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Endogenous production of fibronectin is required for self-renewal of cultured mouse embryonic stem cells

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Abstract

Pluripotent cells are attached to the extracellular matrix (ECM) as they make cell fate decisions within the stem cell niche. Here we show that the ubiquitous ECM protein fibronectin is required for self-renewal decisions by cultured mouse embryonic stem (mES) cells. Undifferentiated mES cells produce fibronectin and assemble a fibrillar matrix. Increasing the level of substrate fibronectin increased cell spreading and integrin receptor signaling through focal adhesion kinase, while concomitantly inducing the loss of Nanog and Oct4 self-renewal markers. Conversely, reducing fibronectin production by mES cells growing on a feeder-free gelatin substrate caused loss of cell adhesion, decreased integrin signaling, and decreased expression of self-renewal markers. These effects were reversed by providing the cells with exogenous fibronectin, thereby restoring adhesion to the gelatin substrate. Interestingly, mES cells do not adhere directly to the gelatin substrate, but rather adhere indirectly through gelatin-bound fibronectin, which facilitates self-renewal via its effects on cell adhesion. These results provide new insights into the mechanism of regulation of self-renewal by growth on a gelatin-coated surface. The effects of increasing or decreasing fibronectin levels show that self-renewal depends on an intermediate level of cell-fibronectin interactions. By providing cell adhesive signals that can act with other self-renewal factors to maintain mES cell pluripotency, fibronectin is therefore a necessary component of the self-renewal signaling pathway in culture.

Keywords

extracellular matrix; fibronectin; integrin; self-renewal; embryonic stem cell

Introduction

Mouse embryonic stem (mES) cells, derived from the embryonic day 3.5 inner cell mass, are grown in culture as self-renewing, pluripotent cells that retain the ability to differentiate into any cell type in culture and contribute to all adult tissues upon re-injection into blastocysts [1]. Undifferentiated mES cells are characterized by a specific set of markers, including the surface marker SSEA-1 [2], the signaling molecule Stat3 [3], and the transcription factors Nanog [4, 5] and Oct4 [6, 7], that are down-regulated upon differentiation. The self-renewal state of mES cells is controlled by a network of signaling pathways that prevents differentiation and promotes cell proliferation [8, 9]. Soluble factors such as the cytokine

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leukemia inhibitory factor [LIF] [10, 11] and various components of serum [12] have been identified as upstream effectors of these pathways, and often have pleiotropic effects [13].

In addition to these soluble factors, reports have indicated that stem cell behavior can also be influenced by the composition of the physical substrate on which cells are grown [14, 15]. Physical interactions with the cellular microenvironment primarily involve adhesion to the extracellular matrix (ECM), a three-dimensional protein network that is essential for functional arrangement of cells in tissues [16]. Most studies examining the role of ECM-dependent signaling in stem cell fate decisions have focused on its effects on differentiation [17]; indeed, protocols for lineage-specific differentiation often use growth on specific ECM substrates to achieve maximum efficiency [12, 17–19]. By contrast, the role of the ECM in mES cell self-renewal remains poorly defined. Though protocols for propagation of undifferentiated mES cells often call for a gelatin-coated substrate [20], the involvement of endogenously produced ECM proteins in ES cell adhesion and self-renewal signaling has not been thoroughly explored.

One of the most ubiquitous ECM proteins is fibronectin, a modular protein that is assembled into a fibrillar matrix and contains binding sites for cells and other ECM proteins (Figure 1A) [21, 22]. Fibronectin matrix plays a major role in cell rearrangements that are crucial for embryogenesis. Fibronectin is initially found in the pre-implantation embryo, localized between cells of the inner cell mass [23, 24]. Fibronectin production and assembly into a matrix are also required during later stages of development, especially for cellular movements during and after gastrulation [25, 26]. In mice, null mutations in fibronectin or its major integrin receptor α5α1 are embryonic lethal [25–28].

Due to its presence in the *in vivo* environment from which mES cells are derived, and its demonstrated impact on signaling in a plethora of other cell types [29], we set out to define the contribution of the ECM protein fibronectin to maintenance of the self-renewal state in feeder-free cultures. We demonstrate that the amount of fibronectin on the substrate provides a crucial signal to control the mES cell choice between self-renewal and differentiation.

Materials And Methods

Cell culture and reagents

Mouse CCE mES cells [30, 31] and Nanog-GFP mES cells [32] were gifts of Dr. Ihor Lemischka (Mt. Sinai Medical School). Cells were cultured on 0.1% gelatin (Chemicon) in mES cell medium (Dulbecco's Modified Eagle Medium [DMEM], 15% mES-cell screened Fetal Bovine Serum (FBS, Hyclone Laboratories, Logan UT), 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium-pyruvate, 100 U/ml penicillin/10 μg/ml streptomycin (all GIBCO), 1.3×10^{-4} M 1-thioglycerol (Sigma), in the presence of 1000 U of LIF/ml (Millipore). Cells were passaged every other day and replated at a density of 2.6×10^4 cells/cm^2, for no more than 15 passages. Where indicated, cells were plated in Knockout medium in which FBS is replaced with 15% Knockout Serum Replacement (GIBCO). Knockout Serum Replacement contains various growth factors but no fibronectin or other adhesive ECM proteins (GIBCO, personal communication), and is able to maintain self-renewal of mES cells under standard culture conditions.

Immunofluorescence and microscopy

Phase contrast images of cells growing in culture were taken using a Nikon TMS microscope equipped with a Photometrics CoolSnap camera. For immunofluorescence, cells were plated on the indicated surfaces coated on glass coverslips. For gelatin coating, cover slips were incubated in 0.1 mg/ml Poly-D-Lysine (Sigma) for 5 minutes, washed in sterile

water, and dried for 1 hour at 37°C, at which point they were coated with 0.1% gelatin for 45 minutes at room temperature. Cells were fixed and permeabilized at room temperature in 3.7% (w/v) formaldehyde (Sigma) in PBS + 0.5 mM $MgCl_2$ for 15 minutes followed by 0.5 % NP40 (Calbiochem) in PBS + Mg^{2+} for 15 minutes. Primary and secondary antibodies were used in 2% BSA-PBS at the following dilutions: R457 rabbit anti-fibronectin [33] (1:100), mouse anti-vinculin (Sigma, 1:300), rhodamine goat anti-rabbit IgG (Mole cular Probes, 1:400), and rhodamine goat anti-mouse IgG (Molecular Probes, 1:400). For experiments with FUD, cells were grown on gelatin-coated dishes for 48 hrs in LIF containing KO medium supplemented with III-11C or FUD at a concentration of 2.4 μg/ml (0.3 μM). Floating colonies were allowed to settle in a microfuge tube to reduce colony clumping that can occur with centrifugation. Floating and attached cells were in fixed in 4% paraformaldehyde in PBS for 10 min. Post- fixation immunofluorescence staining was performed as described above. Coverslips were mounted on slides using Fluoroguard Anti-Fade reagent (BioRad) and sealed with nail polish. Visualization and image capture were performed with a Nikon TE2000U microscope equipped with a Cooke SensiCamQE High Performance camera. Images were adjusted identically in Adobe Photoshop.

Quantification of cell areas

Cell spreading on gelatin and on increasing concentrations of fibronectin was analyzed in at least four experiments. Cell areas were measured using IP Lab software. Changes in cell area are represented as % change from gelatin. For each condition, the graph represents averaged aggregate data from five unique fields [AVE], while error bars represent relative standard error. To determine relative standard error, the following formula was used: SEMRELx = (SEMx/AVEx + SEMGel/AVEGel) × (AVEx/AVEGel), where × = condition [FN0.1, FN1, or FN10]. For each condition, the standard error of the mean was determined by dividing the standard deviation of the average by the square root of the number of samples (SEMx = STDEVx/√nx).

Flow cytometry

Cells were collected, spun down, and resuspended in PBS containing 2% FBS (Hyclone). To disperse non-adherent cell aggregates, collected cells were suspended in 1 ml Accumax (Innovative Cell Technologies) and placed on a Labquake rotator for 5 minutes at room temperature. Dispersed cells were collected and resuspended in PBS plus 2% FBS to a density of 10^7 cells/ml. For staining, cells were blocked in 2% goat serum (Sigma), and stained with 0.8 μl of SSEA-1 (Developmental Studies Hybridoma Bank, 0.1 mg/ml) followed by 1 μl rat anti-mouse IgM-Phycoerythrin [PE] (Beckman Coulter, 0.1 mg/ml), each in 100 μl PBS plus 2% FBS. Flow cytometric analysis was performed using a LSRII flow cytometer (BD Biosciences, San Jose, CA). Excitation of fluorochromes was at 488 nm. Fluorescence emission was collected through a 525/50 bandpass filter for GFP, and a 575/26 bandpass filter for PE. The mean fluorescence intensity (MFI) values were determined using FACSDiVa software (BD Biosciences, San Jose, CA). MFIs for a representative experiment with Nanog-GFP cells (Figure 3) were 1784 on gelatin, 1698 on FN0.1, 1626 on FN1, and 1645 for cells on FN10. For SSEA-1 staining, MFIs were 7349 on gelatin, 6415 on FN0.1, 6433 on FN1, and 5734 on FN10. In Figure 6, MFIs averaged 2276 on $III_{9–10}$, 2113 with FN, 2336 at day 0, and 1385 without added FN. A minimum of 20,000 events was collected for each sample.

Cell lysis and immunoblotting

Cells were washed with cold PBS and lysed with modified RIPA buffer as described [34]. Total protein concentration was determined using a BCA protein assay (Pierce Chemical Co., Rockford, IL). Normalized, reduced samples were separated by SDS-PAGE, transferred to nitrocellulose, and blocked overnight in buffer A (25 mM Tris-HCl pH 7.5, 150 mM

NaCl, 0.1% Tween-20). Blots were incubated with primary, then secondary antibodies at the following dilutions in buffer A: rabbit anti-pFAK-Y^{397} (Invitrogen, 1:2000), rabbit anti-FAK (Millipore, 1:1000), rabbit anti-Stat3 (Cell Signaling, 1:1000), mouse anti-pStat3-Y705 (Upstate, 1:2000), horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, 1:20000), horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, 1:10000). Blots were developed using ECL reagents (Pierce). Where indicated, blots were placed in stripping solution (62 mM Tris-HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and incubated at 65°C with gentle agitation for 20 minutes. Stripped blots were washed in buffer A overnight, and then re-probed as indicated. Blots were scanned using a Chemi-Doc system and bands were quantified using Quantity One® software (Bio-Rad). Quantities were averaged over multiple experiments and the Student's t-test was used for statistical analyses.

Quantitative reverse transcriptase-PCR

RNA was isolated from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Purified RNA was resuspended in water, and concentrations were determined using a ND-1000 Spectrophotometer (NanoDrop). 1 μg of total RNA was reverse transcribed using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). cDNA was then used as a template for real-time RT-PCR using primers constructed as described by Ivanova et al. [8]. Quantitative RT-PCR was performed using the Mx3000P QPCR System (Stratagene). cDNA was added to a reaction mix containing Brilliant® II SYBR QPCR Low Rox Master Mix (Stratagene) and 100 nM of each primer. PCR reaction conditions were: 10 minutes at 95°C, followed by 45 cycles of 30 seconds at 95°C, 60 seconds at 60°C, 60 seconds at 72°C. Data analysis was performed using MxPro™ QPCR Software (Stratagene). All data values were normalized to GAPDH.

siRNA transfection

Transfections were carried out following the manufacturer's protocol for mouse mES cells using Lipofectamine-2000 (Invitrogen). siRNAs used were either SMARTpool siRNAs against mouse fibronectin, or ON-TARGET*plus* Non-targeting siRNAs that do not target any known mouse gene (Dharmacon). Mock (Lipofectamine-2000 in Opti-Mem) and untransfected (Opti-Mem only) controls behaved identically to non-targeting controls (data not shown). Cells were trypsinized, counted, spun down, and resuspended in Knockout medium lacking antibiotics to a density of 2×10^5 cells/ml. Two ml of cells were plated on gelatin in the presence of LIF in a 35 mm dish. 1 μg/ml of rat plasma fibronectin in CAPS buffer [10 mM CAPS, pH 11, 150 mM NaCl] was added to the medium.

After 24 hours, siRNA-treated cells were trypsinized and replated at 2.6×10^4 cells/cm^2 on gelatin. For inclusion of exogenous fibronectin, rat plasma fibronectin was added to the medium to a final concentration of 1 μg/ml; otherwise, an equivalent volume of CAPS buffer was added. After 1 day, non-adherent cells were transferred to polystyrene dishes and cultured in suspension for the duration of the experiment. Cells plated in the presence of exogenous fibronectin were passaged every other day. To test the effects of the $III_{9–10}$ integrin binding domain, cells were replated onto surfaces coated with a solution of 50 μg/ml GST-$III_{9–10}$, and passaged every other day.

Quantification of cell adhesion

A mES cell colony was defined as any cluster of 5 or more contiguous cells. The number of colonies per non-overlapping microscopic field was counted in multiple fields ($n \geq 4$) to give total colonies. Each field encompassed ~10% of the total well surface area. Medium, including any floating clusters, was removed by aspiration, PBS was added and after gentle rocking, PBS was aspirated. The number of colonies/field was counted in multiple fields ($n \geq 4$) to determine the number of attached colonies. Graphs represent average number of

colonies/field for two independent experiments. Error bars represent one standard deviation from average value.

Fibronectin purification, metabolic labeling, and ELISA

Fibronectin was purified from rat plasma using gelatin-Sepharose chromatography [35]. The fibronectin 70 kDa fragment was expressed in insect cells and purified as described [33]. The fibronectin $III_{9–10}$ domain was expressed in bacteria as a GST-fusion protein, and purified by glutathione-agarose affinity chromatography (GE Healthcare Life Science) according to the manufacturer's instructions. Proteins were stored in CAPS buffer. For coating surfaces, purified proteins were diluted to the appropriate concentration in CAPS buffer. Wells were coated for two hours at 37° C, then blocked in 1% BSA in PBS for 30 minutes at 37° C. E. coli expressing recombinant His-tagged FUD or His-III-11C were kindly provided by Dr. Jane Sottile (Rochester University Medical Center). Proteins were purified as described [36].

Where indicated, cells were metabolically labeled for 24 hours with 50 μCi [^{35}S]Methionine (MP Biomedicals)/ml of medium. Fibronectin was isolated from labeling medium using gelatin-Sepharose binding, separated by SDS-PAGE under reducing conditions, and quantified using a Storm 860 PhosphorImager system (GE Healthcare Life Sciences). Samples were normalized before loading by total protein concentration determined using a BCA protein assay (Pierce Chemical Co., Rockford, IL).

For the fibronectin ELISA, cells were plated on a gelatin-coated surface or on tissue culture plastic for 24 hours, then released from the substrates using Versene (GIBCO) and gentle pipetting. Wells were blocked with 1% BSA in PBS and then incubated with either R457 or R184 rabbit anti-fibronectin polyclonal antiserum [33] (1:1000 in 1% BSA-PBS) for two hours at room temperature followed by a 1 hr incubation with biotinylated goat-anti-rabbit IgG (GIBCO, 1:2000 in 1% BSA-PBS). Bound antibodies were detected with streptavidin β–galactosidase (Invitrogen, 1:5000 in 1% BSA) and nitrophenyl-B-D-galactopyranoside (Sigma, 1 mg/ml in substrate buffer [50 mM Na_3PO_4 pH 7.2, 1.5 mM $MgCl_2$] as recommended by the manufacturer. The reaction was stopped by addition of 0.5 M Na_2CO_3. Samples were quantified by absorbance at 405 nm on an ELx800 Plate Reader (BioTek Instruments), and values were quantified relative to empty tissue culture plastic wells.

Results

mES cells synthesize and assemble fibronectin

Mouse CCE mES cells remain in an undifferentiated state when grown on gelatin-coated substrates in the presence of serum and LIF [1]. Under these conditions, cells grow in compact colonies and express self-renewal markers like SSEA-1 (data not shown), as well as the α5α1 integrin receptor [27, 28] that mediates fibronectin matrix assembly. Analysis of secreted proteins demonstrated that mES cells produce fibronectin (see Figure S2), while immunofluorescent staining confirmed assembly of fibronectin into a fibrillar matrix (Figure 1B).

The assembly of fibronectin matrix by undifferentiated mES cells suggests that fibronectin may contribute to stem cell self-renewal. By contrast, previous reports have demonstrated that growth on fibronectin induces differentiation of mES cells [15, 17]. However, these earlier reports tested the effects of fibronectin at a single protein concentration and did not investigate cell-autonomous production of fibronectin. To reconcile these previous reports with our observations, we first determined the effects of fibronectin substrate levels on self-renewing mES cells by comparison to cells grown on a gelatin substrate. Cells were plated in medium containing LIF on gelatin or on substrates prepared by coating with fibronectin

solutions of 0.1, 1.0 and 10 μg/ml [FN0.1, FN1, FN10, respectively]. FN10 approximates the coating concentration previously shown to induce differentiation (~2 $\mu g/cm^2$) [17]. ELISA of surfaces 24 hours after cell plating showed similar amounts of surface fibronectin on gelatin and on FN0.1 substrates (with relative ELISA signals of 1.0 and 0.7, respectively) and significantly more fibronectin on FN1 and FN10 substrates (ELISA signals of 2.1 and 2.4, respectively).

mES cell phenotypic differences with increasing fibronectin concentration were readily observed within a few hours of plating, and could be quantified by measurements of cell areas. The majority of cells grown on FN0.1 remained rounded with few discernible vinculin-positive focal adhesions, similar to cells grown on gelatin (Figure 2A, B). When grown on either FN1 or FN10, cells were well spread and showed peripherally localized vinculin staining indicative of focal adhesions (Figure 2C, D). Quantification of cell areas showed that, on average, cells grown on FN1 or FN10 were significantly more spread than those on gelatin or FN0.1 (Figure 2E). Therefore, mES cell shape and size increase with the amount of fibronectin on the substrate, showing that the effects of fibronectin on mES cell behavior are concentration-dependent.

Increased substrate fibronectin induces loss of mES cell self-renewal

Undifferentiated mES cells are rounded and grow in compact colonies on gelatin (see Figure 1B), while differentiation is accompanied by changes in cell shape [1]. To determine whether changes in morphology with fibronectin substrate concentration correlate with changes in self-renewal, we monitored GFP expression under the control of the Nanog promoter, using a Nanog-GFP mES cell line [32] (Figure S1A). Like mES cells, these GFP-expressing cells grow as colonies on gelatin in the presence of LIF, and express self-renewal markers such as Nanog, Oct4, and SSEA-1. Importantly, they also make fibronectin and assemble it into a fibrillar matrix (Figure S1B). Nanog-GFP cells were grown on increasing amounts of fibronectin or on gelatin. GFP expression was quantified by flow cytometry within 6 days of plating in order to capture changes that occur early in the cell response to fibronectin substrates. As expected, control cells grown on gelatin maintained a GFP expression profile identical to the starting Nanog-GFP mES cell population (Figure 3A). A nearly identical GFP distribution was observed for cells grown on FN0.1. However, when cells were grown on FN1 or FN10, we observed a shift of the expression profile toward decreased GFP expression (Figure 3A). A shift to lower expression was also observed for SSEA-1 (Figure 3B).

Self-renewal efficiency was determined by quantifying GFP and SSEA-1 expression levels. As expected, for cells grown on either gelatin or FN0.1, the proportion of self-renewing, GFP-positive or SSEA-1-positive cells was virtually identical to the starting population (Table S1). For cells grown on FN1 or FN10, we observed a statistically-significant reduction in the proportion of the population expressing these markers, indicating reduced numbers of self-renewing cells (Table S1). To confirm loss of self-renewal efficiency, we analyzed endogenous levels of the self-renewal markers Nanog and Oct4 by quantitative RT-PCR. Similar to our observations by flow cytometry, we found that cells grown on FN1 or FN10 had statistically-significant reductions in expression of these self-renewal markers (Figure 3C). Our results with FN10 agree with previous studies showing that fibronectin at a concentration of ~2 $\mu g/cm^2$ induces loss of self-renewal [15]. Furthermore, fold changes in Nanog and Oct4 expression mirror observations made in previous studies of mES cell differentiation [37, 38]. Notably, with FN0.1 substrates, we show that fibronectin at a lower concentration (~0.02 $\mu g/cm^2$) maintains the self-renewal state.

Increased fibronectin increases integrin signaling and inhibits self-renewal signaling

Integrin-dependent signaling is mediated by focal adhesion kinase (FAK) [39]. We found that, on FN10 substrate in the presence of the self-renewal factor LIF, cells were maximally spread (see Figure 2E) and levels of activated phospho-Y397 FAK were significantly increased by 2.1-fold compared to cells on gelatin or FN0.1 (2.1 ± 0.7, $p < 0.01$, n=6). Total FAK was unchanged (Figure 3D). Together, these data demonstrate that increasing cell-matrix interactions increased integrin signaling. Since expression of self-renewal markers was inversely correlated with cell areas, this result argues that increasing cell-fibronectin interactions enhances integrin signaling to promote loss of self-renewal, even in the presence of LIF. LIF affects self-renewal through Stat3 [3, 40], and activation of Stat3 by phosphorylation on tyrosine 705 (pStat3-Y705) is indicative of self-renewal [3]. The difference between pStat3-Y705 on gelatin and FN0.1 was not significant. However, pStat3-Y705 levels decreased significantly, more than 4-fold on FN10 compared to gelatin. Average pStat3 levels on FN10 were 0.24 ($\pm$ 0.14, $p < 0.02$, n=3) versus 1.0 on gelatin (Figure 3E), showing that LIF does not maintain Stat3 activation when fibronectin levels are high. Thus, increasing the number of mES cell-fibronectin interactions induces integrin signaling and loss of self-renewal possibly by effects on LIF signaling downstream of FAK.

Fibronectin knockdown inhibits mES cell adhesion

The effects of fibronectin substrate concentration suggest that the extent of cell adhesion and spreading may determine mES cell-fate decisions between self-renewal and differentiation. Furthermore, the fact that undifferentiated mES cells produce fibronectin suggests that endogenous protein may play an active role in promoting self-renewal. Having demonstrated that increased concentrations of substrate fibronectin induced loss of self-renewal, we next set out to determine if interactions with fibronectin are necessary for self-renewal. Therefore, mES cells were treated with siRNAs against fibronectin to eliminate its production (Figure S2). Treated cells grown in the presence of LIF were unable to adhere to the gelatin-coated surface when plated in Knockout medium that does not contain serum-derived ECM proteins. Instead, cells formed floating cell clusters reminiscent of differentiating embryoid bodies (Figure 4A). Adhesion was quantified by counting total and attached cell colonies; virtually no colonies were attached after fibronectin knockdown (Figure 4E). Trypan blue and propidium iodide stainings confirmed that the non-adherent cells had not undergone anoikis (data not shown). Identical treatment using control non-targeting siRNAs, which do not target any known mouse gene, did not impair cell adhesion to gelatin (Figure 4B, E), suggesting that the effect is specific to knockdown of fibronectin.

Fibronectin mediates ES cell adhesion to the gelatin substrate

Cell adhesion to gelatin could be restored to cells treated with fibronectin siRNA by adding purified fibronectin to the medium (Figure 4C, E), demonstrating that under these conditions, fibronectin is both necessary and sufficient for cell adhesion to the gelatin substrate. Adhesion was also rescued by plating siRNA-treated cells on surfaces coated with the $III_{9–10}$ fragment of fibronectin (see Figure 1A) containing the RGD and synergy sites for $\alpha5\beta1$ integrin binding (Figure 4D, E), indicating that cells adhere to fibronectin using integrins. The inability of cells to adhere to gelatin in the absence of fibronectin demonstrates that gelatin alone is insufficient to promote cell adhesion. Therefore, we wondered if a gelatin substrate was necessary under self-renewal conditions. In contrast to adherent cells on gelatin substrates (Figure 5A-i), mES cells plated on tissue culture plastic without a gelatin coating were unable to attach to the surface (Figure 5A-ii).

Fibronectin binds to gelatin [41], and analysis of surface composition by ELISA with anti-fibronectin antibodies showed 3-fold more fibronectin bound to a gelatin-coated substrate than to uncoated tissue culture plastic (Figure 5B). Furthermore, analysis of media collected

from cells plated on uncoated and on gelatin-coated surfaces showed that significantly more fibronectin was found in the medium for cells plated on tissue culture plastic than on gelatin (Figure 5B *inset*), indicating that fibronectin was indeed depleted from the medium by binding to the gelatin coating. Further support for a role for fibronectin binding to gelatin in mES cell adhesion was provided by adding proteins that can affect interactions between fibronectin and gelatin. The N-terminal 70 kD fragment of fibronectin [33] contains the gelatin-binding site (see Figure 1A) and can compete with fibronectin for binding to gelatin. The FUD peptide from the F1 protein of *S. pyogenes* binds to fibronectin at its gelatin-binding site and can compete with gelatin for binding to fibronectin [42, 43]. 70kD or FUD were added to culture medium at a final concentration of 0.7 μM and 0.3 μM, respectively. Compared to the adhesion on gelatin of untreated cell colonies (Figure 5C-i), mES cells grown in the presence of excess 70 kD fragment showed a dramatic reduction in adhesion to gelatin (Figure 5C-ii). A more pronounced effect was observed in the presence of FUD where no attached colonies were detected (Figure S3A). Addition of FUD also significantly reduced the amount of mES cell fibronectin bound to gelatin as detected by ELISA (Figure S3B). In addition to affecting fibronectin-gelatin binding, both 70 kD and FUD inhibit fibronectin matrix assembly [22, 36]. Examination of attached and floating colonies for differences in fibronectin fibril numbers showed that mES cells treated with III-11C control peptide had fibrillar matrix while cells grow in the presence of FUD showed few if any fibrils (Figure S3C). These results suggest that 70 kD fragment and FUD inhibit cell adhesion by blocking the fibronectin-gelatin interaction and argue that a major role for gelatin is to immobilize fibronectin on the substrate to promote cell adhesion. Furthermore, changes in fibronectin matrix that correlate with loss of cell adhesion to gelatin suggest that matrix assembly may be needed for maximal adhesion of mES cell colonies.

Combined, these observations demonstrate that, under normal self-renewal conditions, the use of a gelatin coating serves to bind fibronectin secreted by mES cells, thus providing a fibronectin substrate for integrin-dependent mES cell adhesion.

Loss of adhesion induces loss of self-renewal capacity

Growth of mES cells in suspension induces formation of embryoid bodies and is commonly used to induce differentiation [44]. To determine whether fibronectin siRNA-treated, non-adherent mES cell colonies were differentiating, GFP expression was measured by flow cytometry of siRNA-treated Nanog-GFP cells plated on gelatin in Knockout medium, with or without exogenous fibronectin. Knockdown of fibronectin caused a shift in the GFP profile toward lowered expression in the non-adherent cells (Figure 6A), despite the presence of LIF. This shift was prevented by addition of exogenous fibronectin to the siRNA-treated cells (Figure 6A). Cells treated with non-targeting siRNAs retained their GFP expression profiles, both in the presence and absence of exogenous fibronectin (data not shown). Fibronectin siRNA-treated cells replated onto $III_{9–10}$ also maintained GFP expression over time (Figure 6A), confirming a role for integrin-dependent adhesion in self-renewal.

Quantification of flow cytometry profiles confirmed that the % GFP-positive cells decreased over time with fibronectin siRNA treatment, but did not change if fibronectin was added to the culture medium (Table S2). Likewise, no decrease in % GFP-positive cells was observed if cells were plated on $III_{9–10}$ (Table S2), or for cells treated with control siRNAs (data not shown). Endogenous levels of the self-renewal markers Nanog and Oct4 were also reduced in non-adherent cells, relative to cells plated in the presence of exogenous fibronectin (Figure 6B). Observed reductions in these self-renewal markers are consistent with previous reports for mES cell differentiation into embryoid bodies [37, 38]. Thus, loss of integrin-mediated adhesion to fibronectin induced loss of self-renewal markers even in the presence of LIF, indicating defects in mES cell self-renewal.

Self-renewal is correlated with adhesion-dependent integrin signaling

As expected, we found that integrin signaling was affected in non-adherent, fibronectin siRNA-treated mES cells. Analysis of pFAK-Y397 levels showed that activation of FAK was, on average, 1.7-fold higher in adherent cells compared to FAK in non-adherent cell aggregates, while total FAK levels were not changed (Figure 6C). Our earlier experiments using different concentrations of substrate fibronectin demonstrated that changes in integrin signaling were correlated with changes in LIF-dependent self-renewal signaling pathways. We therefore hypothesized that the loss of self-renewal marker expression in non-adherent mES cells lacking fibronectin could result from decreased signaling from LIF through Stat3. Surprisingly, when we analyzed pStat3-Y705 levels, we found no significant difference in fibronectin siRNA-treated cells with or without fibronectin added (average pStat3 levels of 0.84 and 1.0, respectively) (Figure 6D), indicating that LIF stimulation of this pathway occurs in the absence of cell-fibronectin adhesion. These results show that FAK activation correlates with mES cell adhesion and self-renewal and suggest that adhesion signals cooperate with other signaling pathways to control mES cell self-renewal and differentiation in this system.

Discussion

By analyzing the role of adhesion in mES cell self-renewal, we have found that mES cell production of, and interaction with, the ECM protein fibronectin plays a crucial role in maintaining pluripotency in cell culture. Mouse ES cells self-renew when cultured on a gelatin substrate in medium containing LIF and other soluble factors. Our results show that gelatin captures fibronectin produced by mES cells, generating a surface that supports cell adhesion and self-renewal. Increasing substrate fibronectin levels induced mES cell spreading with formation of focal adhesions and stimulation of FAK activity. Concomitantly, expression of Nanog, Oct4, and SSEA-1 self-renewal markers was down-regulated, and phospho-Stat3 levels were reduced even though the cells were cultured in the presence of LIF. Conversely, reducing fibronectin expression by siRNA knockdown caused mES cells cultured in LIF-containing medium to become non-adherent and to reduce FAK signaling and expression of self-renewal markers. Loss of self-renewal capacity was prevented by addition of exogenous fibronectin to restore adhesion. Our results therefore suggest that fibronectin regulates a switch between mES cell self-renewal and differentiation through its effects on cell adhesion.

These findings suggest that maintenance of mES cell self-renewal depends on an intermediate level of cell-fibronectin interactions. At the optimal concentration of fibronectin, cell-ECM interactions synergize with signals from other sources including LIF to support self-renewal. A decrease in fibronectin below a certain threshold leads to loss of ECM signals and FAK signaling, and promotes differentiation reminiscent of embryoid body formation, while an increase in cell-fibronectin interactions increases cell spreading, FAK activity, and differentiation. Variations in fibronectin levels may work to shift cells to a non-committed state between undifferentiated and differentiated committed states mES cells can exist in a “pre-differentiated” state, where down-regulation of Nanog does not induce irreversible loss of self-renewal capacity, but merely pre-disposes mES cells toward differentiation [45]. Nanog levels have been shown to fluctuate within a mES cell population [46]. Therefore, changing the level of interaction with fibronectin may not be fully inducing differentiation, but rather promoting the expansion of cells expressing lower levels of Nanog, thus changing mES cells to a non-committed state in which they are primed to differentiate. While we observed differential expression of lineage-specific differentiation markers under conditions that promoted loss of self-renewal, no specific lineage was consistently induced (unpublished observations), suggesting that lineage-specific commitment to differentiation requires additional instructive cues.

ES cell production of fibronectin might provide an adhesive gauge that integrates with signals from other pathways to help mES cells decide between self-renewal and differentiation. The ability of the level of cell interactions with fibronectin to impact self-renewal parallels the concentration effects of Oct4, a known regulator of pluripotency. Oct4 must be maintained at an appropriate level for self-renewal, and any increase or decrease from the optimum level was found to induce differentiation [47]. In addition to cell-ECM interactions, mES cell-cell interactions mediated by E-cadherin also contribute to pluripotency. E-cadherin has recently been shown to play a critical role in somatic cell reprogramming [48]. Modulation of E-cadherin-mediated cell-cell adhesion impacted cell survival and self-renewal of human ES cells [49]. Therefore, it seems likely that self-renewal depends on a balance of cell-ECM and cell-cell adhesion. In fact, mES cell-assembled fibronectin matrix may also contribute important cell adhesive signals since we observed a correlation between loss of mES cell adhesion and reduced fibronectin matrix. Coordinated changes in adhesive signals from the ECM and from other cells could shift the balance, and this could then prime stem cells to respond to other extracellular cues that induce lineage-specific differentiation.

Maintenance of self-renewal appears to depend on synergistic effects of myriad self-renewal factors. Removal of LIF induces differentiation [10, 11, 50], but the presence of a LIF signal is insufficient for self-renewal in the absence of serum [12], implying that signaling from both is necessary but neither is sufficient for self-renewal. Fibronectin has the ability to affect numerous signaling pathways, some of which have been linked to mES cell self-renewal and proliferation. Growth on fibronectin leads to activation of PI-3 kinase [51] and c-Myc [52], both of which are necessary for proliferation of undifferentiated mES cells [9, 53, 54]. Fibronectin has been linked to mES cell proliferation through a Rho GTPase-caveolin-1 pathway [54]. Moreover, in certain cell lines, fibronectin activates Stat3 [55], linking cell-ECM interactions to this key pathway for mES cell self-renewal.

Paradoxically, pathways that participate in self-renewal also promote differentiation. For example, LIF, in addition to activating Stat3 self-renewal signaling, activates the MAP kinase pathway, which promotes differentiation [56]. MAP kinase is also activated by fibronectin signaling through FAK [57], and we found that FAK activation in mES cells increased with higher substrate levels of fibronectin. Fibronectin also activates the Rho GTPase/ROCK pathway, which controls cytoskeletal organization and cell shape [58]. Rho-mediated cell shape changes have been linked to cell proliferation and differentiation [59, 60]. Differentiating cells on FN1 or FN10 were much more spread than undifferentiated cells grown on gelatin, indicating that the effects of fibronectin on differentiation might occur, in part, through cell shape changes. Non-adherent cells forming three-dimensional clusters clearly experience different mechanical forces than adherent cells. Morever, we did observe that mES cells grown on increasing concentrations of $III_{9–10}$ did not spread or lose expression of self-renewal markers (unpublished observations), suggesting that mechanical forces, mediated through integrin-based adhesion, are in fact necessary to induce loss of self-renewal.

Our results provide new insights into the role played by integrin-mediated adhesion in pluripotency in culture. Mouse ES cells isolated from the inner cell mass were initially maintained on a feeder layer of embryonic fibroblasts [61, 62], which were subsequently shown to produce LIF [10, 11, 50]. However, the presence of LIF is insufficient for self-renewal in the absence of adhesion [63, 64], as cells grown on tissue culture plastic without feeders formed non-adherent, differentiating embryoid bodies [61, 62]. Primary fibroblasts used as feeder layers for mES cells assemble a matrix rich in fibronectin so, under those culture conditions, fibronectin is present as a substrate for mES cell adhesion.

mES cells express integrin receptors for other ECM proteins, including laminin, and self-renewal can be maintained by growth on a laminin-coated surface [65]. Indeed, we found that culturing mES cells on increasing concentrations of laminin-111 produced the same down-regulation of self-renewal factors as was observed for fibronectin (unpublished observations). However, laminin does not bind to gelatin, and was therefore unable to compensate for loss of cell adhesion upon siRNA-induced fibronectin knockdown. Thus, under the standard feeder-free/gelatin mES cell culture conditions, laminin does not support adhesion or self-renewal. The use of gelatin in feeder-free conditions allows for propagation of undifferentiated mES cells via its ability to uniquely bind fibronectin (from either endogenous or exogenous sources), thereby presenting an adhesive substrate that promotes self-renewal.

Conclusion

Interaction with fibronectin is an essential component of the self-renewal program for feeder-free culture of mES cells. Fibronectin has a unique ability to bind to gelatin and its immobilization on gelatin-coated surfaces provides the appropriate level of adhesion to support ES cell pluripotency. Either increasing or decreasing fibronectin levels leads to mES cell differentiation even in the presence of LIF. These findings suggest that signals generated by mES cell-produced fibronectin work along with signals from soluble factors to promote self-renewal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ECM	extracellular matrix
mES	mouse embryonic stem
LIF	leukemia inhibitory factor

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Highlights

- Mouse ES cells express fibronectin and assemble a fibronectin matrix
- Mouse ES cell self-renewal in feeder-free culture depends on fibronectin
- Fibronectin promotes ES cell adhesion to the gelatin substrate
- ES cell differentiation is induced by increasing or decreasing fibronectin levels
- Fibronectin expressed by ES cells provides an adhesive signal for self-renewal

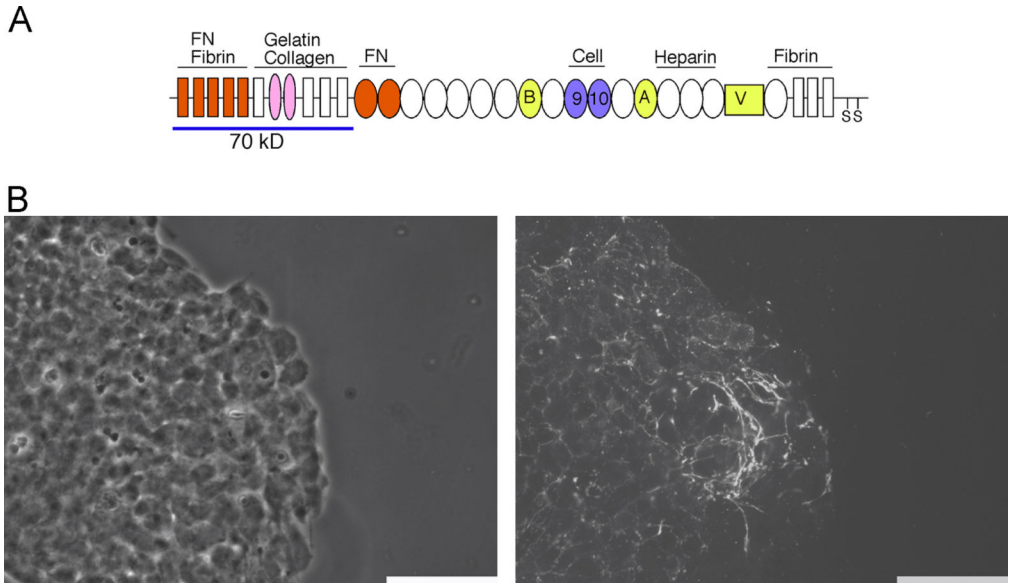

Figure 1.

Fibronectin is produced and assembled by undifferentiated mES cells. (A) Domain structure of fibronectin (adapted from [21]). (B) mES cells grown on gelatin in mES cell medium in the presence of LIF were fixed, stained with R457 anti-fibronectin antiserum followed by rhodamine-goat anti-rabbit IgG, and analyzed by phase contrast (left) and fluorescence (right) microscopy. Scale bars = 50 μm.

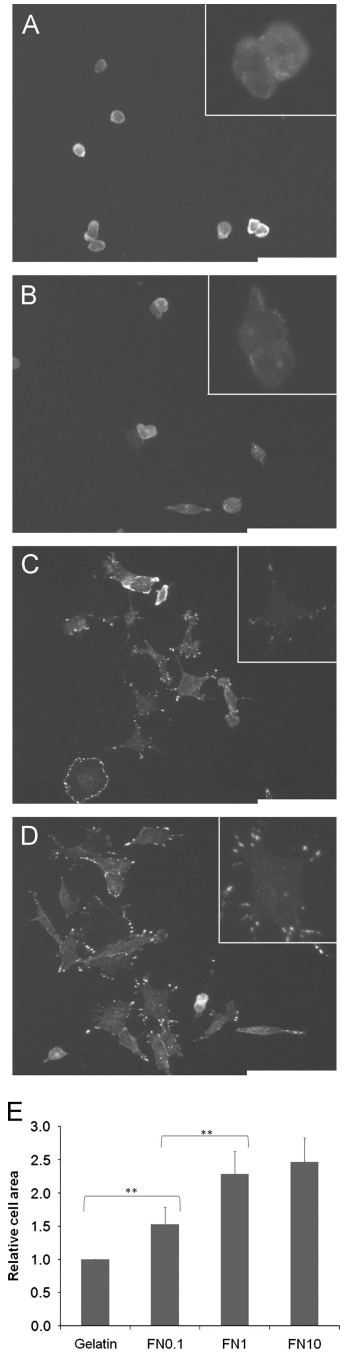

Figure 2.

Increased substrate fibronectin induces mES cell spreading. (A–D) mES cells were plated in the presence of LIF on a gelatin-coated surface (A) or on surfaces coated with fibronectin [FN] at 0.1 (B), 1 (C), or 10 (D) μg/ml. After 6 hours, cells were fixed, permeabilized, and stained with anti-vinculin monoclonal antibody followed by rhodamine-goat anti-mouse IgG. Scale bars = 50 μm. Insets show magnified images of cells. (E) Cell areas were quantified as described in Materials and Methods for mES cells grown on the indicated ECM coatings in the presence of LIF for 4 hours. Bars represent average cell areas relative to average area of cells on gelatin and are from a representative experiment. Error bars

represent standard error in the mean for each condition, relative to that for gelatin. Statistical analyses were performed relative to Gelatin. ** $p < 0.01$.

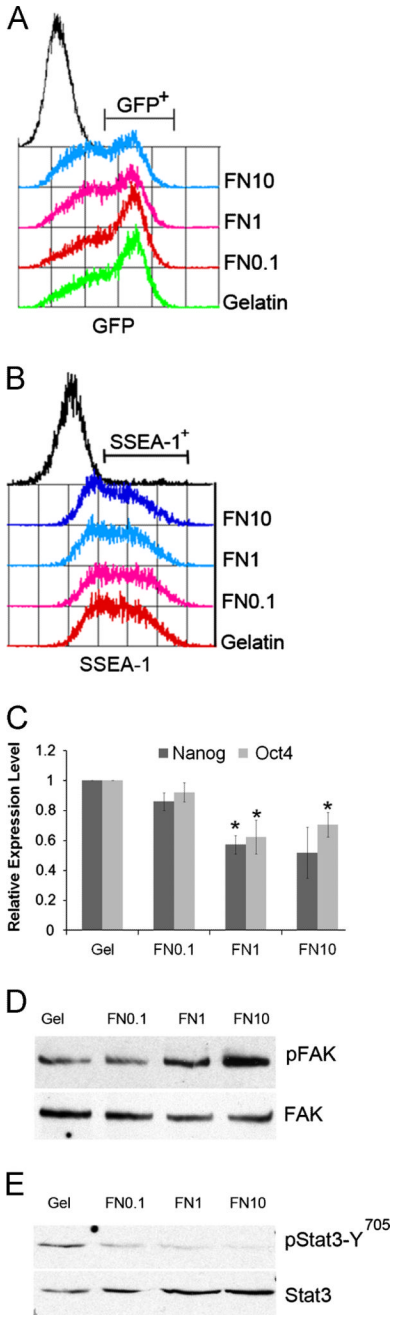

Figure 3.

Fibronectin affects self-renewal markers and FAK phosphorylation. (A, B) Nanog-GFP mES cells were grown on the indicated surfaces for six days, passaging every other day. After six days, expression of GFP (A) or SSEA-1 (B) was quantified by flow cytometry. SSEA-1 was detected with anti-SSEA-1 antibody and fluorescent secondary antibody. Black profile is from non-fluorescent mES cells (A) or unstained mES cells (B). (C) RNA was extracted from cells grown as in (A–B), and quantitative RT-PCR was performed using primers for Nanog and Oct4. Values for fibronectin samples were normalized to gelatin [Gel] (set at 1). Graphs represent the average of triplicate samples from two independent experiments, and error bars indicate +/− one standard deviation from the mean. * $p < 0.05$. (D, E) mES cells grown on indicated substrates were lysed after 6 days, equal amounts of

protein were separated by SDS-PAGE, proteins were detected by immunoblotting with anti-pFAK-Y397, anti-FAK, or anti-pStat3-Y705 antibodies. pStat3 blots were stripped and re-probed with anti-Stat3 antibodies. Blots are representative of at least two experiments.

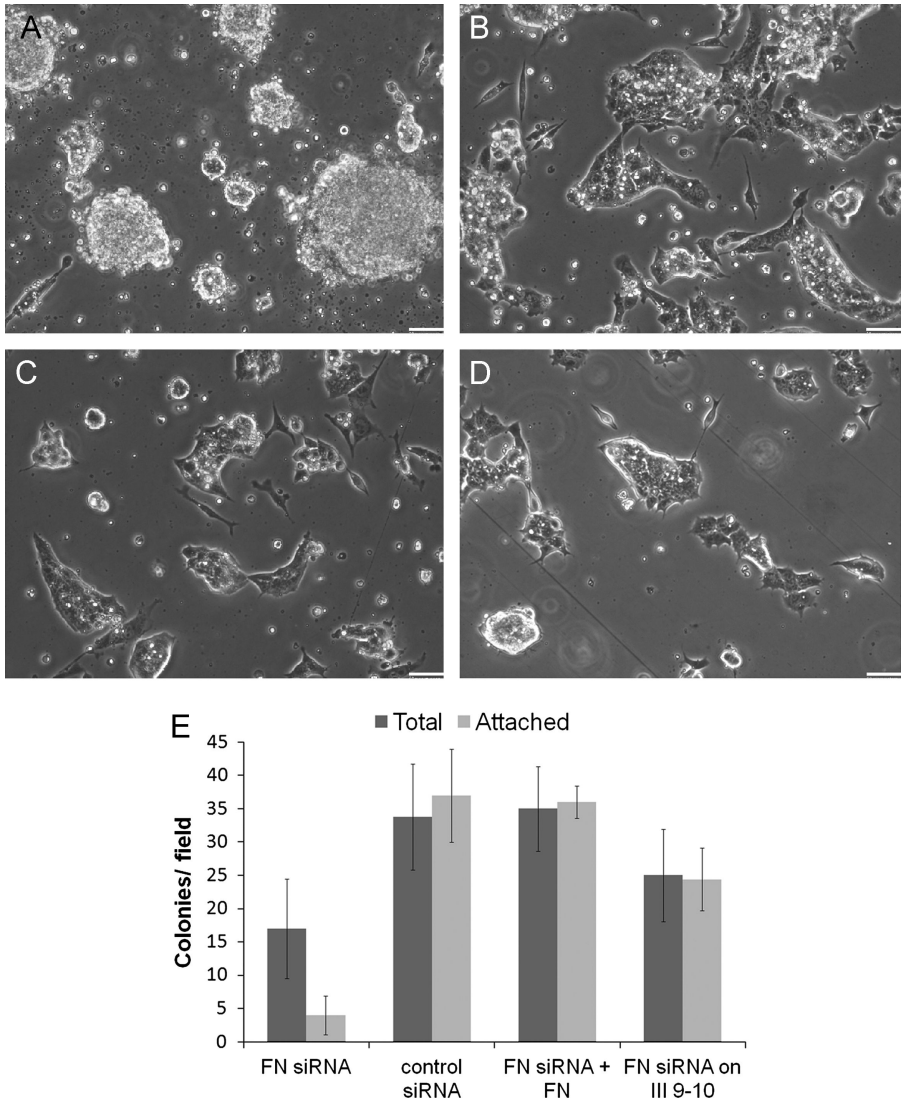

Figure 4.

siRNA knockdown of fibronectin causes loss of cell adhesion mES cells were treated with fibronectin (FN) siRNAs (A) or control siRNAs (B) followed by plating on gelatin in the presence of LIF in Knockout medium. (C) As in A, but with addition of 1 μg FN/ml to culture medium. (D) mES cells were treated with fibronectin siRNAs and plated in the presence of LIF on tissue culture plastic coated with 50 μg/ml III9–10 protein. Phase contrast images were taken 24 hours after plating. (E) Total colonies were counted in multiple microscopic fields (n > 4), wells were washed with PBS, and attached colonies were counted in multiple fields (n > 4). Numbers of colonies per field were averaged. Graphs represent average number of colonies/field for two independent experiments. Error bars represent one standard deviation from average value. Scale bars = 50 μm.

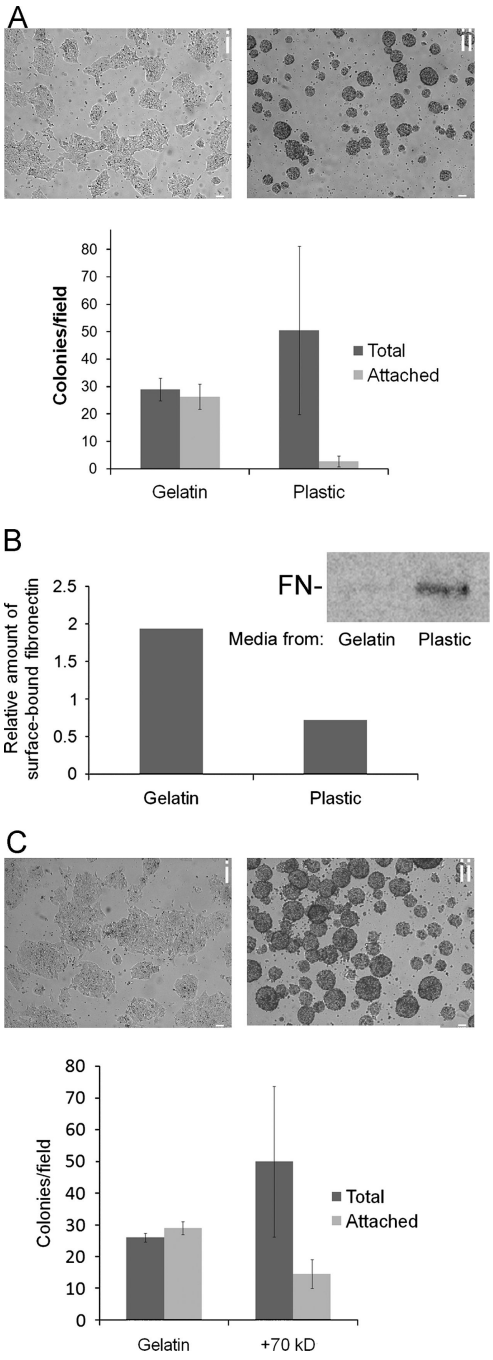

Figure 5.

Fibronectin binds to the gelatin substrate. (A) Phase contrast images of mES cells plated on gelatin-coated (*i*) or uncoated (*ii*) tissue culture plastic. The graph shows total and attached mES cell colonies on gelatin-coated (Gelatin) or uncoated (Plastic) surfaces 24 hours after plating. Averages for two independent experiments were calculated as in Figure 4 legend. (B) mES cells grown for 24 hours were removed from the indicated surfaces by treatment with EDTA. An ELISA was performed to quantify surface-bound fibronectin using R457 anti-fibronectin antiserum. (B, *inset*) mES cells on either uncoated or gelatin-coated tissue culture wells were labeled with ^{35}S-methionine for 24 hours. ^{35}S-labeled fibronectin was isolated from the media, separated by SDS-PAGE, and analyzed using a phosphorimager.

FN indicates location of fibronectin band. (C) Phase contrast images of mES cells plated on gelatin, either in the presence of CAPS buffer (*i*) or in the presence of 0.7 μM (50 μg/ml) 70 kD fibronectin fragment (*ii*). Graph shows total and attached cell colonies without [Gelatin] and with 70 kD [+70 kD], averaged from two independent experiments.

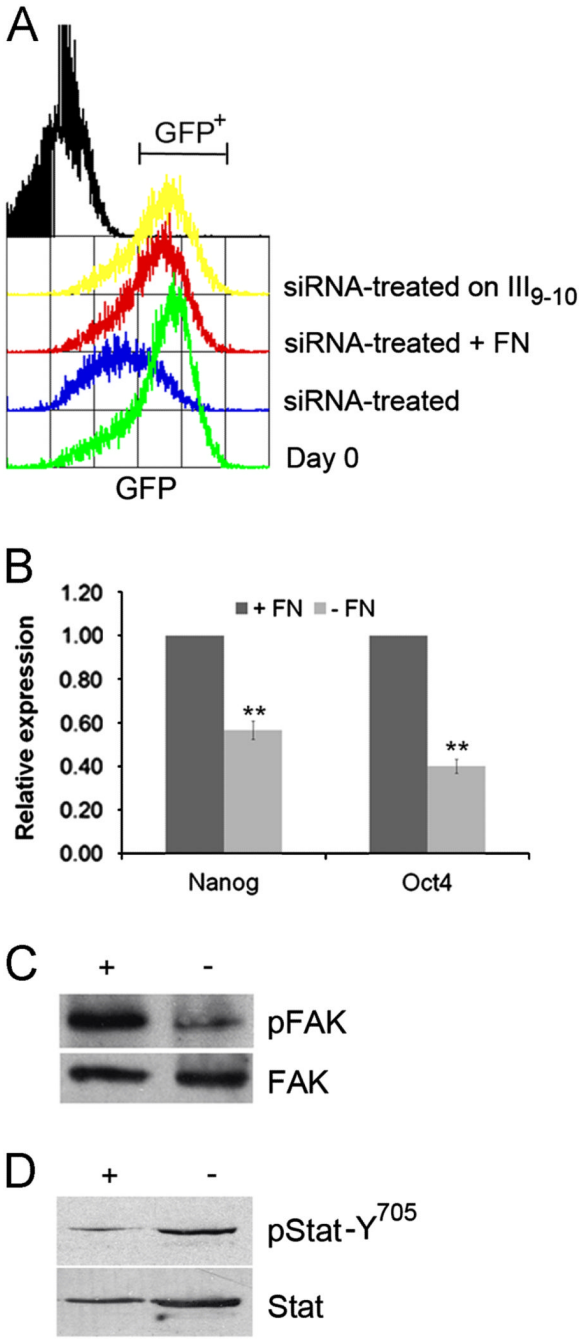

Figure 6.

Fibronectin knockdown results in loss of self-renewal. (A) Nanog-GFP mES cells were treated with siRNAs against fibronectin and then grown under the indicated conditions in the presence of LIF in Knockout medium. GFP expression was monitored by flow cytometry on day 6. Day 0 profile is of siRNA-treated cells before replating. Black profile is of non-fluorescent mES cells. (B) Nanog-GFP mES cells were treated with siRNAs against FN, and then grown either in the presence (+FN) or absence (-FN) of exogenous fibronectin. RNA was extracted from cells on day 6, and quantitative RT-PCR was performed using primers for Nanog and Oct4. Values for each sample are normalized to siRNA-treated cells plated + FN. Graphs represent the average of triplicate samples in two independent experiments, and

error bars indicate +/− one standard deviation from the mean. ** $p < 0.01$. (C, D) Cells grown as in (B) with (+) or without (−) fibronectin added were lysed on day 6. Equal amounts of protein were separated by SDS-PAGE, and proteins were detected by immunoblotting with anti-pFAK-Y397 or anti-FAK (C) or with anti-pStat3-Y705 (D) antibodies. pStat blots were stripped and reprobed with anti-Stat3 antibodies (D). Blots are representative of at least two experiments.