Integrin-Associated Focal Adhesion Kinase Protects Human Embryonic Stem Cells from Apoptosis, Detachment, and Differentiation

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INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent stem cells that exhibit epithelial-like features resembling the epiblast epithelium of the post-implantation embryo (Nichols and Smith, 2009). Similarly to epithelial cells, hESCs are dependent on E-cadherin-mediated cell-cell contacts and anchorage to the extracellular matrix (ECM) via integrin receptors (Ohgushi et al., 2010; Braam et al., 2008). Various studies have established the efficacy of integrin engagement with ECM substrates in supporting hESC self-renewal and pluripotency (Braam et al., 2008; Baxter et al., 2009; Miyazaki et al., 2012; Soteriou et al., 2013; Rodin et al., 2014). However, the specific nature and role of downstream signaling from integrins in hESCs remains largely unexplored.

One of the key functions of the ECM in epithelial cells is to prevent a common form of apoptosis, anoikis, or “homelessness” of cells that have lost contact with the matrix (Frisch and Francis, 1994). Anoikis is executed via the mitochondrion and results in activation of caspase downstream of integrin-associated pathways (Gilmore et al., 2000). ECM-integrin interaction initiates signaling, promoting the assembly of cytoplasmic scaffold and kinase proteins at focal adhesions near active integrin clusters (Giancotti and Ruoslahti, 1999). Focal adhesion kinase (FAK), a protein tyrosine kinase, is one of the principal integrin signaling regulators, containing three domains: the protein 4.1, ezrin, radixin, moesin (FERM) domain, the kinase domain, and the focal adhesion targeting domain (Frame et al., 2010). Upon integrin activation FAK localizes at the adhesion site where structural changes displace the inhibitory FERM, allowing autophosphorylation of the Tyr397 (Y397) site, leading to the activation of its intrinsic kinase function and the formation of docking sites for multiple downstream signaling molecules (Frame et al., 2010). Several signaling players directly interact with the Y397 site, e.g., Src, which in turn phosphorylates FAK, promoting further activation, or p130Cas, Grb2, and phosphatidylinositol 3-kinase (PI3K), involved in controlling cytoskeletal rearrangements, cell cycle, and survival (Parsons, 2003). FAK is crucial in preventing anoikis through direct activation of PI3K, via the Y397 site, in turn promoting the pro-survival AKT cascade (Gilmore et al., 2000; Xia et al., 2004). FAK can also leave focal adhesions and act in a kinase-independent manner by localizing in the nucleus where the FERM scaffolds the AKT target MDM2 for ubiquitination of pro-apoptotic p53, leading to its protein degradation (Lim et al., 2008).

Among the repertoire of integrins, the β1-integrin subunit mediates the attachment of hESCs to fibronectin via the α5β1 heterodimer (Baxter et al., 2009), as well as other commonly used ECM (Braam et al., 2008). Although hESCs cultured on ECM have been shown to express active FAK and AKT (Miyazaki et al., 2012; Rodin et al., 2014; Wrighton et al., 2014), the functional contribution of the FAK pathway to hESCs has not been dissected.

SUMMARY

Human embryonic stem cells (hESCs) can be maintained in a fully defined niche on extracellular matrix substrates, to which they attach through integrin receptors. However, the underlying integrin signaling mechanisms, and their contribution to hESC behavior, are largely unknown. Here, we show that focal adhesion kinase (FAK) transduces integrin activation and supports hESC survival, substrate adhesion, and maintenance of the undifferentiated state. After inhibiting FAK kinase activity we show that hESCs undergo cell detachment-dependent apoptosis or differentiation. We also report deactivation of FAK downstream targets, AKT and MDM2, and upregulation of p53, all key players in hESC regulatory networks. Loss of integrin activity or FAK also induces cell aggregation, revealing a role in the cell-cell interactions of hESCs. This study provides insight into the integrin signaling cascade activated in hESCs and reveals in FAK a key player in the maintenance of hESC survival and undifferentiated state.

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Here, we show that integrin activation in hESCs is transduced by FAK to regulate adhesion and prevent the onset of anoikis or differentiation via an AKT/MDM2/p53 cascade. Together, our results reveal a critical role for FAK in the control of hESC fate, as a mediator of integrin signaling crosstalk with key hESC regulatory players.

RESULTS

Matrix-Integrin Binding Activates FAK Signaling Upstream of AKT

To characterize integrin signaling in hESCs cultured on fibronectin, we investigated FAK activation. Immunofluorescence analysis of phosphorylation sites marking FAK activity showed widespread expression of the autophosphorylation Y397 site, induced upon integrin engagement in OCT4-positive cells (Figure 1A). Other phosphorylated residues, created by Src kinase binding to FAKY397 during adhesome assembly, were expressed in a small proportion of cells (Figure S1A) showing that hESCs display active FAK signaling. Importantly, hESCs express high levels of active β1-integrin and the focal adhesion marker paxillin but in a diffuse or punctate distribution, while upon differentiation focal adhesions are visible (Figure S1B). Next, we asked whether FAKY397 is a transducer of fibronectin/β1-integrin binding. hESCs grown on fibronectin had active FAKY397 and its downstream PI3K target AKT Ser473 (S473) (Figure 1B). Conversely, plating hESCs on a non-integrin-activating substrate, Poly-L-Lysine, or blocking β1-integrin selectively with antibody (MAB13), which we previously showed induces hESC detachment from fibronectin (Baxter et al., 2009), reduced FAK and AKT activity (Figures 1C and 1D). We then applied a selective FAK inhibitor, PF562271, which reduced pFAKY397 in a dose-dependent manner (Figures S1C and S1D). Pharmacological inhibition of 80% of FAKY397 with PF562271 at 2 μM (FAKi) was comparable with blocking β1-integrin in reduction of FAK activity (Figures 1C and 1D) without affecting the potential off-target CDK1 (Figure S1E). Moreover, after FAK inhibition there was a reduction of pAKT similar to that seen following integrin inhibition or on Poly-L-Lysine (Figures 1C and 1D). In summary, our data indicate that integrin engagement in hESCs is transduced through FAKY397 and its downstream kinase, AKT (Figure 1E).

Inhibition of FAK Signaling Induces Cell Blebbing and a Caspase-Dependent Anoikis

Autophosphorylation of FAK is crucial for the transduction of survival signals by recruiting PI3K that in turn induces the AKT cascade (Xia et al., 2004). Since our data suggested that FAKY397 transduces integrin activation to AKT, we next determined whether FAK kinase activity supports survival of hESCs. hESCs responded to FAK kinase block with PF562271 by detaching from the matrix (Figure 2E) and undergoing apoptosis in a dose-dependent manner (Figure 2A). At the same time, we excluded the possibility that the FAK/Src complex mediated survival, since inhibition of Src did not induce cell detachment and dephosphorylation of AKT even if its target FAKY861 was decreased (Figures S2A and S2B). Similarly, inhibition of the integrin-associated pseudokinase ILK, reported to target AKT in differentiating hESCs (Wrighton et al., 2014), did not affect cell attachment or survival (Figure S2C). Furthermore, inhibition of ILK did not affect pluripotency markers over time (Figure S2D).

To further confirm that the inhibition of FAK kinase is responsible for apoptosis of hESCs, we tested in parallel two other selective FAK inhibitors in both hESCs and human induced pluripotent stem cells (hiPSCs) with a high-throughput assay that measures early caspase activation. All tested FAK inhibitors induced caspase activity in proportion to dose in both hESCs (Figure 2B) and hiPSCs (Figure S2E). In addition, selective inhibitors of AKT induced caspase activity in a similar manner (Figure 2C), supporting AKT as an effector of FAK. Finally, by measuring together caspase activity, viability, and cytotoxicity after FAKi, we found that caspase activity was induced without nonspecific cytotoxicity (Figure S2F).

To validate whether FAK-dependent apoptosis relies on caspase activity, we applied the caspase inhibitor Z-VAD-FMK to hESCs treated with FAKi. Immunostaining confirmed that FAKi induced cleaved caspase-3 expression that was inhibited by Z-VAD-FMK (Figure 2D). Floating dead cells, normally present after FAKi, were abolished by Z-VAD-FMK but instead, attached single and groups of hESCs, with prominently blebbing membranes, were observed (Figure 2E). The effects of FAKi on survival, cell blebbing, and caspase activation were confirmed on vitro-nectin (Figures S2G and S2H).

Cell blebbing is an indicator of cytoskeletal contraction, commonly a result of caspase-3 cleavage of ROCK during apoptosis, leading to increased contractility (Coleman et al., 2001). Our data indicate that FAK inhibition induced cell blebbing independently of caspase, a unique mechanism reported in hESCs after cell-cell dissociation (Ohgushi et al., 2010), but also observed by us in groups of cells. However, caspase activity is required to complete detachment from the ECM, a key feature of anoikis. Moreover, Z-VAD-FMK also rescued FAKi-dependent early (Annexin V) and late (Annexin V/7-AAD) apoptosis, increasing the proportion of live cells, suggesting a suppression of hESC caspase-dependent turnover (Figure 2F). Overall, these results show that FAK kinase activity is required to suppress a caspase-dependent anoikis in hESCs.
Loss of Total FAK Disrupts hESC Attachment and Leads to Cell Aggregation

Since FAK is a modular protein that functions as a scaffold during adhesome assembly linking integrins to the actin cytoskeleton, we investigated the effect of substantially reducing total FAK on hESCs. Knocking down FAK protein by 70% using small interfering RNA (siFAK) (Figure 2G) induced visible changes in morphology and decreased hESC attachment to fibronectin. Cells formed aggregates similar to those seen after blocking β1-integrin with antibody (Figure 2H), showing that integrin/FAK is required for the transmission of attachment cues in hESCs. Moreover, cell aggregates were also produced in hESCs grown on vitronectin, after blocking its receptor αvβ5-integrin.
with antibody (Figure S2I). Similarly to the effect of MAB13, pFAK activity was downregulated after inhibition of vitronectin-integrin binding (Figure S2J), suggesting that a common downstream FAK signaling may be activated. Furthermore, apoptosis was increased by both MAB13 and siFAK (Figures 2K and 2l), although late apoptotic cells are fewer in the latter (Figure 2J), strongly supporting the role of β1-integrin and FAK in survival of hESCs. In conclusion, loss of β1-integrin activity or FAK produces a shift from matrix-cell adhesion to cell-cell adhesion with lowered survival, highlighting the role of integrin/FAK signaling in the maintenance of pro-survival adhesion cues in hESCs.

**FAK Localizes in the Nucleus of hESCs and Regulates MDM2/p53 Levels during Anoikis**

The results reported in Figures 1C and 2A link FAK inhibition with the downregulation of AKT and a subsequent apoptotic response. It has been reported that de-adhesion or kinase inhibition promotes nuclear accumulation of FAK (Lim et al., 2012), which exercises kinase-independent functions, including scaffolding MDM2 ubiquitination of p53 as an additional mechanism to support survival (Lim et al., 2008). To test whether FAK participates in this process in hESCs, we first determined its cellular location. Immunofluorescence of hESCs with total FAK antibody revealed a diffuse distribution in the cytoplasm but also in the nucleus, while FAKY397 localized at the cell surface (Figure 3A). Accordingly, FAK was repeatedly found in the nuclear fraction of hESCs (Figure 3B). After 6 hr of integrin blocking or FAK inhibition, total FAK did not accumulate in the nucleus, but was reduced in the cytoplasm and also slightly so in the nucleus (Figures 3C and S3A). Immunofluorescence also showed a widespread loss of FAK after 1 hr from integrin/FAK inhibition (Figure 3D). Moreover, inhibition of integrin/FAK deactivated pMDM2 while upregulating p53 (Figure 3E). The elevation of p53 protein was detectable already after 1 hr of treatments (Figure S3B), and we observed its increase mainly in the nucleus (Figure 3F). Furthermore, p53 upregulation appears linked to the reduced MDM2 ubiquitin ligase activity, since FAK inhibition reduces the poly-ubiquitin chains associated with p53 (Figure 3G). In conclusion, attachment of hESCs to the ECM supports FAK kinase and total protein expression, which keeps MDM2 active and p53 low, ultimately preventing a caspase-dependent anoikis.

**hESCs Avoid Anoikis by Exiting Their Undifferentiated State**

We have demonstrated that integrin signaling regulates survival of hESCs, but does it play a role in maintaining the undifferentiated stem cell state? First, we found that after 24 hr of FAKi the entire population of apoptotic hESCs retained the pluripotency-associated marker NANOG (Figure 4A). However, when hESCs were cultured in the presence of FAKi for 3 days, a subpopulation of cells that remained attached and escaped anoikis had acquired a differentiated morphology (Figures 4B and S4A). Indeed, the cells were NANOG negative and had elongated nuclei after 2 or 1 μM of FAKi (figures 4C and S4C). Similar results were obtained on vitronectin and laminin (Figure S4D). Strikingly, FAKi-differentiated cells dramatically downregulated FAK and NANOG proteins while pSMAD2 was slightly increased (Figure 4D). At the gene-expression level, FAKi reduced both NANOG and OCT4 pluripotency-associated genes (Figures 4E and S4B). In parallel, early differentiation genes were upregulated (Figure 4E). Consistent with the role of FAK downstream of integrins, blocking β1-integrin also induced differentiation in a similar fashion (Figure 4F). Integrin-blocked aggregates were comparable with embryoid bodies (EBs) in the induction of differentiation, although EBs showed a greater induction of...
differentiation markers at day 3 (Figure 4F), but similar to MAB13 inhibited aggregates at day 5 (Figure S4E). Together, these data show that hESCs undertake one of two routes after inhibition of integrin/FAK signaling: they die through anoikis, remaining undifferentiated, or survive but differentiate, losing characteristic hESCs morphology and markers. Our data highlight a model of hESC regulation by integrin signaling (Figure 4G) with FAK as a major transducer of integrin cues for attachment, survival, and maintenance of stem cell identity.

**DISCUSSION**

Here we shed light on the events downstream of integrin activation in hESCs and reveal in FAK the mediator of this signaling and a positive regulator of survival, adhesion, and stem cell maintenance. Indeed, hESCs respond to FAK inhibition by exiting the stem cell state through either anoikis or differentiation. We found that integrin activation in hESCs is transduced by FAKY397 to activate AKT and MDM2 and suppress p53 and caspase activation. This FAK-dependent survival pathway is consistent with that reported in adult cell types (Lim et al., 2008), but in the context of hESCs we reveal how integrin signaling supports pluripotency circuits, since AKT and p53 are well known to regulate the balance between self-renewal and differentiation (Singh et al., 2012; Jain et al., 2012). We found no evidence that the loss of FAK signaling in pluripotent cells biases differentiation to a particular lineage, but rather the resultant switch-off of PI3K/AKT and switch-on of p53 can shift hESCs out of pluripotency (Singh et al., 2012; Jain et al., 2012).

Our data confirm and extend previous work reporting FAK and AKT phosphorylation in hESCs (Miyazaki et al., 2012; Rodin et al., 2014; Wrighton et al., 2014). However, a recent paper reported that FAK is activated only upon differentiation (Villa-Diaz et al., 2016), similarly to murine ESCs where stem cell maintenance inversely correlates with integrin activation (Toya et al., 2015). This discrepancy with our and previous reports on hESCs may be explained by our data showing that the integrin signaling players are indeed active in hESCs but are only assembled into obvious focal adhesions upon differentiation. Clearly, hESCs manipulate complex integrin machinery for different purposes in different environments. For example, it was shown that ILK inhibition but not FAK inhibition increased endoderm differentiation in the presence of activin A (Wrighton et al., 2014), whereas we saw no effect of ILK on survival or differentiation when added to hESC media.

We found that hESCs require FAK for maintenance of substrate adhesion, which is consistent with its role in transmitting forces from integrins to the cytoskeleton (Huveneers and Danen, 2009). Double inhibition of FAK kinase and caspase revealed signs of cytoskeletal hypercontraction, similar to the unique and lethal hESC-response to single-cell dissociation (Oghushi et al., 2010), but also visible in groups of mutually adhering cells. Thus, our results suggest that integrin signaling is an essential yet distinct cue from cell-cell adhesion for suppression of cytoskeleton contraction and apoptosis. However, integrin signaling may crosstalk with cell-cell adhesion. Indeed, we observed formation of cell aggregates after blocking β1-integrin or FAK knockdown.

Finally, we discovered that hESCs possess a nuclear pool of FAK that does not accumulate but reduces after FAK inhibition, unlike in adult cells (Lim et al., 2012). We propose that hESCs utilize non-canonical FAK mechanisms to quickly respond to defective adhesion, which could involve undiscovered roles for nuclear FAK in the context of pluripotent cells.

In conclusion, this study shows that the ECM exerts extensive control over feeder-free hESCs via a FAK-dependent cascade linking integrins to intrinsic regulatory players, supporting survival and maintenance of stem cell identification.
identity. This study advances our understanding of the underlying integrin signaling controlling hESCs, of particular importance for their culture on ECM for both basic and translational research.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

hESCs HUES1 (Harvard University), MAN5 (North West Embryonic Stem Cell Center), Shef-1 (University of Sheffield), and hiPSCs Sendai J (Neusentis) were cultured in defined mTESR1 (Life Technologies) medium on 50 μg/ml fibronectin as previously described (Baxter et al., 2009). Cells were passaged with TryPLE express (Gibco) at a ratio of 1:3. HUES1 was used as lead line throughout the study; MAN5 was used when specified, and Shef1 and SendaiJ to confirm compound screening. For selected experiments, hESCs were plated on 5 μg/ml of vitronectin-N (Life Technologies) or laminin 521 (Biolamina).

**Integrin Blocking**

hESCs were dissociated and seeded at a density of 0.7 × 10⁴ cells/cm² in hESC media supplemented with 10 μg/ml MAB13 anti β1-integrin antibody (M. Humphries, University of Manchester) or rat immunoglobulin G (IgG) isotype (Sigma) and plated on fibronectin. For cells cultured on vitronectin, αVβ5-integrin was inhibited with 10 μg/ml MAB1916z blocking antibody (Millipore) and incubated for 5 hr at 37°C. The ApoToxGlo Triplex assay (Promega) was used according to the manufacturer’s instructions (Lonza). hESCs HUES1 (Harvard University), MAN5 (North West Embryonic Stem Cell Center), Shef-1 (University of Sheffield), and hiPSCs Sendai J (Neusentis) were cultured in defined mTESR1 (Life Technologies) medium on 50 μg/ml fibronectin as previously described (Baxter et al., 2009). Cells were passaged with TryPLE express (Gibco) at a ratio of 1:3. HUES1 was used as lead line throughout the study; MAN5 was used when specified, and Shef1 and SendaiJ to confirm compound screening. For selected experiments, hESCs were plated on 5 μg/ml of vitronectin-N (Life Technologies) or laminin 521 (Biolamina).

**Compound Screening**

Cells were prepared 24 hr before the assay by passing in presence of Y-27632 (10 μM) (Sigma-Aldrich) for 1 hr and nucleofected with Amaxa Nucleofector following the manufacturer’s instructions (Lonza), with target-specific FAK siRNA (sc-29310, Santa Cruz Biotechnology) at a final concentration of 200 nM, β-2-microglobulin siRNA control (Life Technologies), or pmaxGFP control vector (Lonza). Medium was changed the following day and experimental analysis performed 66 hr later.

**siRNA Knockdown**

hESCs were treated with 10 μM Y27632 (Sigma-Aldrich) for 1 hr and nucleofected with Amaxa Nucleofector following the manufacturer’s instructions (Lonza), with target-specific FAK siRNA (sc-29310, Santa Cruz Biotechnology) at a final concentration of 200 nM, β-2-microglobulin siRNA control (Life Technologies), or pmaxGFP control vector (Lonza). Medium was changed the following day and experimental analysis performed 66 hr later.

**Statistics**

Data were analyzed with Student’s t-test using GraphPad Prism. p values of <0.05 were considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.07.006.

**AUTHOR CONTRIBUTIONS**

L.V. designed and performed the experiments; M.B. performed integrin analysis; B.I. performed the Src and integrin αVβ5 experiments; P.W. supervised the compound screening; S.J.K. designed and supervised the study; L.V. and S.J.K. co-wrote the manuscript.

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**Figure 4. hESCs Avoid Anoikis by Exiting Their Undifferentiated State**

(A) Quantification of Annexin V/NANOG double-positive hESCs treated for 24 hr with DMSO or FAKi. *p < 0.05.

(B) Phase images of hESCs treated with DMSO or FAKi for 3 days. Scale bar, 100 μm.

(C) Immunofluorescence images of hESCs treated with DMSO or FAKi for 3 days. Cells were co-stained with DAPI and antibody against NANOG. Scale bar, 50 μm.

(D) Immunoblot of FAK, pSMAD2, and NANOG in hESCs treated with DMSO or FAKi for 3 days.

(E) Gene expression showing fold change for pluripotency-associated markers NANOG and OCT4 and early differentiation markers PAX6, SOX17, Goosecoid, and FOXA2 in hESCs treated with DMSO or FAKi for 3 days. *p < 0.05 relative to DMSO.

(F) Gene expression fold change for pluripotency-associated and early differentiation markers [as in (E)] in hESCs treated with 10 μg/ml of MAB13 or IgG control for 3 days. *p < 0.05, MAB13 relative to IgG and EBs relative to hESCs; n.s., not significant.

(G) Proposed model for FAK signaling in hESCs: ECM-integrin binding activates FAK, which induces PI3K upstream of AKT/MDM2 survival cascade leading to suppression of p53. In absence of FAK activity, the concomitant switch-off of AKT and elevation of p53 induces a caspase-dependent anoikis or downregulation of hESCs core genes and differentiation.

Data represent mean ± SEM (n = 3 experiments). See also Figure S4.
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Supplemental Information

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SUPPLEMENTAL FIGURES

FIGURE S1

A

B

C

D

E

Dose-response PF562271

pFAK activation (relative densitometry)

log[PF562271], μM
Figure S1. Matrix-Integrin Binding Activates FAK Signaling Upstream of AKT
(Related to Figure 1)

(A) Immunofluorescence images of hESCs cultured on fibronectin and stained with antibodies against OCT4 and pFAKY407, pFAKY576, pFAKY577 or pFAKY861. Scale bars 50 µm

(B) Immunofluorescence image of undifferentiated and differentiated hESCs cultured on fibronectin and stained with antibodies against Paxillin, Actin and active β1-integrin. For differentiation, cells were grown in hESCs media (Baxter et al., 2009) without NT4 and AA for 7 days. Scale bar 50 µm

(C) Immunoblot of pFAKY397 and FAK after increasing concentrations of PF562271 inhibitor added for 1 h.

(D) Dose-response curve of pFAKY397 relative to PF562271 concentration added for 1 h. Average of 3 independent experiments. *indicates p<0.05.

(E) Immunoblot of CDK1 and β-tubulin after 3 hr of 2 µM PF562271.

Data represented as mean +SEM
FIGURE S2

A. DMSO + 10nM pp60 + 25nM pp60

1h 2h 1h 2h 1h 2h 24h 1h 2h 1h 2h 24h

pSrc 416
GAPDH

DMSO + pp60

pSrc Y416
pFAK Y861
pAKT

C. DMSO + ILK inhibitor (cpd22)

Detached cells (x 10^6)

DMSO + ILK inhibitor cpd22

Dead cells (x 10^6)

D. OCT4

ILK inhibitor (cpd22)

DAPI

E. FAK inhibition (hiPSCs)

Caspase activity (Luminescence)

Inhibitor concentration Log_{10} (µM)

FAKi ZVAD-FMK

F. Viability

(Fluorescence)

hESC on vitronectin

G. DMSO

FAKi

FAKi ZVAD-FMK

H. Cleaved caspase 3 positive cells (%)

I. control

+ IgG Ab

+ blocking αVβ5 Ab (MAB1916z)

45 min

24 hours

J. Control

+ MAB1916z

pFAKY576

Tubulin

cpd22

n.s.
Figure S2. Inhibition of FAK Signaling Induces Cell Blebbing and a Caspase-Dependent Anoikis (Related to Figure 2)

(A) Top: Immunoblot of pSrc416 and GAPDH in hESCs treated with DMSO or Src inhibitor pp60 at 10nM or 25nM for 1h, 2h or 24hr. Bottom: Immunoblot of pSrc416, pFAKY861, pAKT and β-tubulin in hESCs treated with DMSO or pp60 (25nM) for 1 and 2 hr.
(B) Phase images of hESCs plated on fibronectin for 24 hr in presence of Src inhibitor pp60 (25 nM) or DMSO control. Scale bars 100 µm.
(C) Quantification of cell detachment (cell in suspension) and survival (dead cells stained positive with trypan blue) in hESCs treated for 24 hr with 0.4 µM of ILK inhibitor cpd22. (n=3 independent experiments).
(D) Immunofluorescence images of hESCs treated with DMSO or 0.4 µM cpd22 for 3 days and stained with antibody against OCT4.
(E) Caspase activity in hiPSCs treated for 5 hr with the indicated concentrations of FAK inhibitors. *indicates p<0.05 (n=3 independent experiments).
(F) Scatter plots of viability, cytotoxicity and caspase activity measured in parallel in hiPSCs treated with DMSO or 2 µM of PF562271 for 5 hr. Each dot represents a well of a 96-well plate.
(G) Phase images of hESCs cultured on vitronectin substrate and treated for 24 hr with DMSO, FAKi only or with Z-VAD-FMK. Inserts from left to right show: hESCs colony, cells undergoing anoikis and cell blebbing. Scale bar 50 µm.
(H) Cleaved-caspase 3 expression in hESCs cultured on vitronectin and treated with DMSO, FAKi only or with Z-VAD-FMK for 24 hr. *indicates p<0.05 relative to DMSO (n=3 independent experiments).
(I) Phase images of hESCs replated on vitronectin for 45 mins or 24 hr in presence of 10 µg/ml of MAB1916z or mouse IgG control versus untreated control. Scale bars 100 µm.
(J) Immunoblot of pFAKY576 or β-tubulin in hESCs cultured on vitronectin from 1 to 3 days (3d) with 10 µg/ml of IgG control or MAB1916z.

Data represented as mean +SEM.
Figure S3. FAK Localizes in the Nucleus of hESCs and Regulates MDM2/p53 Levels during Anoikis (Related to Figure 3)

(A) Immunoblot of FAK in the nuclear and cytoplasmic fraction of hESCs treated with IgG control or MAB13 for 6 hr. β-tubulin serves as cytoplasmic marker (C) and PARP as nuclear marker (N).

(B) Immunoblot of p53 and GAPDH in control hESCs versus cells treated with FAKi or MAB13 for 1 hr.
FIGURE S4

A  Day3 treated Man5 hESCs

DMSO  FAKi

B  Day 3 treated MAN 5 hESCs

DMSO  FAKi

C  DMSO  FAKi (1μM)

NANOG  OCT4  DAPI

D  Vitronectin

Laminin

FAKi

E  Day 5 treated HUES1 hESCs

OCT4  NANOG

PAX6  SOX17

FOXA2  SOX1
Figure S4. hESCs Avoid Anoikis by Exiting their Undifferentiated State (Related to Figure 4)

(A) Phase images of hESCs MAN5 treated with DMSO or 2 µM of PF562271 (FAKi) for 3 days. Scale bar 100 µm.
(B) Gene expression fold-change of pluripotency markers \textit{NANOG} and \textit{OCT4} in hESC MAN5 treated with DMSO or FAKi for 3 days. * indicates p<0.05 (n=3 independent experiments).
(C) Immunofluorescence images of hESCs cultured on fibronectin and treated with DMSO or 1 µM PF562271 for 3 days. Cells were costained with DAPI and antibodies against NANOG and OCT4. Scale bars 200 µm.
(D) Immunofluorescence images of hESCs cultured on vitronectin or laminin 521 and treated with DMSO or 2 µM PF562271 for 3 days. Cells were costained with DAPI and antibodies against NANOG and OCT4. Scale bars 200 µm.
(E) Gene expression fold-change of pluripotency markers \textit{NANOG}, \textit{OCT4} and differentiation markers \textit{PAX6}, \textit{SOX17}, \textit{FOXA2} and \textit{SOX1} in hESC HUES1 treated with DMSO, FAKi, 10µg of MAB13 or rat IgG for 5 days. * indicates p<0.05. MAB13 relative to IgG, EB relative to hESCs (n=3 independent experiments)

Data represented as mean ±SEM
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Apoptosis assay and Flow Cytometry:
Apoptosis measurements were performed using the Annexin V-PE and 7-AAD kit (BD Pharmigen) and acquired on a Lab Quanta SC flow cytometer (Beckman Coulter). For Annexin V/NANOG double staining, samples were pre-stained with Annexin V for 15 mins and washed 3 times with PBS 1X prior to fixation. For intracellular staining, cells were fixed in 4% paraformaldehyde at room temperature and permeabilized in 70% MeOH at 4°C. Samples were probed with Nanog-PE or AlexaFluor 488 Isotype control (both from BD Pharmigen) for 30 mins and analysed. Data were analysed using Summit 4.3 (Beckman Coulter).

Immunoprecipitation:
incubated in 50 µl of NER buffer for 40 mins on ice. The remaining nuclear pellet was washed twice vortexed for 15 mins, incubated 2 mins on ice, vortexed 5 sec and spun at 500 g for 7 mins. Cytoplasmic proteins were collected from the supernatant. The remaining nuclear pellet was washed twice with CERI buffer plus protease and phosphatase inhibitors (ROCHE) for 10 mins on ice. CERII buffer was added to the hESC pellets were lysed in NER buffer for 10 mins on ice. Cells were incubated with primary antibodies dilutions containing 1% serum overnight at 4°C. Cells were washed and incubated with secondary antibodies conjugated with Alexa fluorophores (Invitrogen) and nuclei stained with DAPI (Sigma). Images were taken with an Olympus 1X71 microscope (Olympus) with a Q-imaging Retiga SRV camera and processed using QCapture Pro (QImaging) and ImageJ 64.

Subcellular fractionation:
hESC pellets were lysed in NER-PER extraction reagents (Thermo Scientific). Cell pellet was resuspended in 200 µl of CERI buffer plus protease and phosphatase inhibitors (ROCHE) for 10 mins on ice. CERII buffer was added to the solution, vortexed for 15 mins, incubated 2 mins on ice, vortexed 5 sec and spun at 500 g for 7 mins. Cytoplasmic proteins were collected from the supernatant. The remaining nuclear pellet was washed twice in PBS, spun at 500 g for 7 min and incubated in 50 µl of NER buffer for 40 mins on ice, vortexed every 10 mins. Nuclear proteins were collected in the supernatant after a 16,000 g centrifugation for 10 mins at 4°C.

Immunoprecipitation:
hESC pellets corresponding to 5 x 10^6 cells was used for each immunoprecipitation condition following the p53 immunocapture kit (ab154470, Abcam). For each immunoprecipitation, 10 µl of p53 beads slurry were used in a 1.5 ml tube incubated under rotation for 16 hr at 4°C.

Immunofluorescence:
hESC were fixed in 4% paraformaldehyde, blocked with 10% serum matching the species of the secondary antibody and permeabilised with 0.1% TritonX-100. Cells were incubated with primary antibodies dilutions containing 1% serum overnight at 4°C. Cells were washed and incubated with secondary antibodies conjugated with Alexa fluorophores (Invitrogen) and nuclei stained with DAPI (Sigma). Images were taken with an Olympus 1X71 microscope (Olympus) with a Q-imaging Retiga SRV camera and processed using QCapture Pro (QImaging) and ImageJ 64.

Widefield restoration microscopy with Delta Vision:
hESC were grown and fixed on glass-bottom culture dishes (35.0/10mm Grainer Bio-one) coated with fibronectin. Images were acquired on a Delta Vision Olympus 1X71 microscope (Applied Precision) restoration microscope using a 100x/140 Uplan S Apo objective and the Sedat Quad filter set. The images were collected using a Coolsnap HP (Photometrics) camera with a Z optical spacing of 0.2 µm.

Antibodies and reagents:
Immunofluorescence antibodies were used against: Paxillin (BD Bioscience), F-AntI (Fluorescein phallloidin, Invitrogen), activated β1-integrin 12g10 (gift from M.Humphries, Wellcome Matrix Centre, The University of Manchester), NANOG (R&D Systems); OCT4 (BD Biosciences); FAK clone 4.47 (Millipore); pFAK397, pFAK407, pFAK576, pFAK577 and pFAK861 (Invitrogen); p53 (ab131442, Abcam), Cleaved-caspase3 (Asp175, Cell Signalling). Western blotting antibodies were against: FAK, pFAK397, pFAK576, pSrc416, pAKTSer473, p53, pMDM2, NANOG (all from Cell Signalling); pFAK861 (Invitrogen); CDK1 (R&D); GAPDH 71.1 (Sigma Aldrich); AKT (Invitrogen); pSMAD2 (Millipore); Multiubiquitin Chain MoAb (clone FK2, Cayman chemical); β-tubulin (Li-COR). Secondary antibodies: IRDye 680LT Goat anti-Mouse IgG; IRDye 800CW Goat anti-Rabbit IgG (Li-COR), IRDye 680LT Donkey anti-Goat IgG. Small molecules: FAK inhibitors PF-562271 (Roberts et al., 2008), PF-06405780 (Bunnage et al., 2011), PF-04557611 (Lovering et al., 2012); AKT inhibitors PF-05094152 (Heerding et al., 2008), PF-04907559 (Heerding et al., 2007) and PF-04943582 (Zhu et al., 2007) were all provided by Pfizer. ILK inhibitor cpd22 from Calbiochem. Src inhibitor pp60 (521-533) from Tocris. Caspase inhibitor Z-VAD-FMK from Abcam.

Protein extraction and immunoblotting:
Adherent cells were lysed on ice in RIPA buffer (Sigma) with added protease and phosphatase inhibitors (ROCHE). Protein extracts were quantified using the BCA protein detection kit (Thermo Scientific). Equal protein amounts were separated on 12% poly-acrylamide gel and transferred to a nitrocellulose membrane (Whatman). Membranes were blocked in Odyssey buffer (Li-COR), incubated overnight at 4°C with primary antibody, washed and incubated with IRDye conjugated secondary antibodies for 2 hr at room temperature. Membranes were scanned with the Odyssey detection system (Li-COR).

Subcellular fractionation:
hESC pellets were lysed in NER-PER extraction reagents (Thermo Scientific). Cell pellet was resuspended in 200 µl of CERI buffer plus protease and phosphatase inhibitors (ROCHE) for 10 mins on ice. CERII buffer was added to the solution, vortexed for 15 mins, incubated 2 mins on ice, vortexed 5 sec and spun at 500 g for 7 mins. Cytoplasmic proteins were collected from the supernatant. The remaining nuclear pellet was washed twice in PBS, spun at 500 g for 7 min and incubated in 50 µl of NER buffer for 40 mins on ice, vortexed every 10 mins. Nuclear proteins were collected in the supernatant after a 16,000 g centrifugation for 10 mins at 4°C.

Immunoprecipitation:
hESC pellets corresponding to 5 x 10^6 cells was used for each immunoprecipitation condition following the p53 immunocapture kit (ab154470, Abcam). For each immunoprecipitation, 10 µl of p53 beads slurry were used in a 1.5 ml tube incubated under rotation for 16 hr at 4°C.
**Real-time PCR:**
Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and converted to cDNA with the M-MLV reverse transcriptase (Promega). For qPCR mastermixes, power SYBR Green Master Mix (Applied Biosystems) was used and plates were run using the CFX96 Real-Time System (Bio-Rad). mRNA levels were normalized to GAPDH expression and relative expression calculated as fold-change from the untreated control.

Table S1. Primer set used in this study, Related to Figure 4

| GENE       | FORWARD                  | REVERSE                   |
|------------|--------------------------|---------------------------|
| OCT4       | AGACCATCTGGCGCTTTGAG     | GCAAGGGGCGGCAGCTT         |
| NANOG      | GGCTCTGTTTTGCTATATCCCCCTAA | CATTACGATGCAGCAAATACAAGA  |
| GAPDH      | ATGGGGAAAGGTGAAGGTCG      | TAAAAGCGACCCCTGGGTGACC    |
| PAX6       | CTGGCTAGCGAAAGCAACAG     | CCCGTCAAACATCCTAGTTATCA   |
| SOX17      | AGAGATTTGTTTCCATAGTTGATT | TGTGGGGACACATCAAGCT       |
| SOX1       | GCGGTAACAATACAAAAAACTTGTAA | GCGGAGCTCGTGCGCATT       |
| FOXA2      | TTCAGGCCGGCTAACTCT      | AGTCTCGACCCCCACCTTGCT     |
| Goosecoid  | GATGCTGCCCTACATGAACGT    | GACAGTGCACTGTTGAGAAG      |
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