Thus, pluripotency appears to be linked to unique colony organization and adhesion structure.

INTRODUCTION

Focal adhesions (FAs) are multifaceted organelles that link the extracellular matrix (ECM) to the cell's contractile actin cytoskeleton to determine adhesion strength and mechanosensing and to regulate cell polarity, survival, and mitosis (Tseng et al., 2012). The contribution of mechanotransduction and cell contractility to developmental processes is evident in vivo (Wozniak and Chen, 2009). In addition, recent studies have demonstrated the importance of ECM stiffness in pluripotent stem cell (PSC) specification (Chowdhury et al., 2010; Przybyla et al., 2016b) and the significance of Rho-ROCK-myosin signaling in human PSC (hPSC) maintenance (Ohgushi and Sasai, 2011), highlighting the crucial role of the contractile actin cytoskeleton in stem cell biology. However, the organization and significance of FAs, the cell's mechanotransducing units, and their actin linkage have remained unstudied in hPSC colonies.

In the pluripotent state, stem cells adopt tight colony morphology with low adhesive strength (Singh et al., 2013), in stark contrast to the parental somatic cells (e.g., fibroblasts) used to generate induced PSCs (iPSCs). Thus, substantial remodeling of cell adhesions is a prerequisite for reprogramming and, accordingly, cell-ECM interactions represent a barrier toward reprogramming (Qin et al., 2014). In culture, hiPSC survival and pluripotency requires appropriate adhesion to ECM (Chen et al., 2014), suggesting that integrin-mediated FAs play an important role in the maintenance of pluripotency in vitro.

RESULTS

Large FAs and a Prominent Actin Fence Encircle Pluripotent Colonies

Parental fibroblasts, used for the reprogramming of the hiPSC lines, demonstrated 5- to 10-fold more robust adhesion to Matrigel (MG), vitronectin (VTN), and laminins (LM-521, LM-511) compared with hiPSCs (Figure S1A), prompting us to examine further the FAs and cytoskeletal network within these two cell types. We plated parental fibroblasts and hiPSCs on VTN and imaged cell-ECM contacts 24 hr post attachment, within 100–200 nm of the matrix interface, using high-resolution total internal reflection fluorescence (TIRF) microscopy. To assess FAs across the entire colony without compromising resolution, we focused our attention on relatively small hiPSC colonies (1–35 cells). As expected, FAs in hiPSCs were remarkably different from the parental fibroblasts. Surprisingly, hiPSCs displayed large, PAXILLIN-positive FAs at the edges of the colonies ("cornerstone" FAs) in stark contrast to the smaller and more uniformly distributed FAs in the strongly adherent parental fibroblasts (Figure 1A). In addition, while the parental fibroblasts displayed multiple thin actin stress fibers aligned predominantly along the long axis of the cell and connected to FAs at their distal ends, akin to dorsal stress fibers (Naumanen et al., 2008), hiPSC colonies exhibited thick actin stress fibers parallel to the colony edge and anchored to FAs at both ends, in accordance with features of ventral stress fibers (VSFs) (Naumanen et al., 2008). This phenotypic difference was apparent in hiPSCs

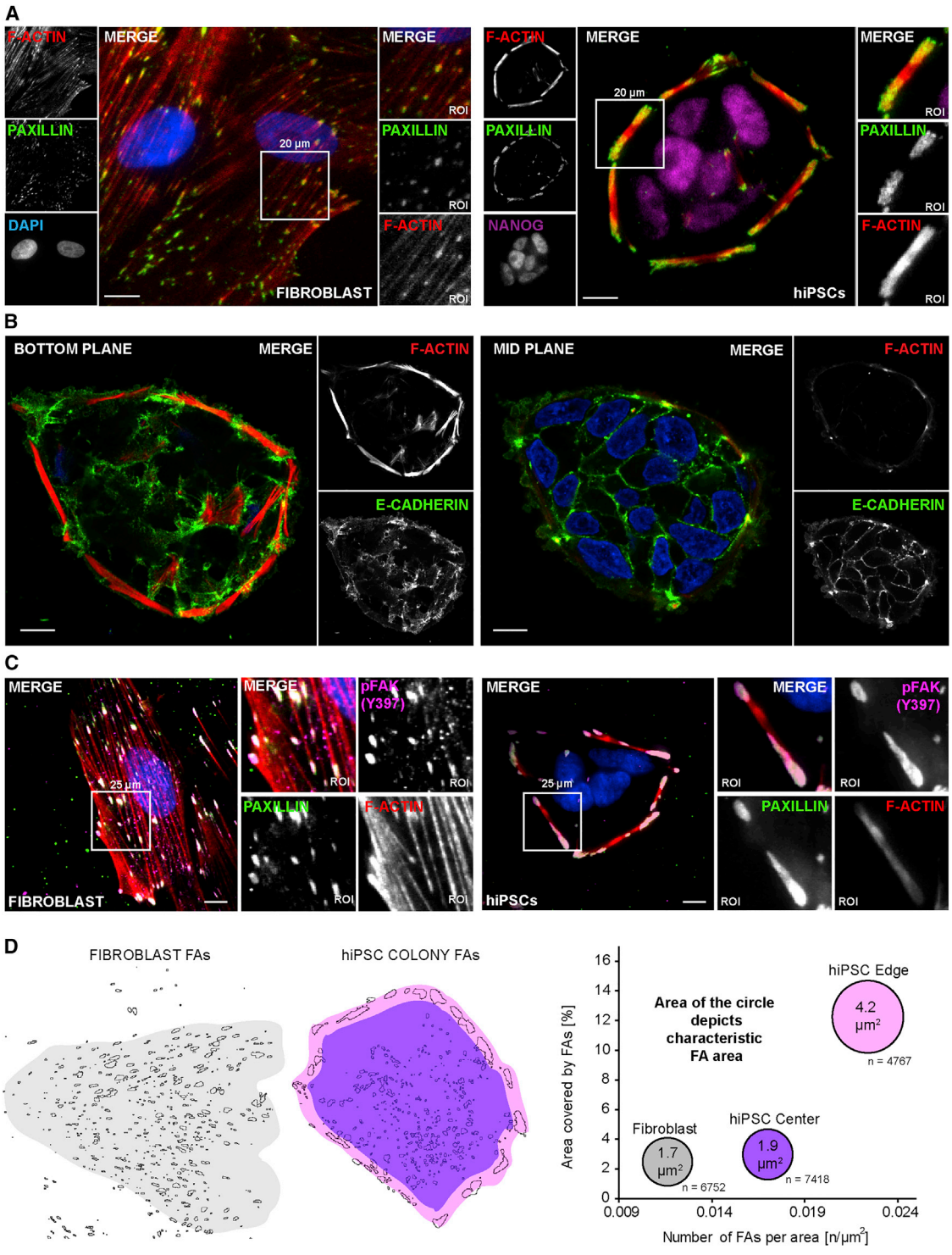


Figure 1. Prominent FAs and Actin Stress Fibers Define the hiPSC Colony Edge

(A and C) TIRF images of parental fibroblasts (left) and hiPSC colony (right) stained as indicated, DAPI/NANOG (mid plane).

(B) Confocal stacks of hiPSC colony (bottom and mid plane) stained as indicated, DAPI (blue). (D) A representative illustration of FAs detection areas and quantification of FA size, coverage, and density in parental fibroblasts (n = 6,752) and in the center (n = 7,418) and edge of the hiPSC colony (n = 4,767).

Data are from more than three biologically independent experiments. Scale bars, 10 µm. See also Figure S1.

cultured on VTN, LM-521, and MG, and in human embryonic stem cells (hESCs) (Figure S1B). E-cadherin-dependent cell-cell adhesions are also important for pluripotency, survival, and colony formation of hESCs (Li et al., 2012). Interestingly, E-CADHERIN was concentrated in close proximity to the cell-ECM interface in between the VSFs at the colony edge (Figure 1B), suggesting that cell-cell junctions contribute to the actin fence integrity alongside FAs.

HiPSC FAs appeared to be functional signaling platforms positive for focal adhesion kinase pFAK (Y397) and pPAXILLIN (Y118) (Figures 1C and S1C), suggesting a role beyond simple anchorage for these adhesions in maintaining hiPSC cultures (Mitra and Schlaepfer, 2006). FAs located at the hiPSC colony edge had a larger characteristic area (4.2 μm^2), coverage (12.3%), and density (0.022/μm^2), compared with FAs visible in the center of the colonies (1.9 μm^2, 3%, 0.017/μm^2) or FAs in parental fibroblasts (1.7 μm^2, 2.5%, 0.012/μm^2) (Figures 1D and S1D). Further analysis revealed that in small colonies consisting of $\leq$10 cells large FAs were most predominant; however, the density and number of FAs remained distinct between the edge and the center irrespective of colony size (Figure S1E). Although the presence of large FAs in weakly adhering hPSC colonies is counterintuitive, these results imply that hPSC-ECM interactions may be predominately mediated by the colony edge. Interestingly, characteristic FAs and the actin fence appeared to assemble rapidly and to be subsequently stable, as 2 hr post colony fragmentation and replating these features were already prominent (Figure S2A). The FAs grew in size after plating for up to 20 hr, whereas the number of FAs remained relatively constant (Figures S2B and S2C).

Cytoskeletal Mechanical Stress Depends on the Angle of Ventral Stress Fibers

VSFs are contractile actin bundles (Naumanen et al., 2008), and accordingly we observed MYOSIN IIA and phosphorylated myosin light chain (pMLC) co-localization with the thick actin fibers at the hiPSC colony edge (Figures 2A and 2B). In addition, the actin bundling protein α-ACTININ demonstrated clear overlap with actin fibers and the FAs (Figure 2C), further confirming these actin structures to be VSFs.

VSF contraction is known to impact on FA size, composition, and overall cell adhesion (Katoh et al., 2011). Remarkably, hPSC colony organization and FA size, observed in cells plated on glass/polystyrene, were not significantly altered on 30- or 10-kPa VTN-functionalized hydrogels, and only modestly reduced on 3 kPa. However, reduced stiffness did correlate with gradual decrease in FA number (Figure S2D). Using traction force microscopy (TFM), we assessed the traction stress applied by individual hPSC colonies. We selected 10-kPa rigidity to achieve optimal balance between retained colony morphology and sufficient compliancy for detecting cell-ECM force mediated bead displacement. hPSCs were found to exert strong inward-directed mechanical forces on the ECM at specific locations coinciding with VSFs at the colony edge (Figure 2D). These data imply a role for the VSF actin fence in tight packaging of hPSC colonies. Accordingly, increasing cell numbers correlated with significantly smaller individual cell area and higher overall cell density within colonies (Figure S2E). In addition, hiPSCs had, on average, 34 FAs per cell compared with 66 in fibroblasts. Therefore, high compaction results in decreased ECM contact area and reduced FA number per cell, possibly explaining the overall weak adhesion of hiPSC colonies.

Interestingly, small hiPSC colonies with acute inter-fiber angles exerted significantly higher stress on the ECM per cell than larger colonies where VSFs were aligned linearly one after another and the opposing mechanical stress of adjacent FAs appeared to counteract each other (Figures 2D and 2E). In single hiPSCs, VSFs had the sharpest angles and formed a triangular-shaped fence around the cell. In two-cell clusters, the inter-fiber angles were increased to 90° (Figure 2F). This pattern of VSF organization suggests that single stem cells experience the highest mechanical stress, and that upon expansion colonies adopt a rounder morphology to accommodate wider inter-fiber angles and ultimately reduce the mechanical stress exerted by individual cells at the colony edge. These results may begin to explain why cellular tension and actomyosin contractility, while important for hESC colony survival and proliferation (Harb et al., 2008; Ohgushi et al., 2015), are lethal to single dissociated hESCs (Ohgushi and Sasai, 2011).

Rho Signaling Controls Cornerstone FAs

Inhibition of actomyosin contraction triggers disassembly of stress fibers and mature FAs (Katoh et al., 2011). Accordingly, blebbistatin (myosin heavy chain ATPase inhibitor), Y-27632 (Rho kinase inhibitor; ROCKi) treatment, or cytochalasin-D-mediated ablation of the actin cytoskeleton triggered disassembly of actin and actin-associated FAs in hiPSC colonies (Figures 3A and S3A). Furthermore, ablation of FAs with a short exposure to CDK1 inhibitor RO-3306 (Robertson et al., 2015) led to rapid loss of large FAs and the colony-encircling actin fence (Figure S3B). These findings validate that actomyosin contractility and cornerstone FAs are indispensable for the formation of the VSF actin fence.

Intact VSFs and FAs also appeared to be essential for colony morphology and compaction, as blebbistatin and ROCKi treatments triggered loss of the typical pluripotency-associated sharp-edge colony morphology and reduced cell density (Figure 3B). In addition, blebbistatin, ROCKi, or RO-3306 treatment resulted in expansion of

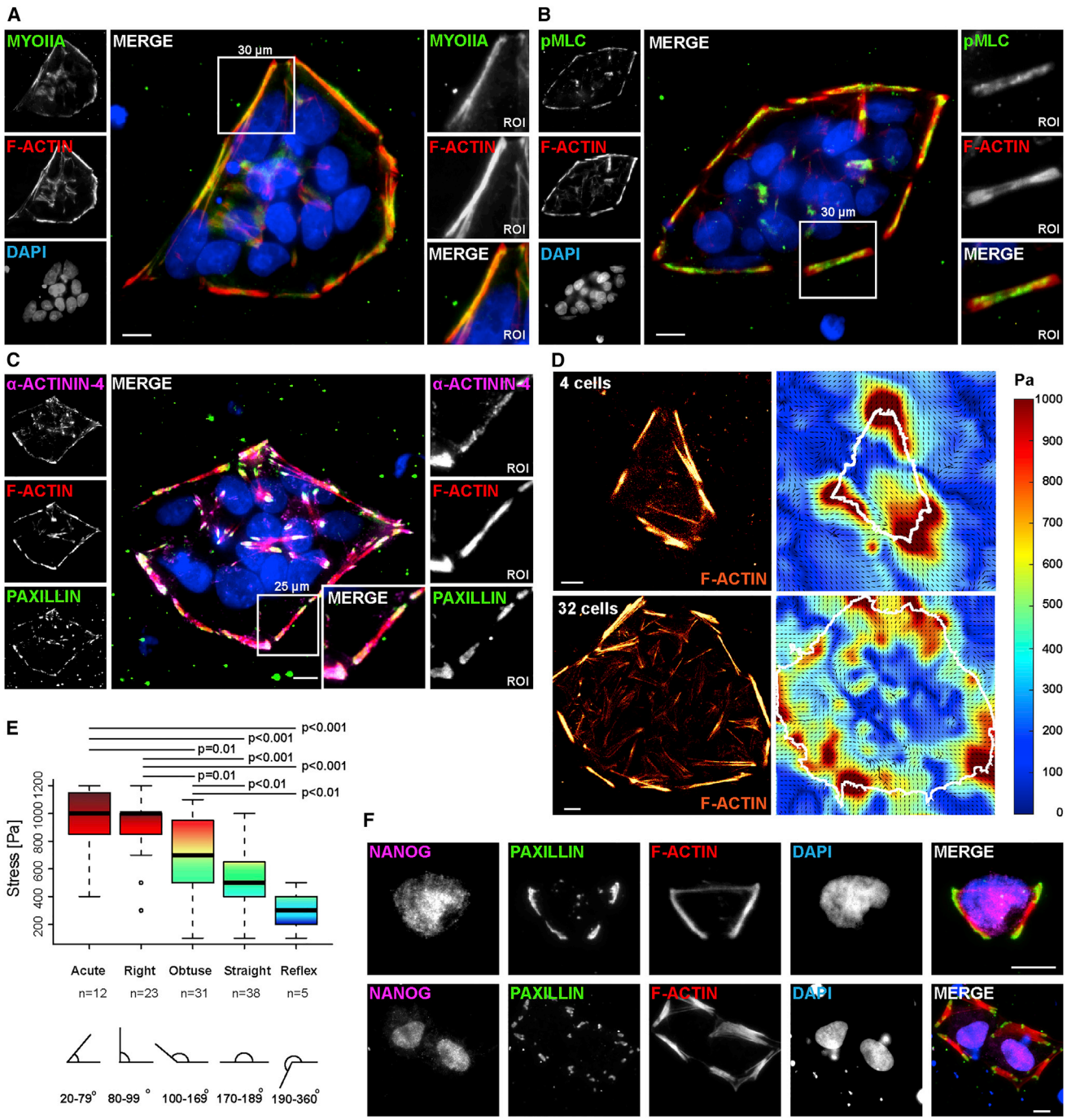


Figure 2. Mechanical Stress Depends on the Angle of Ventral Stress Fibers

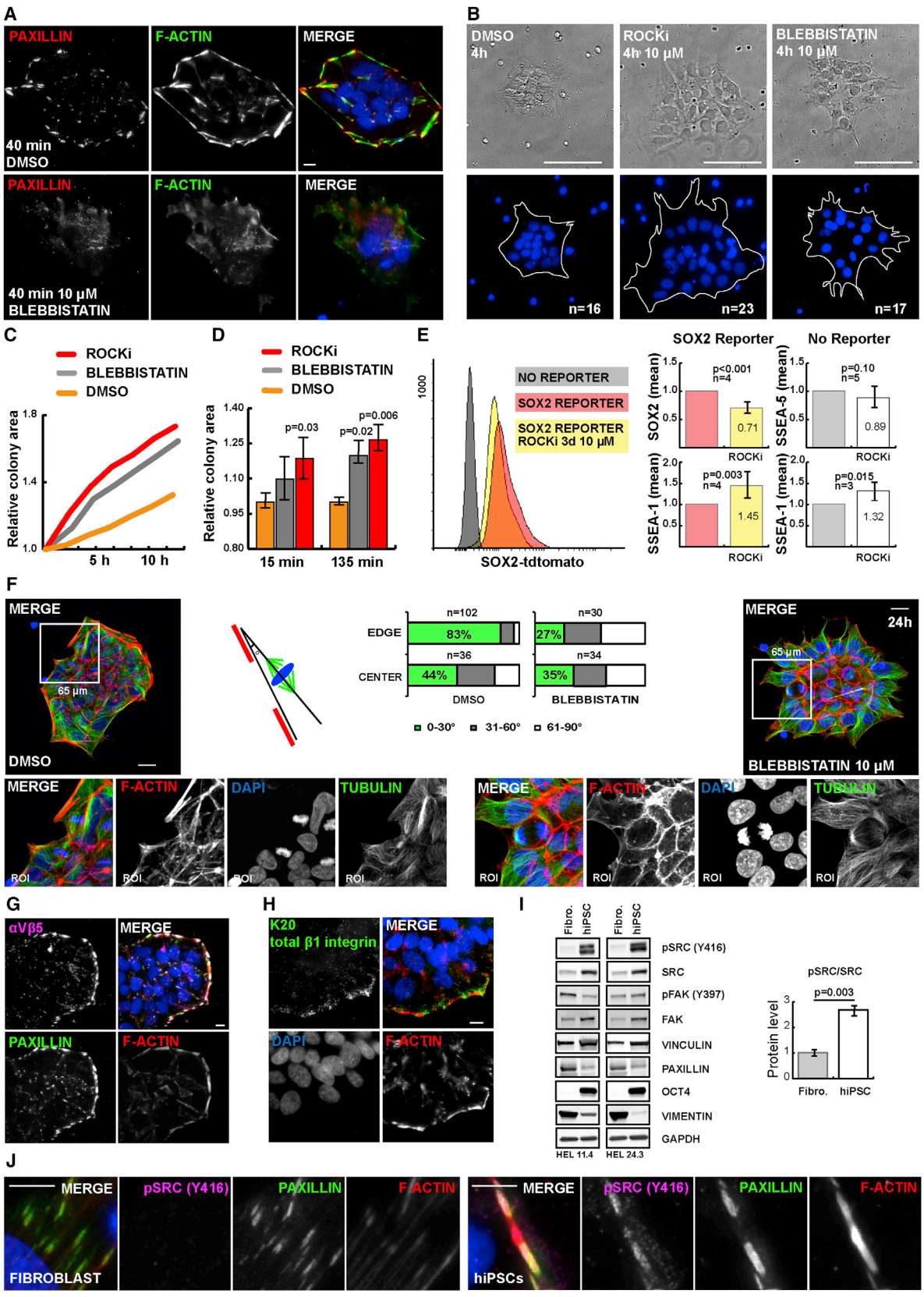
(A–C) TIRF images of hiPSC colonies stained as indicated.

(D) TFM stress maps and representative confocal images of F-ACTIN (SiR-actin) in hiPSC colonies plated on ~10-kPa VTN-functionalized polyacrylamide gels. White lines depict the colony edge.

(E) Quantification of mechanical stress at FAs relative to inter-VSF angle (n = 3 biologically independent experiments; median IQR: 25th–75th percentile; whiskers: 1.5 × IQR; ANOVA, Turkey's HSD).

(F) TIRF images of one (top) or two (bottom) hiPSCs stained as indicated. DAPI/NANOG (mid plane).

Scale bars, 10 μm. See also Figure S2.



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cell area (Figures 3C, 3D, and S3C), indicating loss of colony compaction.

Contractile Actin Fence and Large FAs Reinforce Pluripotency

Next, we studied the inter-relationship between the actin fence and pluripotency using a SOX2-td-Tomato reporter hiPSC line. Following ROCKi exposure 3 days after plating, SOX2 expression, although positive in all cells, was significantly reduced compared with control (Figure 3E). Levels of the pluripotency linked marker SSEA-5 were also decreased, but these data did not reach significance. Conversely, expression of the SSEA-1 differentiation marker was induced in the presence of ROCKi. Thus, ROCKi affects pluripotency, indicating a role for the actin fence and large FAs in force-dependent regulation of the core pluripotency network.

Colony Morphology Is Maintained by Edge-Oriented Cell Division

Time-lapse imaging demonstrated that during colony expansion mitosis occurred throughout the colony (Movie S1). However, at the colony periphery mitotic spindles were predominantly (83%) aligned parallel (0°–30°) to the VSFs, compared with a more random orientation in the colony center. The parallel positioning was dependent on an intact contractile actomyosin border as treatment with blebbistatin triggered random mitotic spindle orientation throughout the colony (Figure 3F). These data suggest that daughter cells located on the colony edge retain their localization and assemble prominent FAs and actin bundles to reform the tight edge upon colony expansion.

FAs of hiPSCs Display High SRC Activity

Integrins play important roles in mechanosensing (Atherton et al., 2016). The main integrin subunits expressed at the protein level in hPSCs are α3, α5, α6, αv, β1, and β5 (Meng et al., 2010; Rowland et al., 2010; Vuoristo et al., 2009; Xu et al., 2001). Notably, we detected prominent αvβ5 integrin staining at FAs associated with VSFs (Figure 3G) and β1 integrin at the periphery of the colonies (Figure 3H), implicating these integrins in cornerstone FA signaling.

Interestingly, in hiPSC colonies the expression of FA signaling proteins appeared to be distinct from parental fibroblasts (Figures 3I and S3D). Levels of a major force-related FA protein VINCULIN (Atherton et al., 2016) were slightly higher in hiPSCs, whereas PAXILLIN levels were reduced. Total FAK levels were elevated but the pool of pFAK remained unchanged, indicating proportionally lower FAK activity in hiPSCs. In contrast, both SRC expression and active pSRC (Y416) were significantly elevated in hiPSCs. Furthermore, pSRC was highly abundant in hiPSC and nearly absent in fibroblast FAs (Figure 3J). As Rho signaling can direct SRC to FAs at stress fiber termini (Timpson et al., 2001), this could account for the strong pSRC localization observed in hiPSC FAs.

FAK and SRC, in particular, have been implicated in Rho signaling from FAs (Huveneers and Danen, 2009; Schiller and Fässler, 2013) and in the regulation of cell proliferation and survival in different cell types (Mitra and Schlaepfer, 2006). FAK inhibition disrupted hiPSC colony morphology and induced cell detachment within a few hours (Figure S3E and Movie S2). Similarly, PP2-mediated inhibition of SRC family kinases triggered cell detachment originating from the periphery of the colonies (Figures S3F and S3G; Movie S3). Thus, both FAK and SRC signaling appear to be essential for hiPSCs.

Pluripotency-Associated VSFs and Large FAs Are Lost in Differentiation

Retinoic acid (RA)-driven differentiation, verified by loss of pluripotency markers SOX2, NANOG, and SSEA-3 and induction of differentiation marker SSEA-1 (Figures S4A and S4B), resulted in the loss of typical colony morphology (Figure S4C) and pluripotency-associated VSFs, and prompted

Figure 3. Rho Signaling Controls Large FAs with High SRC Activity, Reinforces Pluripotency, and Dictates Orientation of Mitosis

(A–D) hiPSC colonies treated with blebbistatin (10 μM, 40 min [A] or 4 hr [B]), ROCKi (Y-27632, 10 μM, 4 hr [B]), or DMSO. (A) TIRF images of colonies stained as indicated. (B) Bright-field and DAPI-stained hiPSC colonies (white lines depict colony edge). (C and D) Relative colony area scored from live imaging in different time points (n = 3 biologically independent experiments, mean ± SD, t test).

(E) Flow-cytometric analysis of SOX2:td-Tomato and SSEA-1 in the SOX2 reporter hiPSC line and SSEA5 and SSEA-1 in non-reporter hiPSC line after ROCKi exposure (10 μM, 3 days) relative to control (n > 3 biologically independent experiments, mean ± SD, t test).

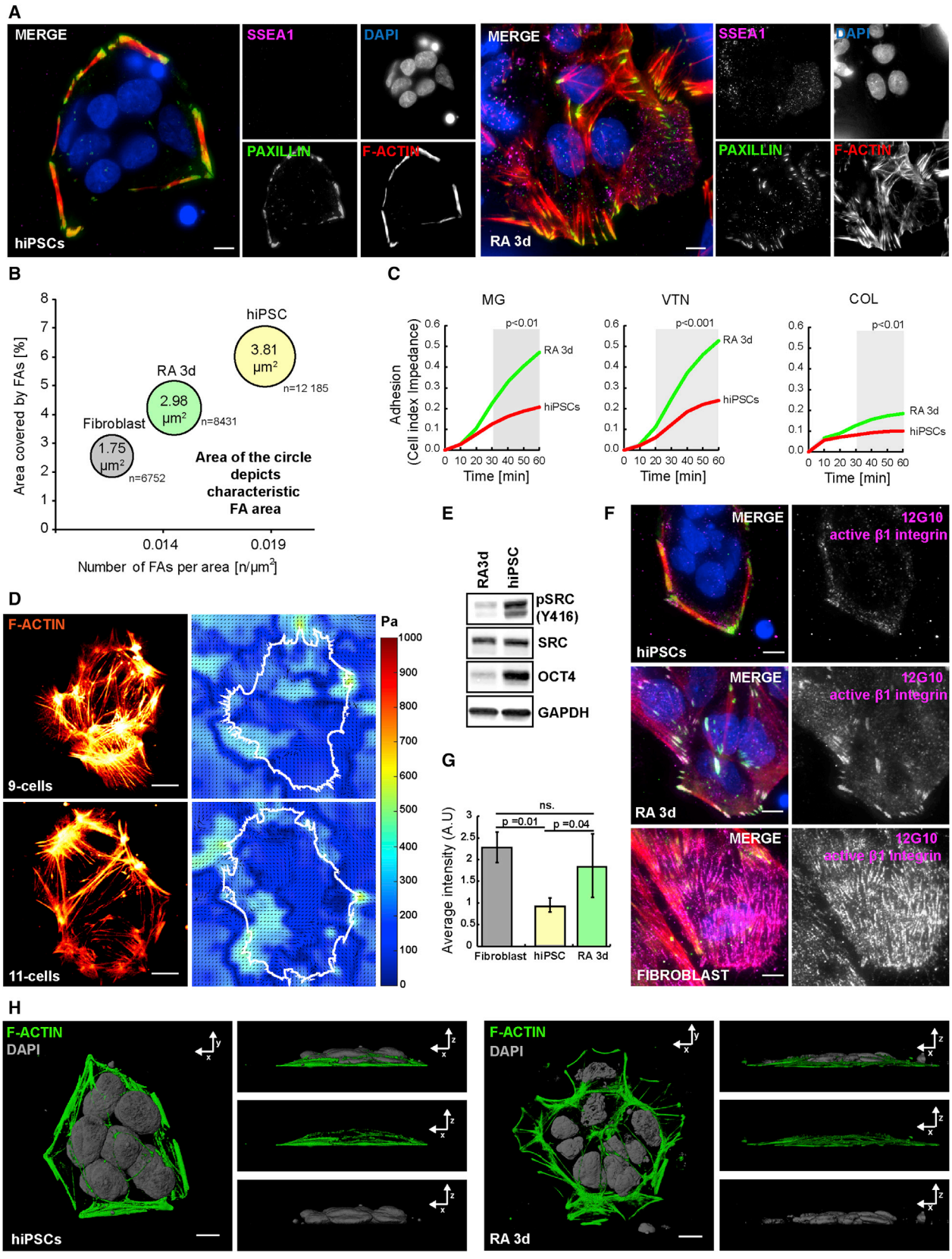
(F) Confocal images of DMSO-treated (left) or blebbistatin-treated (10 μM, 24 hr; right) hiPSC colonies and quantification of mitotic spindle orientation (angle between mitotic spindle and colony edge). Data are from three biologically independent experiments.

(G and H) TIRF images of hiPSC colonies stained as indicated, DAPI (blue).

(I) Western blot analysis of FA components and OCT4 (pluripotency marker), VIMENTIN (fibroblast marker), and GAPDH (loading control) in fibroblasts and in two different hiPSC lines HEL11.4 and HEL24.3, and quantification of pSRC and total SRC ratio (n = 3 biologically independent experiments, mean ± SD, t test).

(J) TIRF images of parental fibroblasts and hiPSCs stained as indicated.

Scale bars, 10 μm (A, G, H, J), 100 μm (B), and 20 μm (F). DAPI images are taken in the mid plane. See also Figure S3.



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a perpendicular switch in the orientation of FAs and actin fibers (Figures 4A, S4D, and S4E). In addition, differentiation was accompanied by a striking reduction in FA size (Figure 4B), further validating that large FAs correlate with pluripotency.

The differentiation-associated switch in actin and FA organization was accompanied by significantly enhanced cell ECM adhesion (Figure 4C). In addition, altered stress fiber alignment correlated with colony flattening and increased cell-surface area (Figure S4F), which was not due to an increase in cell size (Figure S4G), and resulted in a marked loss of colony compaction (Figure S4H). In conjunction with the cytoskeletal changes, differentiation triggered a substantial loss in mechanical stress exerted by cells on the ECM and a uniform distribution of forces across the colony (Figure 4D) in contrast to spatially restricted mechanical forces observed in pluripotent colonies (Figure 2D). In line with reduced FA size and mechanical stress, we noted significant downregulation of adhesion signaling, in the form of reduced pSRC, in differentiated cells (Figures 4E and S4I).

In somatic cells, adhesion to the ECM is controlled by regulation of integrin activity (Bouvard et al., 2013). In hPSC, β1 integrin is essential for supporting colony interactions with various ECMs (Braam et al., 2008; Prowse et al., 2010; Rowland et al., 2010; Vuoristo et al., 2009). We found that active β1 integrin expression (detected with 12G10, an activation state-specific antibody), primarily restricted to cornerstone FAs in hPSC colonies, was significantly lower in hiPSCs than in parental fibroblasts. Differentiation increased β1 integrin activity at FAs and at the cell surface (Figures 4F, 4G, S4J, and S4K) in accordance with increased cell adhesion, suggesting that tight control over integrin activity is coupled to the regulation of the pluripotent phenotype.

Finally, and in agreement with the TIRF data, three-dimensional reconstruction of confocal microscopy images, depicting the entire colony, further validated the presence of VSFs concentrated at the colony-ECM interface in hiPSCs and a clear transition in actin morphology upon differentiation (Figure 4H and Movie S4). These results imply that pluripotency associated FAs and VSFs at the colony edge, in addition to tightly controlled integrin and SRC activity, are critical for regulation of adhesion strength and maintenance of hPSC colonies and that stem cell differentiation involves significant alterations in FA composition, mechanotransduction, and rearrangement of the actin cytoskeleton.

DISCUSSION

hPSCs self-organize into sharp-edged colonies in culture (Ginis et al., 2004). Our discovery of a unique FA-dependent actin fence encircling hPSC colonies that contributes to pluripotency provides a high-resolution view into the physical constraints of these colonies.

Recently, congruent with our findings, hESC colonies were reported to display strong ECM interactions and enhanced myosin activity at the colony edge (Przybyla et al., 2016a; Rosowski et al., 2015). Interestingly, other work has suggested 10-fold higher cell-ECM tractions in human versus mouse ESC colonies (Chowdhury et al., 2010). Force involves the activation of the Rho-ROCK-myosin signaling cascade and affects FA size, composition, and position (Katoh et al., 2011), so the exceptionally large hPSC FAs are likely to be linked to the strong traction stress produced by the connecting VSFs.

Long-term culturing with ROCKi improves cloning efficiency, but is believed not to affect pluripotency. Based on our data, ROCKi-treated cells remain pluripotent but display lower levels of SOX2, in line with the ability of ROCKi to limit pluripotency and prime hiPSCs for epithelial-to-mesenchymal transition and mesodermal differentiation (Maldonado et al., 2016). In addition, differentiation is particularly apparent at the colony edge (Rosowski et al., 2015; Warmflash et al., 2014), suggesting that mechanical properties may influence the outcome of differentiation cues. These findings support the notion that tight colony morphology is required for complete pluripotency.

Figure 4. Differentiation Triggers Actin Reorientation, Altered FA Size, and Increased Integrin Activation

hiPSC colonies were left untreated or were differentiated in the presence of retinoic acid for 3 days (RA 3d, 10 μM).

(A) TIRF images stained as indicated, SSEA1 was used as marker of differentiation, DAPI (mid plane).

(B) Comparison of FA size, coverage, and density in total hiPSC colony area (n = 12,185), post RA differentiation (n = 8,431) and in fibroblasts (n = 6,752). Data are from more than 3 biologically independent experiments.

(C) Real-time adhesion of single cells on MG, VTN, and collagen (COL) over 1 hr (n = 3 biologically independent experiments, mean, t test).

(D) TFM stress maps and representative confocal images of F-ACTIN (SiR-Actin) on 10 kPa VTN-functionalized gels. White lines depict the colony edge.

(E) Western blot analysis of pSRC (Y416), SRC, OCT4, and GAPDH.

(F) TIRF image of active β1-integrin (12G10); for PAXILLIN and F-ACTIN see Figure S4.

(G) Quantification of active integrin levels in (F). Mean ± SD, ANOVA, Tukey's HSD.

(H) Three-dimensional Imaris reconstruction.

All scale bars represent 10 μm. See also Figure S4.

SRC activity maintains the undifferentiated phenotype and regulates the cell cycle of pluripotent cells (Annerén et al., 2004; Chetty et al., 2015), and β1-integrin-dependent SRC activity protects hESCs from mesodermal differentiation on stiff gels (Przybyla et al., 2016b). Given that active SRC localizes to FAs in hiPSC colonies and SRC inhibition disturbs colony morphology and adhesion, force-directed SRC signaling in FAs might have an important role in supporting pluripotency.

We find that integrin activity is dramatically increased upon differentiation, in line with a recent observation that forced activation with Mn^{2+} can induce differentiation (Villa-Diaz et al., 2016). The underlying mechanism restricting integrin activity in hPSCs and potential pluripotency-linked integrin inactivating factors will be an interesting avenue for future investigation. Taken together, our data are supportive of a working model whereby hiPSC colonies exist as an integrated assembly of weakly adhering inner "center" cells, with low β1-integrin activity, surrounded by a border of "edge" cells with large "cornerstone" FAs with high SRC and integrin activity linked by strong contractile VSFs. Together, these structures generate the fence required to maintain compact pluripotent colony morphology.

EXPERIMENTAL PROCEDURES

See also Supplemental Experimental Procedures.

Cell Culture

HiPSC lines HEL24.3, HEL11.4, HEL24.3-SOX2-ntdT reporter, hESC line H9, and parental fibroblasts were obtained from the University of Helsinki. Cells were grown on MG (Corning) or on 5 μg/mL VTN (Life Technologies) in Essential 8 medium (Life Technologies). HiPSCs were differentiated in 10 μM RA (Sigma).

Immunofluorescence and Microscopy

Details of the microscopes used in this study can be found in Supplemental Experimental Procedures. Cells were plated on VTN-coated glass plates and fixed after 24 hr with 4% paraformaldehyde for 15 min, permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min, and stained with the appropriate antibodies, prepared in 30% (v/v) horse serum (Gibco), in addition to DAPI and atto-phalloidin-647 (Sigma) staining.

Traction Force

Cells were plated 24 hr before the experiment onto VTN-functionalized 10-kPa polyacrylamide hydrogels with 0.2-μm FluoroSphere beads (Life Technologies, F881). SiR-Actin (50 nM) (Cytoskeleton) was added to the medium 4 hr prior to imaging. Cells and beads were imaged before and after cell detachment with trypsin-EDTA. Traction stresses were determined using MATLAB (MathWorks) software package following the Fourier transform traction force algorithm.

Adhesion Assay

Adhesion kinetics was measured with a 96-well E-plate (Roche) xCELLigence cell analyzer RTCA (Roche Diagnostics).

Ethical Considerations

HiPSC lines have been derived under the approval of the Coordinating Ethics Committee of Helsinki and Uusimaa Hospital District (statement no. 423/13/03/00/08).

Statistical Analysis

Student's t test and ANOVA were employed. Details of the test and p values are indicated in each figure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.05.021.

AUTHOR CONTRIBUTIONS

Conceptualization, J.I. and E.N.; Methodology, E.N., A.S., C.G., D.B., and M.L.; Formal Analysis, C.G., E.N., and A.S.; Investigation, E.N., A.S., M.B., C.G., and M.S.; Resources, T.O. and J.I.; Writing – Original Draft, E.N. and J.I.; Visualization, E.N., A.S., and C.G.; Supervision, J.I.; Funding Acquisition, J.I.

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