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

Loriana Vitillo,[1,3] Melissa Baxter,[1,4] Banu Iskender,[1,5] Paul Whiting,[2,6] and Susan J. Kimber[1,*]
[1]North West Embryonic Stem Cell Centre, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
[2]Pfizer Neusentis, The Portway Building, Granta Park, Cambridge CB21 6GS, UK
[3]Present address: Wellcome Trust-Medical Research Council Stem Cell Institute, Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge CB2 0SZ, UK
[4]Present address: School of Medicine and Dentistry, University of Central Lancashire, Preston PR1 2HE, UK
[5]Present address: Betul-Ziya Eren Genome and Stem Cell Center, Erciyes University, Kayseri 38039, Turkey
[6]Present address: Alzheimer's Research UK UCL Drug Discovery Institute, University College London, London WC1E 6BT, UK
*Correspondence: sue.kimber@manchester.ac.uk
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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.

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.

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 non-specific 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 vitronectin (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.