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Inhibition of Focal Adhesion Kinase Signaling by Integrin α6β1 Supports Human Pluripotent Stem Cell Self-Renewal

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Key Words. Pluripotent stem cells • Integrins • Self-renewal • Differentiation • Molecular signaling

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Received August 14, 2015; accepted for publication February 1, 2016; first published online in STEM CELLS *EXPRESS* March 1, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2349

ABSTRACT

Self-renewal of human embryonic stem cells and human induced pluripotent stem cells (hiPSCs)—known as pluripotent stem cells (PSC)—is influenced by culture conditions, including the substrate on which they are grown. However, details of the molecular mechanisms interconnecting the substrate and self-renewal of these cells remain unclear. We describe a signaling pathway in hPSCs linking self-renewal and expression of pluripotency transcription factors to integrin α6β1 and inactivation of focal adhesion kinase (FAK). Disruption of this pathway results in hPSC differentiation. In hPSCs, α6β1 is the dominant integrin and FAK is not phosphorylated at Y397, and thus, it is inactive. During differentiation, integrin α6 levels diminish and Y397 FAK is phosphorylated and activated. During reprogramming of fibroblasts into iPSCs, integrin α6 is upregulated and FAK is inactivated. Knockdown of integrin α6 and activation of β1 integrin lead to FAK phosphorylation and reduction of Nanog, Oct4, and Sox2, suggesting that integrin α6 functions in inactivation of integrin β1 and FAK signaling and prevention of hPSC differentiation. The N-terminal domain of FAK, where Y397 is localized, is in the nuclei of hPSCs interacting with Oct4 and Sox2, and this immunolocalization is regulated by Oct4. hPSCs remodel the extracellular microenvironment and deposit laminin α5, the primary ligand of integrin α6β1. Knockdown of laminin α5 resulted in reduction of integrin α6 expression, phosphorylation of FAK and decreased Oct4. In conclusion, hPSCs promote the expression of integrin α6β1, and nuclear localization and inactivation of FAK to supports stem cell self-renewal. STEM CELLS *2016;34:1753–1764*

SIGNIFICANCE STATEMENT

Villa-Diaz et al. describe a new signaling pathway in which the expression of pluripotent transcription factors is linked to the expression of laminin α5 and integrin α6β1 and the inactivation of FAK signaling which allows self-renewal of human pluripotent stem cells.

INTRODUCTION

Human embryonic stem cells (ESC) [1] and induced pluripotent stem cells (iPSC) [2], collectively referred to as human pluripotent stem cells (hPSCs), are able to differentiate into all cell types of the body and are presumed to be capable of indefinite self-renewal in vitro. Although the culture conditions in which these cells thrive are well-documented [3], our understanding of how hPSCs interpret signals from the substrate—mainly composed of extracellular matrix (ECM) proteins—in which they are cultured, is incomplete. Among the ECM proteins that support hPSC self-renewal are fibronectin [4], laminin isoforms 511 and 521 [5], and vitronectin [6]. Therefore, we investigated the role that their main cell-membrane receptors—integrins—may play in self-renewal of hPSCs.

Integrins are heterodimeric transmembrane receptors that cells use to adhere to the ECM [7] and transmit extracellular signaling inside the cells using nonreceptor tyrosine kinases, such as focal adhesion kinase (FAK), and components of the cytoskeleton [8, 9]. At the cellular level integrins play important roles in cell migration, differentiation, and gene expression [7, 10] and are known to be involved in normal organ development and function, cancer metastases, and in the progression of several diseases [11–15]. Integrins are subdivided into α and β families that when combined form at least 24 heterodimer receptor units. These heterodimer receptors have affinity for specific ECM proteins. For example, integrin α5β1 functions to bind fibronectin, α6β1 to laminin, and αVβ5 to vitronectin [8]. In addition, integrins have been used as cell surface markers to

identify different cell populations, including stem cells. However, despite extensive study of these cell membrane receptors in many cell functions, complete understanding of integrin function in regulating self-renewal and differentiation of hPSCs remains elusive.

Here, using flow cytometric analysis (FACS), immunocytochemistry (ICC), and genetic manipulations we identified integrin $\alpha6\beta1$ as the main heterodimer integrin receptor in hPSCs and identified a molecular signaling pathway in hPSCs, in which integrin $\alpha6\beta1$ blocks the activity of FAK, prevents the degradation of pluripotency transcription factors, and maintains self-renewal. Knowledge of this newly identified pathway in hPSCs will contribute to a better understanding of the interactions between the microenvironment and pluripotency transcription factors that regulate self-renewal and differentiation.

Materials and Methods

PSC Culture

All experiments were repeated at least in triplicates and with multiple hESC and hiPSC lines. NIH approved hESCs lines H1, H7, and H9 (WiCell Research Institute, Madison, WI, http://www.wicell.org) and CHB8 and CHB10 (Children's Hospital Corporation, Boston, MA), and three hiPSCs derived in our laboratory [16] (hGF2-iPSCs, hGF4-iPSCs, hFF [human foreskin fibroblasts] iPSCs) were cultured on tissue culture plates (TCP) coated with the synthetic polymer poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] referred to as PMEDSAH-grafted plates (GP), a synthetic polymer that support self-renewal of hESCs and hiPSCs, or on Matrigel hESC-qualified Matrix (Corning, Bedford, MA, http://www.corning.com), human recombinant (hR) laminin-511 (BioLamina, Sundbygerg, Sweden, http:// www.biolamina.com), or hR vitronectin (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) with human-cell-conditioned medium (hCCM, MTI-GlobalStem, Gaithersburg, MD, http://www.mti-globalstem.com) supplemented with 5 ng/ml of hR basic fibroblast growth factor (bFGF; Invitrogen Life Technologies, Grand Island, NY, http://www.thermofisher.com) or StemPro hESC serum-free medium (SFM; Gibco Life Technologies, Grand Island, NY, http://www.thermifisher.com) in incubators with high humidity and 5% CO_2 at 37°C. The medium was replaced every other day. Differentiating cells were mechanically removed with a sterile pulled-glass pipet under a Leica MZ9.5 stereomicroscope (Leica Microsystems, Buffalo Grove, IL, http://www.leica-microsystems.com). Undifferentiated colonies were cut and passed as small cell clusters.

PMEDSAH-GP were prepared and used as described previously [17, 18]. Briefly, PMEDSAH-GP were preincubated with hCCM for at least 48 hours at 37°C in 5% CO_2 before use. Matrigel was diluted at a concentration of 100 μg/ml in cold Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco Life Technologies), added to TCPs and allowed to polymerize during 2 hours at RT. hR vitronectin and laminin-511 were diluted to a concentration of 5 and 10 μg/ml, respectively, in Dulbecco's phosphate buffered saline (D-PBS, Gibco Life Technologies), added to TCPs and allowed to polymerize during half hour at RT. Before plating cells, dishes were washed with D-PBS.

Reprogramming of Human Somatic Cells into iPSCs

hFF (American Type Culture Collection, Manassas, VA, http://www.atc.org) and human embryonic kidney 293T (HEK293T) cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies), 1% nonessential amino acids (NEAA) (Gibco Life Technologies), 1% GlutaMax (Gibco Life Technologies), and 1% penicillin streptomycin (Gibco Life Technologies). This medium was changed during the reprogramming cycle and expansion of hiPS colonies to hCCM supplemented with 4 ng/ml bFGF and 10 μM Y-27632 dihydrochloride (Rock inhibitor; Enzo Life Sciences; Farmingdale, NY, http://www.enzolifesciences.com). The University of Michigan Vector Core generated retroviral vectors carrying Klf4, Sox2, Oct3/4, and c-Myc by transient cotransfection (Addgene plasmids 17217, 17219, 17220, and 17226, and VSV-g envelope plasmid 8454) into Clontech GP2-293 packaging cells using standard protocols. Fibroblasts (150,000) were plated on a 35 mm TCPs in fibroblasts culture medium and infected the following day with 1 ml of 1 × reprogramming cocktail diluted in DMEM medium and supplemented with 10 μg of hexadimethrine bromide (Polybrene; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Eight hours later, the medium was changed, and 72 hours later cells were subcultured on Matrigel coated plates with hCCM supplemented with bFGF and Rock Inhibitor or collected for protein sample and Western blot analysis.

In Vitro Cell Lineage Differentiation

Undifferentiated hESCs were induced to differentiate into neuronal and endodermal lineage using protocols previously described [16, 19]. Briefly, hESCs dissociated into single cells were cultured in basal medium consisting of DMEM/F12 supplemented with 1 × N2, 1 × B27, 2 mM L-glutamine, 1 mM nonessential amino acids (all from Invitrogen Life Technologies), 0.11 mM β-mercaptoethanol, and 0.5 mg/ml bovine serum albumin (BSA, fraction V; both from Sigma Aldrich). To induce neuronal differentiation, 100 ng/ml hR Noggin (Stemgent, Cambridge, MA, http://www.stemgent.com) was added to the basal medium and cells cultured for 8 days. For definitive endoderm differentiation, 100 ng/ml hR Activin A (Stemgent) was added to basal medium and cells cultured for 9 days. To induce differentiation into mesenchymal stem cells (MSC) [20], hESCs were induced into embryoid bodies (EB) and cultured in suspension for 7 days with hCCM in low-attachment culture dishes. Approximately 70 EBs were plated onto 0.1% gelatin-coated dishes in growth medium (α-MEM, 10% FBS, 200 mM L-glutamine, and 10 mM NEAA). Outgrowing cells from EBs were cultured for up to 2 weeks. In subsequent culture, cells were seeded at a density of 7×10^3 per cm^2. To induced osteogenic differentiation, hESC-MSCs at passages 6–7 were incubated in α-MEM with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM β-glycerophosphate (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich), and 50 μM ascorbate-2-phosphate (Sigma Aldrich). Media was changed two times per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with Alizarin Red, pH 4.1 for 20 minutes at RT to verify differentiation.

Flow Cytometry Analysis

Cells were harvested using trypsin 0.25% EDTA and washed in cold BSA 0.5% (w/v) in DPBS and incubated at a concentration of 1×10^6 cells per ml in 1 μg/ml unconjugated goat anti-human IgG (Invitrogen Life Technologies) on ice for 15 minutes, to block nonspecific protein binding. Samples (2.5×10^5 cells) were incubated on ice with optimal dilution of fluorochrome-conjugated monoclonal antibodies in dark, and control samples were incubated with mouse IgG1 κ isotype control. All fluorochrome-conjugated antibodies and isotype controls were of the immunoglobulin G1 (IgG1) isotype and from BD Pharmingen (Sparks, MD, http://www.bd.com) and used at a 1:5 dilution. The following phycoerythrin (PE)-conjugated antibodies were used: CD29, CD49a, CD49c, CD49e, CD49f, SSEA-4, and Tra-1-60. After 30 minutes incubation, cells were washed twice with ice cold 0.5% BSA/DPBS. At least 10,000 events were acquired for each sample using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com), and cell flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson). The value (percentage) of positive cells for each antibody was calculated by subtracting the isotype control value from the detected value of each antibody.

Co-immunoprecipitation, SDS Page Electrophoresis, and Western Blot Analysis

Co-immunoprecipitation (Co-IP) assays, nuclear:cytoplasmic fractionation, and Western blot (WB) analysis were performed in triplicate from different biological samples and were validated in different hPSC lines. IP of 700 μg/sample was performed using ImmunoCruz IP/WB Optima C System (Santa Cruz Biotechnology, Dallas, TX, http://www.scbt.com) following the manufacturer's protocol. Protein extracts were prepared in CHAPS lysis and IP buffer with protease inhibitor and phosphatase inhibitor (all from FIVEphoton Biochemicals, San Diego, CA, http://www.fivephoton.com). Five μg of anti FAK-specific antibody (Clone 4.47; Millipore, Temacula, CA, http://www.millipore.com) and normal mouse IgG (Santa Cruz Biotechnology) were used. Samples were run in a SDS page electrophoresis and analyzed by WB to detect the interaction between N terminal domain of FAK (NT-FAK) and Nanog, Oct4, and Sox2. Whole cell lysates were prepared using Nonidet P40 buffer, while nuclear:cytoplasmic fractions were prepared using reagents and instructions provided by NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, http://www.thermoscientific.com). Protein lysates were separated on 7.5% SDS-polyacrylamide gel and transferred to polyvinylidine flouride (PDVF) membranes. Membranes were incubated with 5% milk in tris-buffered saline and tween 20 (TBST) (w/v) for 1 hour and then incubated with primary antibodies overnight at 4°C. Blots were incubated with peroxidase-coupled secondary antibodies (Promega, Madison, WI, http://www.promega.com) for 1 hour, and protein expression was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The antibodies used were: Oct4 (Santa Cruz Biotechnology), Nanog (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com), Sox2 (Cell Signaling Technology), FAK (Clone 4.47; EMD Millipore, Temacula, CA, http://www.emdmillipore.com), phospho Y397 FAK antibody (Abcam, San Francisco, CA), Integrin α6 (EMD Millipore), Lamin A/C (Santa Cruz Biotechnology), α-tubulin (Santa Cruz Biotechnology), and β actin (Cell Signaling Technology). ImageJ software (http;//rsb.nih.gov/ij) was used for quantification of WB. Relative signal intensity was calculated for all bands and compared to respective control groups after standardization with corresponding loading control. The value for control groups was arbitrary set up with a value of 1 or 0 in the presences/absences of protein expression, respectively. The relative signal intensity is expressed under each blot. For proteome profiler 400 mg protein cell lysate from H9-hESCs per sample were prepared using reagents and instructions provided by Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN, http://www.rndsystems.com).

Activation, Knockdown, and Block of Integrins

To activate integrins, hESCs were treated with 0.5 mM manganese chloride (Mn^{2+}; Sigma Aldrich) or 10 μg of anti-human β Integrin Antibody (Clone TS2/16; Thermo Scientific). As control, cells were treated either with 1 mM calcium chloride (Sigma Aldrich) or with 10 μg of mouse IgG (Santa Cruz Biotechnology), respectively. To knockdown integrin α6 in hESCs, a short hairpin RNA (shRNA) doxycycline (DOX)-inducible hESC lines was prepared. CHB10 cells were infected with lentivirus carrying TRIPZ shRNA-I*TGA6* constructs (GE Dharmacon, Lafayette, CO, http://www.dharmacon.gelifesciences.com). Infected cells were treated with 1 μg/ml or 0.5 μg/ml of Puromycin (Gibco Life Technologies) to select and maintain resistant clonal cell lines, respectively. To knockdown laminin α5 in hESCs, a DOX-inducible cell line containing shRNA sequence for *LAMA5* (Life Technology) was created (WIPO1e-H9ishLAMA5) from H9 cells and provided by Dr. Sean Palecek [21]. shRNA-ITGA6 hESCs and H9ishLAMA5 cell lines were treated with 0.1 mg/ml of DOX (Sigma Aldrich) during 72 hours. To test the role of integrin α6 and β1 in hESC adhesion, cells were treated with integrin-blocking antibodies during passaging to Matrigel and PMEDSAH plates. Briefly, mature colonies growing on PMEDSAH were manually dissociated in small and uniform clusters using the StemPro EZPassage Disposable Stem Cell Tool (Gibco Life Technologies), then 50 clusters of hESCs per group were resuspended in 1 ml of hCCM containing either 5 μg of anti-integrin β1 blocking antibody (Clone P5D2; EMD Millipore), 40 μg of anti-integrin α6 blocking antibody (Clone NKI-GoH3; EMD Millipore) or corresponding amount of IgG, as control group. Cells were subsequently seeded onto new Matrigel coated plates or PMEDSAH-GP and cultured overnight in incubators with high humidity and 5% CO_2 at 37°C. The following day, the number of newly formed colonies and EBs was counted for each group and a mean ± SD of attachment was calculated from three individual experiments.

Immunofluorescence Staining and Confocal Microscopy

Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, incubated in TBS with 0.1% sodium borohydride for 5 minutes and incubated in blocking solution (5% BSA/PBS) for 1 hour, all at RT. Then samples were incubated overnight at 4°C with primary antibodies diluted in 1% BSA. Next day samples were washed three times with PBS, followed by 1 hour exposure to secondary antibodies diluted in 1% BSA. Samples were then incubated for 10 minutes with DAPI, followed by three wash steps

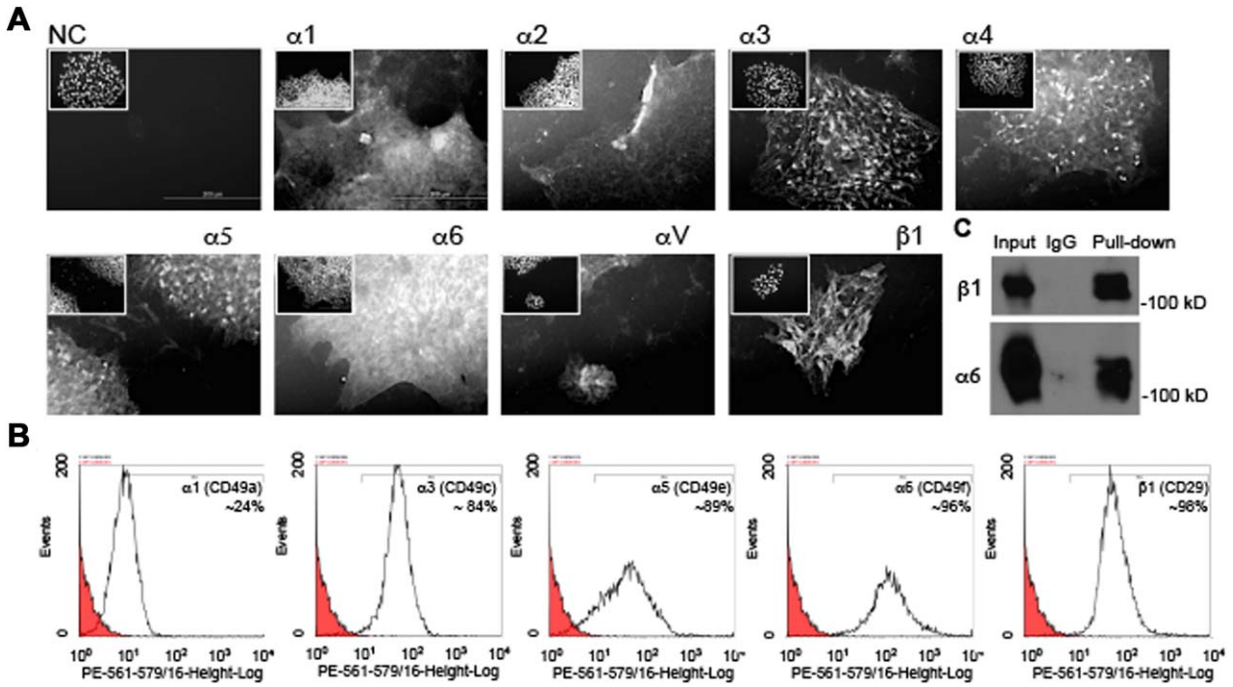

Figure 1. Profiling of integrin isoforms expressed in human embryonic stem cells (hESC). **(A)**: Representative micrographs of undifferentiated H1 hESC colonies growing on Matrigel and immunostained with antibodies specific for integrin isoforms α1, α2, α3, α5, α6, αV, and β1. Inlet micrographs illustrate 4′,6-diamidino-2-phenylindole (DAPI) staining of the corresponding colony. As NC, the first antibody was omitted. Scale bar = 200 μm. **(B)**: Histograms of flow cytometry analysis for specific integrin isoforms expressed in undifferentiated hESCs (H1) cultured on Matrigel indicating the mean ± SEM of positive cells. Control for fluorochrome IgG is shown in each histogram under the white area. **(C)**: Representative immunoblots of co-immunoprecipitation assays showing interaction between integrin β1 and integrin α6 in hESCs. Abbreviations: NC, negative control; PE, phycoerythrin.

with PBS. These steps were at RT and in dark conditions. Samples in glass slides were treated with BD Stabilizing Fixative solution (BD Biosciences) diluted in PBS for 5 minutes, then treated with ProLong Gold Antifade Reagent (Molecular Probes Life Technologies, Grand Island, NY, http://www.thermofisher.com), and mounted with a glass cover slide. Images were captured using a Nikon TE2000-S Epifluorescent microscope with a Nikon DS-Ri1 camera or using a Nikon A-1 Spectral Confocal microscope system. The following antibodies were used: Oct4 (Santa Cruz Biotechnology), Nanog (Cell Signaling Technology), Sox2 (Cell Signaling Technology), FAK (Clone 4.47; EMD Millipore), phospho Y397 FAK (Abcam, San Francisco, CA, http://www.abcam.com), and from Millipore: integrin α2, integrin α3, integrin α5, integrin α6, integrin αV, integrin β1.

RNA Isolation, Preparation, Quantitative Real-Time PCR, and Reverse Transcription PCR

Total RNA was reverse transcribed using the MultiScribe Reverse Transcriptase System (Applied Biosystems, Foster city, CA, http://www.appliedbiosystems.com). The ABI 7300 PCR and Detection System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) were used to conduct real-time polymerase chain reaction (PCR) in triplicate for each sample. Primers used are listed in Supporting Information Table 2. Human *β-ACTIN* was amplified as an internal standard. Relative quantification of gene expression was performed using the comparative C_T Method. For reverse-transcription PCR, total RNA was reverse transcribed using SuperScript One-Step RT-PCR with platinum Taq (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). In a single reaction (25 μl), 0.5 μg of total RNA and 20 pmol of forward (f) and reverse (r) primers were used. Primer sequences are in Supporting Information Table 2. The cDNA synthesis and predenaturation were carried out at 95°C for 2 minutes. The PCR amplification was performed for 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The final extension cycle was run at 72°C for 10 minutes. Finally, 10 μl of PCR reaction product were loaded onto a 1.0% agarose gel.

Statistical Analysis

Experiments were performed in triplicate and data are expressed as mean value ± SEM and analyzed by an unpaired *t* test. Levels of statistical significance were set at $p < .05$.

Results

Integrin α6 Expression Parallels the Undifferentiated State of hPSCs

The expression of integrin subunits α1 (CD49a), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f), αV, and β1 (CD29) was analyzed in undifferentiated H1-hESCs cultured on Matrigel by ICC (Fig. 1A). Quantitative analysis by flow cytometry (FACS) demonstrated that 96% and 98% of cells were positive for integrins α6 and β1, respectively. In addition, it was observed that greater than 80% of cells were α3 and α5 positive, whereas 11% were α1 positive (Fig. 1B). FACS analysis of other hESC cell lines (H9 and CHB10) and a hiPSC lines (hGF2-iPSCs) confirmed that both α6 and β1 integrins are

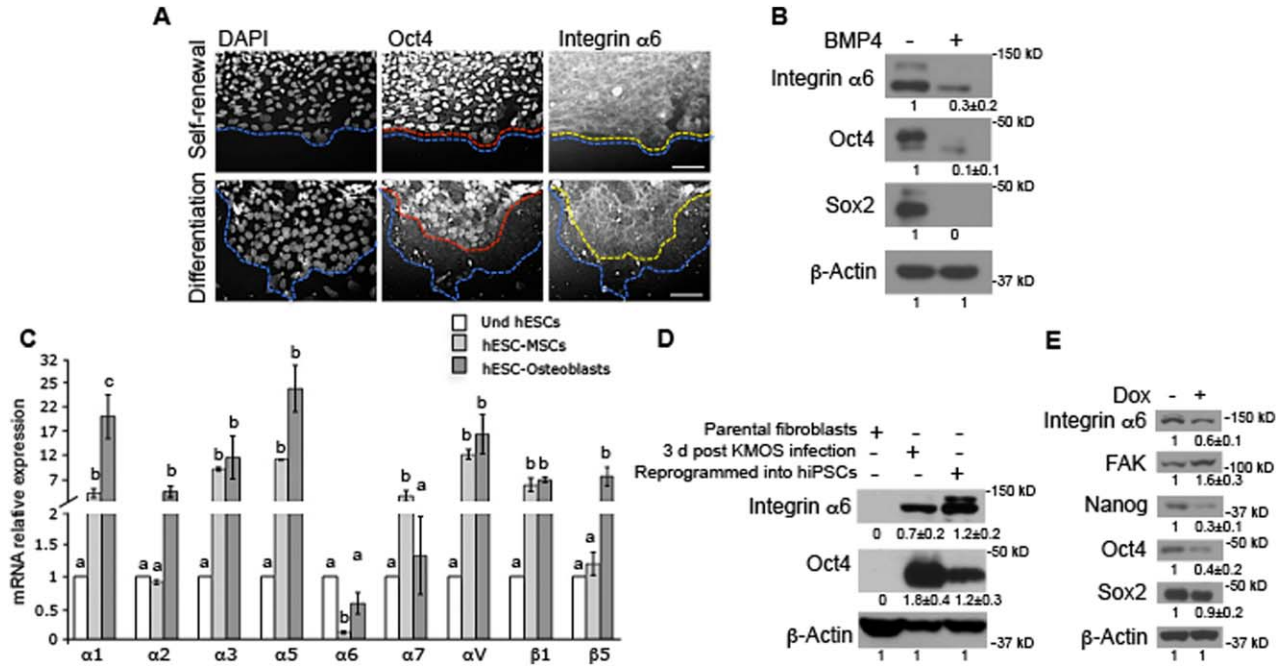

Figure 2. Integrin α6 expression parallels the pluripotent state of human pluripotent stem cells. **(A)**: Panel with representative micrographs of undifferentiated (top) and differentiating (bottom) hESC colonies immunostained with Oct4 and integrin α6 antibodies. DAPI was used to stain the nuclei of all cells in both colonies. The blue dotted lines delineate the borderline of colonies, while the red and yellow dotted lines indicate the borderline of Oct4 + and integrin α6 + cells. Scale bar = 200 μm. **(B)**: Representative immunoblot showing reduction in both Oct4 and integrin α6 protein levels during treatment with/without BMP2. β-Actin was used as the loading control. **(C)**: Graph showing change in RNA levels (mean ± SEM) of specific integrin isoforms expressed in undifferentiated hESCs (white columns) and in hESC differentiated into MSCs (light grey color) and osteoblasts (dark grey color). Quantitative polymerase chain reaction was performed from three independent replicates to calculate the mean ± SEM of the relative mRNA expression for each gene and different letters between columns for a specific integrin isoform indicate significant statistical differences ($p < .05$). **(D)**: Representative immunoblot showing increase in Oct4 and integrin α6 protein levels in protein lysates of parental fibroblasts 72 hours postinfection with Sendai Virus construct for KMOS (middle lane) and in resulting fully reprogram hiPSCs (right lane), compared to protein lysate of parental fibroblasts before infection (left lane). β-Actin was used as loading control. **(E)**: Representative immunoblot showing reduction in integrin α6, Oct4, Nanog, and Sox2 protein levels in lysates from a hESC line with carrying an inducible shRNA construct for integrin α6 and treated during 72 hours with Dox compared to nontreated cells. α-Tubulin was used as loading control. Protein expression in Western blot analysis was calculated in relative signal intensity for each band, and it is indicated below each blot as average ± SEM of three independent replicates. Abbreviations: BMP4, bone morphogenetic protein 4; DAPI, 4′,6-diamidino-2-phenylindole; Dox, doxycycline; FAK, focal adhesion kinase; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; KMOS, Klf4, c-Myc, Oct4, and Sox2; MSCs, mesenchymal stem cells.

predominantly expressed when cultured on defined substrates that support self-renewal, such as hr Laminin-511 [5], hr Vitronectin [6], and the synthetic polymer coating PMEDSAH-GP [17] (Supporting Information Figs. 1-7). By co-IP assays it was determined that integrin α6 interacts with integrin β1 in hESCs, suggesting that they form the α6β1heterodimer (Fig. 1C).

To investigate the role of integrins α6 and β1 in hPSC adhesion, H1-hESCs were incubated separately with their respective blocking antibodies during subculture to Matrigel-coated plates and PMEDSAH-grafted plates. Treatment with α6-blocking antibody did not suppress hESC adhesion to either Matrigel or PMEDSAH-GP, and colonies were formed on both substrates. However, incubation with β1-blocking antibody completely inhibited hESC adhesion and colony formation on both substrates (Supporting Information Table 1), suggesting that β1 integrin, and not the α6 subunit, is primarily involved in hPSCs adhesion to supportive extracellular matrices and PMEDSAH-GP.

To determine the extent to which expression of integrin α6 is associated with the undifferentiated state of hPSCs, the expression of integrin α6 was studied in conditions that induce stem cell differentiation. First, by ICC it was observed that when hESCs were exposed to differentiation medium, cells at the periphery of colonies exhibited reduced expression of both Oct4, a hallmark transcription factor of PSCs, and integrin α6 (Fig. 2A). By immunoblotting it was observed that undifferentiated hESCs and hiPSCs express two isoforms of integrin α6: the A form (higher molecular weight band) and the B form (lower molecular weight band) (Fig. 2B, 2D, 2E). After BMP4 treatment, a well-established inducer of hPSC differentiation [22], the A form band of integrin α6 was no longer detected, while the B form was reduced. Similarly, both Oct4 and Sox2 were no longer detected in the BMP4 treated cells (Fig. 2B). Pluripotent hESCs were also directed to differentiate into either MSCs or osteoblasts to determine how integrin subunits mRNA levels may be altered as a function of stem cell differentiation. Expression of integrin subunits *α1, α2, α3, α5, α7*, and *αV* increased during directed differentiation. In contrast, *α6* was the only integrin subunit that demonstrated significantly reduced RNA expression during differentiation to either MSCs or osteoblasts (Fig. 2C).

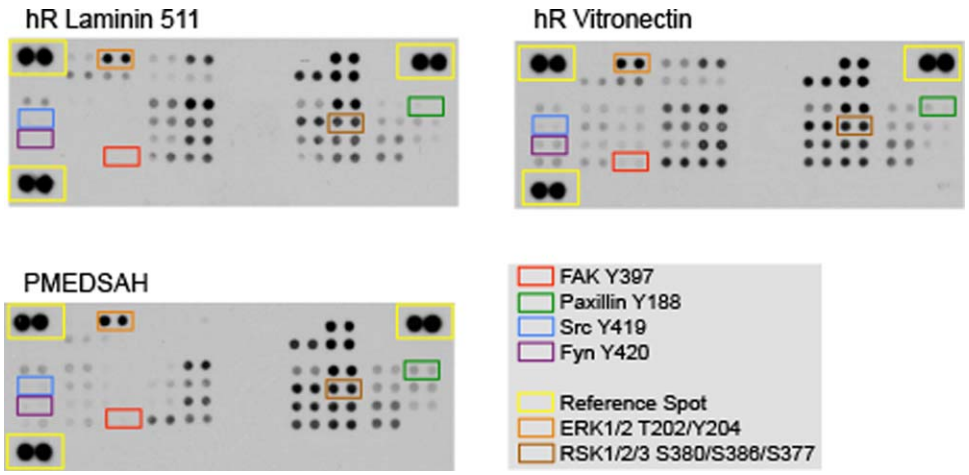

Figure 3. Focal adhesion kinase (FAK) and other kinases of focal adhesions are not phosphorylated in human embryonic stem cells (hESC). Protein lysates from undifferentiated hESCs cultured on hR Laminin 511, hR Vitronectin, and PMEDSAH were analyzed to detect relative levels of protein phosphorylation. In this array the phosphorylation of specific protein kinase was detected in duplicated spots with captured antibodies. Reference spot (highlighted in yellow box) demonstrated the incubation of whole array with Streptavidin-HRP. As a control, the phosphorylation of MAPK family members is highlighted (orange and brown box). Note the lack of phosphorylation in spots specific for FAK (red box), paxilin (green box), Src (blue box), and Fyn (purple box). Abbreviations: hR, human recombinant.

The expression of integrin α6 was studied during reprogramming of human fibroblasts into iPSCs to further explore the specificity of the distinctive changes in integrin α6 in pluripotent cells. hiPSCs were generated by overexpressing Klf4, c-Myc, Oct4, and Sox2 (KMOS) [2, 23] in fibroblasts. WB analysis demonstrated that integrin α6 was not expressed in parental fibroblasts, but the B-form band became detectable three days postinfection with KMOS, and both isoforms of integrin α6 were expressed in fully reprogrammed hiPSCs (Fig. 2D).

To investigate the role of integrin α6 in maintenance of the self-renewal phenotype in hPSCs, the knockdown of this integrin was induced in the CHB10-hESC line with a DOX-inducible shRNA. The induced knockdown of integrin α6 protein in hESCs, as observed by disappearance of A-isoform band and reduction in the B-isoform band, led to a reduction in Nanog, Oct4, and Sox2 levels (Fig. 2E), while treatment with DOX in control cells did not affect the expression of these transcription factors (data not shown). Interestingly, the knockdown of integrin α6 also increased the expression of FAK (Fig. 2E). Taken together, these results indicated that the heterodimer combination of integrin α6β1 is dominantly present in hPSCs, and that the expression of α6 is aligned with the undifferentiated state, since it is upregulated during hiPSCs formation and reduced during cell differentiation. Furthermore, a possible role of integrin α6 in maintaining self-renewal is indicated by the reduction in expression of pluripotent transcription factors in hESCs after its knockdown.

The Integrin-FAK Signaling Pathway is not Active in hPSCs

Because FAK mediates signaling from activated integrins [24, 25], we investigated the activity of this kinase in hPSCs. The activation of FAK is regulated by autophosphorylation at tyrosine (Y)397 [26], which is localized to a linker region between the NT and central kinase domains. The NT domain of FAK is known to repress the catalytic activity of the enzyme by intramolecular autoinhibition. Upon integrin-mediated activation, the carboxyl (C) T domain of FAK is recruited to focal adhesion sites, placing it in proximity and allowing the interaction between the cytoplasmic tail of integrin β subunits and the NT domain of FAK. This action results in relief of the NT domain-mediated autoinhibition and therefore, the activation of FAK catalytic activity [24]. The autophosphorylation at Y397 creates high-affinity binding sites for proteins with SH2 domains, including Src family kinase, which in turn phosphorylate other FAK tyrosine residues that mediate the activation of ERK1/2 and PI-3 kinase. Results obtained from a phosphokinase array analysis of protein lysates from undifferentiated H9-hESCs cultured on hr Laminin-511, hr Vitronectin, or PMEDSAH-GP showed no detectable phosphorylation of FAK at Y397 (Fig. 3). Similarly, no phosphorylation was detected in FYN [27], paxilin [28], and Src [29], proteins associated with FAK signaling and components of focal adhesions. Furthermore, only a small number of cells in undifferentiated H9-hESC colonies exhibited focal adhesions, and those were localized mainly to the colony periphery (Fig. 4 upper panel). However, incubation with an integrin β1-activating antibody induced multiple focal adhesions that were observed throughout the hESC colonies (Fig. 4 lower panel). Interestingly, the expression of Oct4 in the activated-antibody treated group was reduced and localized to the cytoplasm. Similar results were obtained with H1- and CHB10-hESCs and hiPSC lines (data not shown).

To investigate whether FAK is phosphorylated and activated during differentiation of pluripotent cells, H1-hESCs were differentiated into endoderm and ectoderm lineages, and protein lysates were analyzed by WB analysis. The cell lineage differentiation was confirmed by upregulation in mRNA levels of endodermal and ectodermal lineage specific genes, as well as reduction of pluripotent genes (Supporting Information Fig. 8). FAK was detected in undifferentiated hESCs as well as in both endoderm- and ectoderm-induced cells, however the phosphorylation of FAK at Y397 was only detected in lysates from differentiated cells (Fig. 5A). The phosphorylation status of FAK was also determined during induction to the

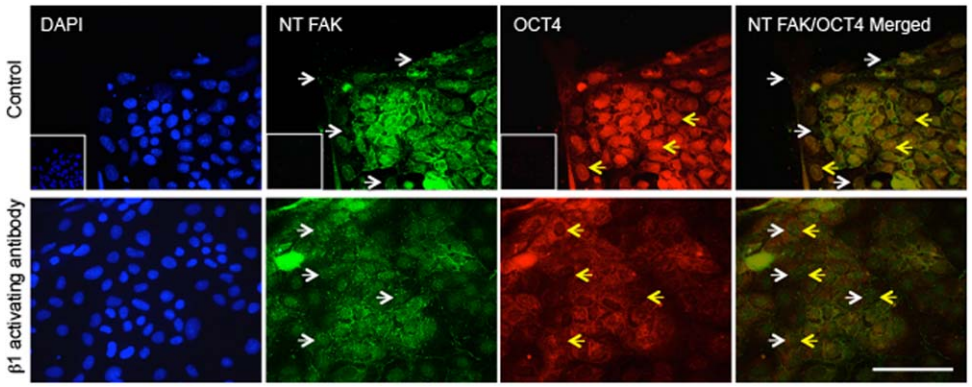

Figure 4. Activation of integrin β1 signaling induces focal adhesions and reduction in Oct4 expression in human embryonic stem cells (hESC). Representative micrographs of a hESC colony (top panel) immunostained with specific antibodies for NT FAK to identified focal adhesion sites (white arrows). Oct4 specific antibody was used to verify the undifferentiated state of hESCs (yellow arrows). Cells were counterstained with DAPI. Treatment with integrin β1 activating antibodies to undifferentiated hESCs (bottom panel) induces the formation of focal adhesion sites. Note this treatment also induced a reduction in nuclear expression of Oct4. Scale bar = 100 μm. Representative micrographs in inserts at the lower left corner of top panel show control immunostaining, in which first antibody was omitted. Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; FAK, focal adhesion kinase; NT, N terminal domain.

pluripotent state by reprogramming human fibroblasts into iPSCs. Phosphorylation of FAK at Y397 was detected in parental fibroblasts but was nondetectable in fully reprogrammed hiPSCs (Fig. 5B).

To investigate a potential role of FAK in self-renewal of hPSCs, undifferentiated H1-hESCs were treated with Mn^{2+}, a strong promoter of integrin function [30]. After 1 hour of Mn^{2+} treatment, phosphorylation of FAK at Y397 was observed, and the intensity of phosphorylation increased after 24 hours treatment (Fig. 5C). In parallel with the activation and phosphorylation of FAK, Oct4 levels were significantly reduced (Fig. 5C). Taken together, these results indicated that in undifferentiated hPSCs both integrin and FAK Y397 signaling are inactive. During differentiation of hPSCs, FAK is phosphorylated, while this kinase is dephosphorylated during reprogramming of human fibroblast into iPSCs. Interestingly, the activation of FAK by either an integrin β1-activating antibody (Fig. 4 lower panel) or Mn^{2+} treatment (Fig. 5C) induced significant reduction of Oct4 in hESCs.

The N-Terminal Domain of FAK is Located in the Nuclei of hPSCs and Interacts with Pluripotent Transcription Factors

Our results in undifferentiated hPSCs indicating inactivity of FAK at Y397 and lack of focal adhesion sites, as well as recent findings that the NT or FERM domain of FAK can be localized in cell nuclei [31–33] prompted us to investigate the immunolocalization of this FAK domain in hESCs. By confocal microscopy, differences in the immunolocalization of the NT domain of FAK between fibroblasts and hESCs were observed. The NT domain of FAK was observed in the cytoplasm but not in the nucleus of fibroblasts (Fig. 6A), while in hESCs, it was localized in both the cytoplasmic and the nucleus (Fig. 6A). Interestingly, a strong colocalization signal was detected between NT FAK and Sox2 in the nucleus of hESCs (Fig. 6A). The nuclear and cytoplasmic localization of NT FAK in hESCs was verified by fractionation studies and WB analysis (Fig. 6B). To further investigate the potential interaction of NT FAK with this pluripotent transcription factor, co-IP assays were performed (Fig. 6C). WB analysis demonstrated that the NT domain of FAK coimmunoprecipitated with both Oct4 and Sox2 in protein lysates from undifferentiated H1-hESCs (Fig. 6C). No co-IP between NT FAK and Nanog was observed in the same lysates (data not shown).

To investigate the extent to which Oct4 and/or Sox2 are able to regulate the nuclear localization of the NT domain of FAK, both transcription factors were overexpressed in HEK293T cells. Immunoblotting results indicated an increase in nuclear NT FAK when Oct4 was overexpressed in HEK293T cells compared to control lysates (Fig. 6D). All together these results indicated that in hPSCs, the NT domain of FAK is localized to the nucleus and interacts with Oct4 and Sox2. The upregulation of NT FAK in the nucleus by overexpression of Oct4 suggests a possible role of this transcription factor in controlling the activity of FAK.

Laminin α5 Maintains Integrin α6 Expression, Inactivity of FAK, and Expression of Pluripotent Transcription Factors in hPSCs

Because integrin α6β1 is the primary laminin receptor [34–36] and the dominant integrin heterodimer in hPSCs when laminin was not provided (i.e., hr Vitronectin and PMEDSAH), we hypothesized these cells might remodel the substrate in which they are growing by secreting and depositing their own laminin. Thus, we investigated whether hESCs secrete and deposit laminin on PMEDSAH-grafted plates. By ICC, laminin α5 was detected in CHB10-hESCs colonies cultured on PMEDSAH-GP (Fig. 7A). Next, we investigated the effects of knocking down laminin α5 in H9-hESCs, and it was observed that *laminin α5 (LAMA5), integrin α6 (ITGA6)*, and *Sox2* mRNA levels decreased compared to control cells in which Dox-induction was omitted. In contrast, *Nanog* and *Oct4* (also known as *POU5F1*) expression did not change (Fig. 7B). At the protein level, knockdown of laminin α5 decreased the expression of integrin α6 and Oct4, while the phosphorylation of FAK Y397 was enhanced (Fig. 7C). These results indicated that hPSCs secret and deposit laminin α5, and the knockdown of this ECM decreases the protein levels of integrin α6, induces the phosphorylation of FAK, reduces Oct4 and induces differentiation.

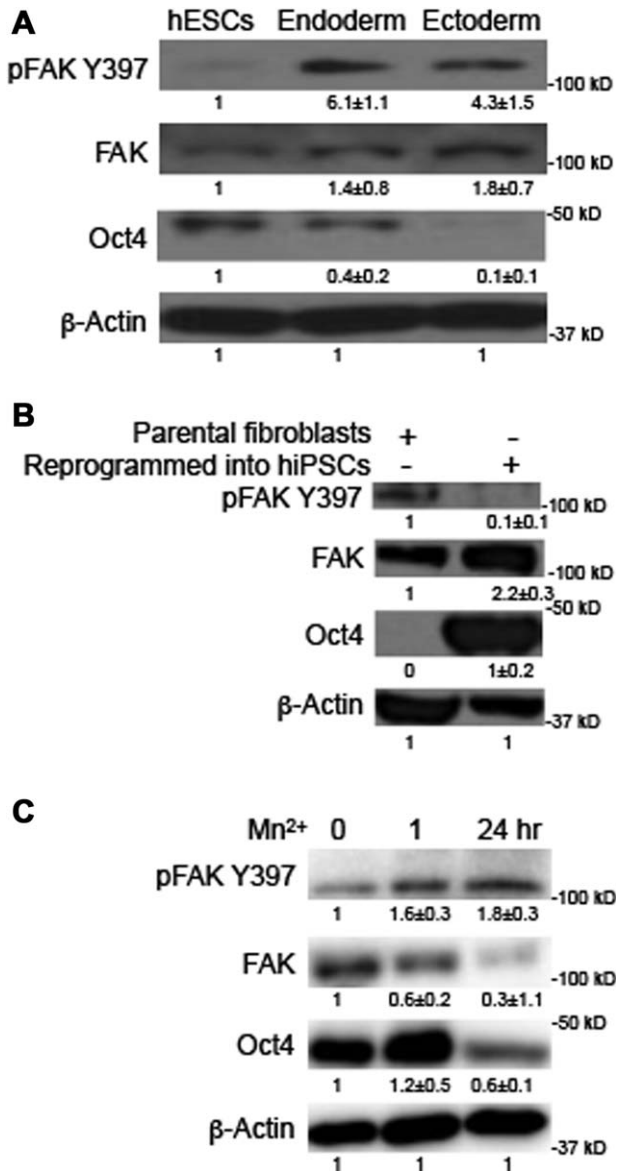

Figure 5. Phosphorylation of FAK correlates negatively with the undifferentiated state of human pluripotent stem cells. **(A)**: Western blot analysis showing changes in FAK phosphorylation at tyrosine (Y) 397 between undifferentiated hESCs and differentiated cells (endoderm and ectoderm derivatives). Oct4 was used to indicate the undifferentiated state of hESCs. **(B)**: Representative immunoblot showing changes in FAK phosphorylation between parental fibroblasts (Oct4-) and resulting human induced pluripotent stem cells (hFF-iPSCs), indicated by Oct4 protein levels. **(C)**: Immunoblot indicating an increase in phosphorylation of FAK in hESCs treated with Mn^{2+} during 1 and 24 hours, which resulted in reduction in Oct4 levels. β-Actin was used as loading control. Protein expression in Western blot analysis was calculated in relative signal intensity for each band, and it is indicated below each blot as average ± SEM of three independent replicates. Abbreviations: FAK, focal adhesion kinase; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells.

Discussion

We identified a correlation of integrin α6 expression and the hPSC state, and confirmed [5, 37–40] that α6β1 is the dominant integrin heterodimer present in undifferentiated hESCs (H1, H7, H9, CHB8, and CHB10) and hiPSCs (hGF2-iPSCs, hGF4-iPSCs, and hFF-iPSCs) in all in vitro cell culture conditions tested here; that is, culture on hr Laminin 511, hr Vitronectin, and PMEDSAH coated plates. When hESCs were differentiated, the expression of integrin α6 decreased, while reprogramming to pluripotency upregulated integrin α6 expression. Furthermore, the knockdown of integrin α6 by shRNA led to decreased expression of Nanog, Oct4, and Sox2 in hESCs, suggesting a role of this integrin in maintaining PSC self-renewal.

To gain molecular insight into the role of integrins in maintaining self-renewal of hPSCs, we examined the activity of FAK, a cytoplasmic kinase that mediates the signaling of active integrins. Surprisingly, the phosphorylation of FAK at Y397 was not observed in undifferentiated hESCs, suggesting inactivity of integrin signaling. Integrins can be present in three main conformational states: ligand-bound, active, and inactive [41], and our results indicated that in hPSCs, subpopulations of integrins co-exist in different activation states. Integrin β1 was present in the ligand-bound state, as was required for cell adhesion of hESCs. Focal adhesion sites were observed mainly at cells at the periphery of colonies, suggesting the presence of a subpopulation of active integrins. However, we found that in the majority of cells from undifferentiated hESC colonies, integrins were diffusely distributed and were present in the inactive state as illustrated by the absence of both focal adhesions and the absence of FAK, paxilin, Src, or Fyn phosphorylation. However, incubation with integrin-activating antibodies or Mn^{2+} resulted in β1 integrin activation, formation of focal adhesions, phosphorylation of FAK, and notably, in reduction in Oct4 expression. These data suggest that in undifferentiated hPSCs the majority of integrins are in an inactive state, but are poised to be activated. When integrins are active, Oct4 expression is reduced in hPSCs. However, the knockdown of integrin α6 in hESCs also induced the reduction of Nanog, Oct4, and Sox2. This suggests that in hPSCs, integrin α6 may be pro-pluripotency, by inhibiting the ability of integrin β1 to phosphorylate FAK at Y397 and with it preventing the repression of pluripotency transcription factors.

The interaction between integrins and signaling molecules is primarily regulated by the β isoform. The cytoplasmic tail of β integrins interacts with cytoskeletal elements and regulates molecules with enzymatic or regulatory functions within focal adhesion sites [9]. However, it has also been established that the cytoplasmic tail of α subunits regulate the localization to focal adhesions and activation of β1 integrin [42, 43]. In particular, evidence in myoblasts suggests that the integrin α6A isoform modulates the activity of integrin β1A and suppresses FAK phosphorylation [44]. This mechanism may also be functioning in hPSCs because phosphorylated FAK and focal adhesions were not detected in undifferentiated cells that express integrin α6. Our results confirm that undifferentiated hPSCs express both the A and B isoforms of integrin α6 and during directed differentiation of hESCs the A form was downregulated at a faster rate than the B form. Thus, we show that undifferentiated hPSCs express isoforms of integrin α6β1 that are known to suppress the phosphorylation and activity of FAK.

Interestingly, new roles for FAK have been identified that involve its localization and function in the nuclei of cells. For

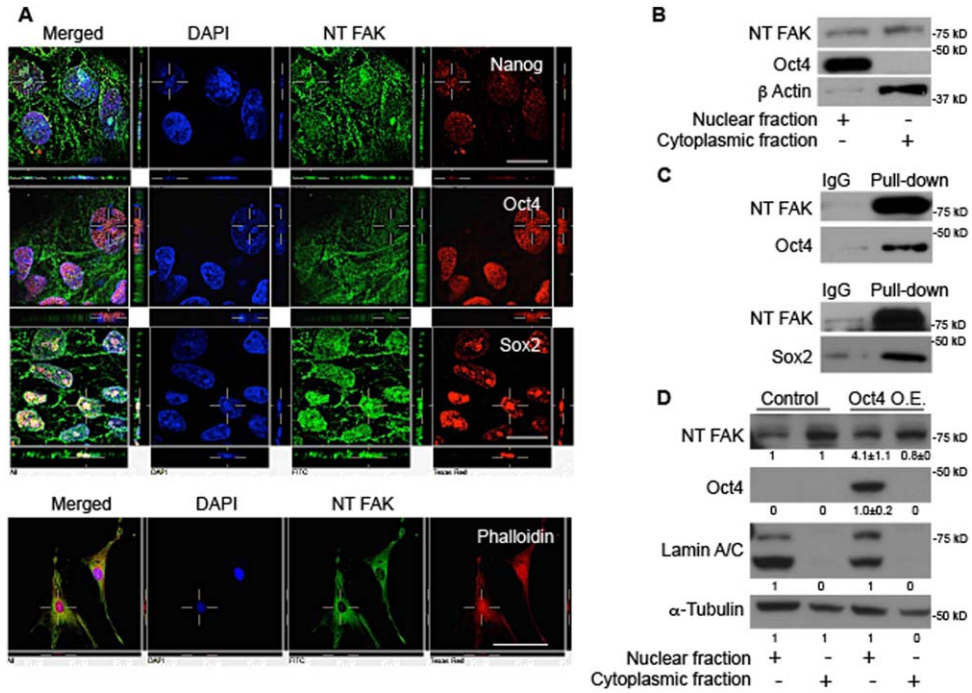

Figure 6. The NT domain of FAK interacts with pluripotent transcription factors in the nuclei of human embryonic stem cells (hESCs). **(A)**: Representative micrographs obtained by confocal microscopy showing nuclear and cytoplasmic immunolocalization of NT FAK in hESCs (top panel) compared to only cytoplasmic immunolocalization in fibroblasts (bottom panel). The undifferentiated state of hESCs is demonstrated by positive expression of Nanog, Oct4, and Sox2. Note the high colocalization signal in merge image between NT FAK and Sox2. Scale bar for top panel = 25 μm, for bottom panel = 100 μm. **(B)**: Representative immunoblot of nuclear and cytoplasmic fractions of hESCs showing detection of NT FAK in both cellular fractions. Oct4 was used to identify both the undifferentiated state of hESCs and the nuclear fraction, while β-actin to identify the cytoplasmic fraction. **(C)**: Representative immunoblots of coimmunoprecipitation assays showing interaction between NT FAK and both Oct4 and Sox2. **(D)**: Representative immunoblot of nuclear and cytoplasmic fractions of HEK 293T cells treated with/without retrovirus to overexpress (O.E.) Oct4. The overexpression of Oct4 increased NT FAK protein levels in the nuclei. Lamin A/C was used to indicate the nuclear fraction, while α-tubulin to show equal protein loading. Protein expression in Western blot analysis was calculated in relative signal intensity for each band, and it is indicated below each blot as average ± SEM of three independent replicates. Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; FAK, focal adhesion kinase; NT, N terminal domain; O.E., overexpression.

example, FAK has been shown to function as a scaffold protein that enhances CHIP E3 ligase-dependent [32] and Mdm2 E3 ligase-dependent [45] ubiquitination and degradation of Gata4 and p53, respectively. Furthermore, interconnections between FAK with pluripotent transcription factors have also been described recently. In glioblastoma cells, Oct4 upregulates the expression of FAK [46], and in 293 cells Nanog directly binds and regulates the NT domain of FAK [47]. Interestingly, both the nuclear localization signal and nuclear export signal sequences of FAK have been identified in its NT domain [48]. Therefore, we investigated the localization of FAK in hPSCs by confocal microscopy. Using an antibody that specifically recognizes the NT domain of FAK, we found the NT domain to be localized mainly in the cytoplasm and nuclei of undifferentiated hESCs, while minimally in focal adhesion sites of cells in the periphery of colonies. However, upon treatment with Mn^{2+} or β1 integrin-activating antibodies, FAK NT was observed in newly formed focal adhesions in cells that also expressed reduced levels of Oct4 in the nuclei. Furthermore, the nuclear localization signal of NT FAK in undifferentiated hESCs strongly colocalized with Sox2, and to a lesser extent with Oct4 and Nanog.

Co-IP studies demonstrated that NT FAK interacts with Oct4 and Sox2, but not with Nanog. This prompted us to investigate whether Oct4 and Sox2 may be involved in the nuclear localization of NT FAK. Overexpression of Oct4 in HEK293T cells led to increased NT FAK in nuclear fractions. This finding suggests that in hPSCs the activity of FAK is indirectly downregulated by Oct4 in two possible mechanisms: first, by promoting the transportation of NT FAK to the nuclei, or alternatively by promoting the expression of specific isoforms of integrins that block the activation of FAK. In fact, it is known that Oct4 and Sox2 bind to promoter regions of *ITGA6* and *ITGB1* [49, 50]. Accordingly, we also showed that during the reprogramming of fibroblasts into hiPSCs, integrin α6 is expressed as early as 3 days postinfection with reprogramming factors.

Another important observation obtained from our studies is that integrin α6β1 is the dominant heterodimer combination expressed in undifferentiated hPSCs, regardless of the supporting substrate on which they were cultured. Integrin α6β1 is the main receptor for laminin [8, 34–36], and it is known that laminins are a major component of Matrigel [51]. Thus, it was not surprising to find high expression of integrin α6β1 in hPSCs cultured on either Matrigel or hr Laminin-511. However, the main receptor for vitronectin is integrin αVβ5 [8, 52], and although αV was also detected, it was not as highly expressed as integrin α6β1 in hESCs cultured on hr Vitronectin. PMEDSAH-GP, in contrast, is a synthetic polymer coating that does not contain motifs that would likely mimic laminin or RGD [17]. However,

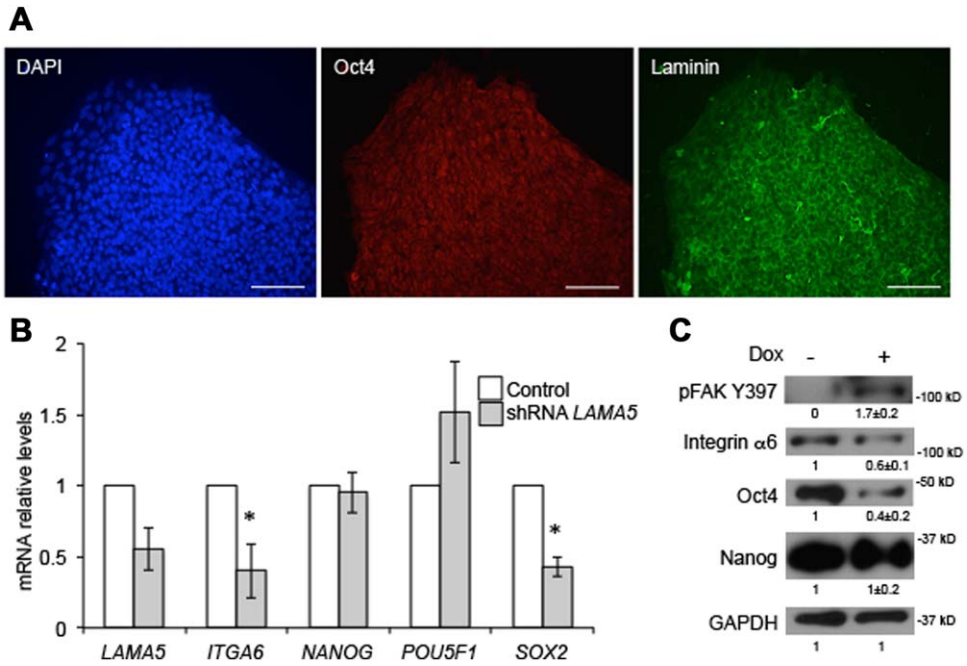

Figure 7. Laminin α5 is secreted and deposited by human embryonic stem cells (hESC). **(A)**: Representative micrographs of a hESC colony cultured on PMEDSAH and immune-stained with laminin and Oct4 antibodies. DAPI was used as counterstaining to show the delimitations of the colony. Scale bar = 150 μm. **(B)**: Graphs showing RNA relative levels (mean fold change ± SEM) in genes expressed in hESCs after knockdown of *LAMA5* by shRNA. Asterisk indicates statistical differences ($p < .05$). **(C)**: Representative immunoblot showing changes in protein levels in hESCs after knockdown of *LAMA5* by shRNA. To induce *LAMA5* knockdown, cells were treated with Dox during 72 hours. Note the increase in phosphorylation of FAK while reduction in integrin α6 and Oct4 in *LAMA5* knockdown cells. GAPH was used as loading control. Protein expression in Western blot analysis was calculated in relative signal intensity for each band, and it is indicated below each blot as average ± SEM of three independent replicates. Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; Dox, Doxycycline; FAK, focal adhesion kinase.

α6β1 integrin was identified as the dominant integrin present in hESCs growing on PMEDSAH-GP. This suggests, that hPSCs may remodel the substrate in which they are cultured, by degrading existing ECM, as well as secreting and depositing new ECM. Recently, it has been demonstrated that hESCs secrete and deposit laminin α5, and that this ECM is necessary for survival and expansion of these cells [21]. Here, we demonstrated that hESCs cultured on PMEDSAH-GP express laminin α5, and the knockdown of this protein resulted in reduction of integrin α6 and Oct4 protein levels, and *Sox2* at RNA levels, while an increase in FAK phosphorylation was observed and Nanog protein levels were not affected. The reduction of Oct4 at protein levels but not at RNA expression suggests a post-transcriptional effect of laminin knockdown on Oct4. However, our data indicated that laminin knockdown does not have an effect in Nanog regulation, as reported previously [21]. These results however are intriguing since knockdown of integrin α6 resulted in reduction on protein levels of both Nanog and Oct4. Further investigation will be required to elucidate whether the lack of laminin effects on Nanog are due to a pathway other than integrin α6. Taken together, these findings suggest a molecular circuit in hPSCs linking laminin α5 deposition, expression and synthesis of integrin α6β1, suppression of FAK activity, and maintenance of pluripotency transcription factors.

Conclusion

In summary, our results demonstrate that undifferentiated hPSCs express high levels of integrin α6 that prevent integrin β1 from phosphorylating FAK at Y397. However, upon activation of β1 integrin by Mn^{2+} treatment or activating antibody, numerous focal adhesion sites are formed, FAK becomes active, and Oct4 nuclear expression is decreased. Other associations between the expression of integrin α6β1, FAK activity, and self-renewal of hPSCs included decreased levels of Oct4 after the knockdown of integrin α6 or laminin α5, which resulted in phosphorylation of FAK. These observations, together with the results indicating that pluripotency transcription factors Oct4 and Sox2 interact and upregulate the expression of N-T FAK in the nucleus, suggest that several mechanisms exist in hPSCs to prevent the phosphorylation and activity of FAK and the exit from the state of self-renewal.

Acknowledgments

We thank Dr. Joerg Lahann and his lab for their assistance in preparing PMEDSAH plates, and Dr. Shinya Yamanaka for Addgene plasmids 17217, 17219, 17220, and 17226. The National Institutes of Health grant R01DE016530-08 supported this work. L.G.V-D. is currently affiliated with Department of Biological Sciences, Oakland University, Rochester, MI.

Author Contributions

L.V.-D.: conception and design; P.K.: financial support; L.V.-D., J.K., A.L., and S.P.: collection and assembly of data; L.V.-D.

and P.K.: data analysis and interpretation and manuscript writing; L.V.-D., J.K., A.L., S.P., and P.K.: final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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