FAK engages multiple pathways to maintain survival of fibroblasts and epithelia – differential roles for paxillin and p130Cas

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Summary
Different cell types interpret their distinct extracellular matrix (ECM) environments to bring about specific cell fate decisions, and can differentiate or undergo apoptosis depending on their local adhesive interactions. Apoptosis in response to an inappropriate ECM environment is termed ‘anoikis’, or homelessness. Several studies, utilising a variety of cell types, have indicated a common, crucial role for focal adhesion kinase (FAK) in suppressing anoikis. A wide range of different integrins can activate FAK, raising the question of how cell type specific effects are regulated. In this study, we have used a constitutively active form of FAK to examine the mechanism of FAK-mediated survival signalling in cell types from distinct embryonic lineages that show differing sensitivities to anoikis. We demonstrate that both fibroblasts and epithelial cells prevent anoikis through FAK activation. We show that FAK activates multiple downstream pathways in order to suppress anoikis. However FAK regulates survival through a more restricted set of pathways in the more anoikis-sensitive epithelial cells. Furthermore, we identify a novel role for paxillin in apoptosis suppression.

Key words: Anoikis, Epithelial cells, Focal adhesion kinase

Introduction
Integrin-mediated adhesion to the extracellular matrix (ECM) is essential for survival of most cells (Meredith and Schwartz, 1997; Reddig and Juliano, 2005). In the absence of an appropriate ECM, normal cells undergo a form of apoptosis termed anoikis (Frisch and Spreauton, 2001; Gilmore, 2005). Loss of sensitivity to anoikis correlates with anchorage-independent tumour growth and the ability of a cell to metastasise and survive in diverse ECM environments (Douma et al., 2004).

Integrins are adhesion receptors that monitor the cellular microenvironment with interactions through ECM proteins (Hynes, 2002). They have a dynamic intracellular signalling role, recruiting numerous enzymes and scaffold proteins to focal adhesions. The nonreceptor tyrosine kinase focal adhesion kinase (FAK) is a key mediator of integrin signalling (Mitra and Schlaepfer, 2006; Parsons, 2003). FAK interacts with signalling and adaptor proteins, including p130Cas, Grb2, paxillin, She and PI-3 Kinase, many of which are involved with cellular survival. FAK is recruited to sites of integrin/ECM attachment via a focal adhesion-targeting (FAT) domain at its C-terminus. FAK recruitment induces autophosphorylation at tyrosine 397, providing a binding site for Src. Src activates upon recruitment to FAK and phosphorylates key tyrosines within both FAK and adaptor molecules such as paxillin and p130Cas, generating multiple binding sites for SH2-domain proteins, and thereby initiating intracellular signalling cascades (Playford and Schaller, 2004).

Shorty after the first descriptions of anoikis, an activated form of FAK was shown to suppress cell death in detached MDCK cells (Frisch et al., 1996b). This effect was dependent upon both FAK kinase activity and its phosphorylation on tyrosine 397. Other studies have shown that disrupting FAK signalling using anti-FAK antibodies, antisense oligonucleotides, or the expression of dominant-negative FAK, induces apoptosis in adherent cells (Gilmore et al., 2000; Hungerford et al., 1996; Ilic et al., 1998; Xu et al., 1996; Xu et al., 1998). Moreover, genetic deletion of FAK sensitises keratinocytes and endothelial cells to apoptosis in vivo (Braen et al., 2006; Essayem et al., 2006). Disruption of FAK signalling not only has effects in normal cells but is also implicated in tumour progression. FAK is overexpressed in approximately 90% of breast tumour cell lines and is amplified in around 80% of primary breast tumours (Agochiya et al., 1999; Lark et al., 2003; Lightfoot et al., 2004; Watermann et al., 2005). Aberrant FAK signalling may facilitate the neoplastic process by providing cells with an anchorage independence, allowing them to survive in an otherwise hostile ECM environment. Thus, understanding how FAK interprets ECM into a survival signal in normal cells is vital in identifying key points at which aberrant signalling in cancer can be targeted. However, current knowledge of which FAK-interacting proteins integrate adhesion with survival pathways is often contradictory (Almeida et al., 2000; Frisch et al., 1996a; Gilmore et al., 2000; Ilic et al., 1998; Khwaja and Downward, 1997).

Here we examine the role of FAK in cell survival utilising a myristoylated form of FAK, (myrFAK). MyrFAK is active independent of adhesion, localises to adhesion complexes and suppresses apoptosis following loss of integrin function. We demonstrate that cells from different embryonic lineages have distinct strategies for interpreting integrin-mediated survival stimuli. Both epithelia and fibroblasts rely on FAK for their survival but...
utilise different yet overlapping sets of FAK adaptors and signalling pathways.

Results

Fibroblasts and epithelial cells show distinct survival requirements for ECM but both transmit this requirement via FAK. Mouse embryonic fibroblasts (MEFs) did not undergo anoikis when they were maintained in suspension in the presence of growth factors, whereas detachment from ECM coupled with withdrawal of growth factors did cause anoikis (Fig. 1a, lower panel) (Hungerford et al., 1996; Meredith et al., 1993). By contrast, mammary epithelial cells (MECs) were sensitive to anoikis regardless of whether growth factors were present (Fig. 1a, upper panel). In response to detachment from ECM, both cell types showed rapid dephosphorylation of FAK and paxillin (Fig. 1b). Adherent mesenchymal and epithelial lineages both showed discrete patterns of tyrosine phosphorylation, with major components at approximately 130 kDa (FAK) and 70 kDa (paxillin). Phosphorylation of both FAK-Y397 and paxillin-Y31 was lost within 15 minutes of detachment from the ECM. Expression of a dominant-negative form of FAK (DN-FAK), consisting of the FAK FAT domain, induced apoptosis in both adherent MECs and MEFs, although the latter also required removal of serum growth factors (Fig. 1c). Thus, despite the distinction between growth factor requirements in MECs and MEFs, both cell types required FAK signalling to provide the ECM-dependent anti-apoptotic signal.

FAK suppresses anoikis via its interaction with p130Cas in rabbit synovial fibroblasts (Almeida et al., 2000; Ilic et al., 1998). We therefore asked whether p130Cas has a role in MEC or MEF survival. We utilised a number of p130Cas deletion constructs (Fig. 2a), all of which expressed at the predicted molecular weights (Fig. 2b). Expression of the CasSH3 domain, previously used as a dominant negative, inhibited the phosphorylation of endogenous p130Cas on tyrosine 410 (Fig. 2c). We next asked whether inhibiting endogenous p130Cas induced apoptosis in fibroblasts or MECs, compared with expression of DN-FAK (Fig. 2d). Both MEFs and MECs were transfected with expression vectors for DN-FAK, FL-p130Cas and p130Cas-SH3. Apoptosis was quantified 24 hours post transfection (MEFs in the absence of serum growth factors). DN-FAK induced apoptosis in both adherent MECs and MEFs, whereas FL-p130Cas did not. By contrast, the dominant-negative p130Cas-SH3 increased apoptosis in MEFs by a small but significant ($P<0.05$) amount. This was notably less than previously seen in rat synovial fibroblasts (Almeida et al., 2000). There was no increase in apoptosis in MECs expressing p130Cas-SH3.

Fig. 1. Anoikis and FAK signalling in MECs and MEFs. (a) MECs and MEFs were detached from ECM for various times in the presence or absence of serum growth factors (GF). Apoptosis was quantified at each time point by examining nuclei stained with Hoechst. Apoptosis occurred in MECs in the presence or absence of GFs. MEFs were significantly protected from anoikis by the presence of GFs. (b) MECs and MEFs were left adherent or detached from ECM for various times. Cell lysates were then separated by SDS-PAGE and immunoblotted for total phosphotyrosine, phospho-FAK tyrosine 397 (Y397), phospho-paxillin Y31, total FAK and total paxillin. (c) MEFs and MECs were transiently transfected with either control vector or pCDNA3DN-FAK. MECs were cultured in the presence of GFs, whereas MEFs were grown in the presence or absence of GFs. Apoptosis was quantified 24 hours post transfection. MEFs were largely protected by GFs from apoptosis induced by DN-FAK expression. Data represent the mean of three experiments. Error bars indicate standard error of the mean.
Together, these data support previously published results indicating that p130Cas has a role in the suppression of anoikis in fibroblastic cells but indicated no similar role in epithelial cells. Furthermore, the much greater amount of apoptosis induced by DN-FAK indicated that in both cell types additional signalling pathways were likely to play a pivotal role in suppression of anoikis.

**Myristoylated FAK suppresses anoikis in MECs and MEFs**

We wanted to identify what other pathways downstream of FAK may be involved in survival. DN approaches can potentially inhibit or sequester key molecules in multiple pathways. We therefore decided to use a constitutively active (CA) FAK to suppress anoikis in detached cells. CA-FAK would remain active following cell detachment, when endogenous adhesion-dependent signalling was turned off.

Myristoylated FAK (myrFAK) was generated by cloning the v-Src myristoylation sequence N-terminal to the FAK coding sequence (Klippel et al., 1996). Autophosphorylation-site (myrFAKY397F) and kinase-inactive (myrFAKK454R) mutants were also expressed (Fig. 3a,b). All three myrFAK constructs localised to focal adhesions in adherent cells (Fig. 3c). MyrFAK was phosphorylated on tyrosine 397 but this was significantly reduced in myrFAKK454R and undetectable with myrFAKY397F (Fig. 3b). Moreover, myrFAK, but not endogenous FAK or myrFAKY397F, remained phosphorylated following loss of cell/ECM attachment (Fig. 3d). Together, these data indicate that although myrFAK is recruited to focal adhesions in adherent cells, its activation and phosphorylation are independent of cell/ECM attachment.

We asked whether myrFAK suppresses anoikis. MEFs and MECs transiently expressing myrFAK, myrFAKY397F or myrFAKK454R were detached from ECM for 24 hours and maintained in the absence of serum (in the case of MEFs), or in complete growth medium (in the case of MECs) (Fig. 3e). Transfected cells were identified by immunostaining with anti-V5, and apoptosis determined by nuclear morphology. In both cell types, the apoptosis occurring as a result of ECM detachment (mock-transfected controls) was rescued significantly in cells expressing myrFAK (P<0.05). The ability of myrFAK to protect against anoikis required both its kinase activity (K454R) and its tyrosine phosphorylation (Y397F).

To determine whether recruiting FAK to the membrane was required for its anti-apoptotic function, we expressed either myrFAK or FAK minus the myristoylation sequence (WT-FAK). Subcellular fractionation demonstrated that although both myrFAK and WT-FAK were detected in the cytosolic fraction, only myrFAK was present in the membrane fraction (Fig. 4a). Both myrFAK and WT-FAK were phosphorylated on tyrosines 397 and 577 in cells detached from ECM, indicating that overexpression alone was sufficient to activate FAK (Fig. 4b). However, when we examined apoptosis in detached cells, although WT-FAK appeared to reduce anoikis in both MECs and MEFs, this was not significantly different (P>0.05) than cells expressing myrFAKY397F (Fig. 4c).

These data show that both activation and recruitment to the membrane is required for FAK to suppress anoikis in both fibroblasts and epithelial cells.

The FAK-p130Cas signalling axis only provides an anti-apoptotic signal in fibroblasts

To further explore the role for p130Cas in survival, we asked whether a form of myrFAK that was unable to bind p130Cas could suppress anoikis. FAK contains two proline rich (PR) domains, which can interact with the SH3-domain-containing proteins (Harte et al., 1996; Hildebrand et al., 1996; Polte and Hanks, 1995). We generated myrFAK constructs lacking either or both PR domains (Fig. 5a). Mutation of the PR domains did not prevent phosphorylation on Y397 or focal adhesion localisation in adherent cells (Fig. 5b,c). We were unable to detect endogenous p130Cas binding, presumably due to the levels of its expression. Thus, to confirm the PR-domain mutations were nonfunctional for SH3-domain binding, we co-expressed either p130CasΔSD (which contains the SH3 domain) or p130CasΔSH3, along with myrFAK and the myrFAK PR-domain mutants. myrFAK was immunoprecipitated with anti-V5 and immunoblotted for the co-expressed p130Cas. Deletion of PR-1 reported as the primary binding site for p130Cas (Harte et al., 1996; Polte and Hanks, 1995), resulted in a noticeable decrease in the amount of p130CasΔSD binding.
binding (Fig. 5d, lane 4). Deletion of both PR domains prevented p130CasΔSD binding (Fig. 5d, lane 6). MyrFAK with PR-2 domain alone mutated was still able to bind overexpressed p130CasΔSD (Fig. 5d, lane 5).

MyrFAK or the myrFAK PR-domain deletion mutants were expressed in MEFs and MECs, cells were detached for 24 hours in the absence (MEFs) or presence (MECs) of serum growth factors, and apoptosis was quantified (Fig. 5e). In agreement with previous studies on fibroblasts (Almeida et al., 2000), mutation of PR-1 abolished the ability of myrFAK to protect MEF anoikis. MyrFAK with a deletion of the PR-2 domain appeared to suppress anoikis to some extent, but this did not reach statistical significance ($P > 0.05$). This may reflect the different ability of each site to interact with p130Cas (Fig. 5d). In marked contrast to the results in fibroblasts, all of the FAK PR-domain mutants suppressed anoikis in epithelial cells to the same extent as the mutated myrFAK ($P<0.001$).

Thus, whereas intact PR-domains are required for FAK-dependent survival in fibroblasts, they do not contribute to epithelial cell survival.

FAK interactions with paxillin and SH2-domain proteins are required for anoikis suppression

p130Cas was not required for MEC survival. Furthermore, using the DN-p130CasSH3 indicated that it was unlikely to be the sole mediator of survival in MEFs. We therefore examined other potential interactions with FAK that may transduce survival signals. The C-terminus of FAK contains two other major interaction sites for signalling adaptors. Tyrosine 925, when phosphorylated, can recruit SH2-domain proteins such as Grb2 (Schlaepfer and Hunter, 1996). The FAT domain contains a binding site for paxillin, consisting of two discontinuous sub domains, in which I936 and I998 are essential (Hayashi et al., 2002). Paxillin shares some features of p130Cas, in that it is an adaptor protein for downstream signals, which interacts with the C-terminus of FAK (Hildebrand et al., 1995; Turner et al., 1990). The FAK/Src complex phosphorylates paxillin at Y31 and Y118, creating binding sites for SH2-domain-containing proteins (Bellis et al., 1995; Deakin and Turner, 2008; Playford and Schaller, 2004).

We generated myrFAK mutants in which either the paxillin binding site or Y925 were disrupted (myrFAK I936E/I998E and
myrFAK/I936E/I998E did not (Fig. 6c, lane 3). We examined binding myrFAKY925F bound to paxillin (Fig. 6c, lanes 2 and 4), whereas Immunoblotting with anti-V5 indicated that both myrFAK and glutathione agarose beads coated with GST-paxillin. myrFAKI936E/I998E or myrFAKY925F were incubated with site, lysates of cells expressing wild-type myrFAK, 6b, lanes 8 and 9). To confirm disruption of the paxillin-binding sites were required for myrFAK to suppress anoikis, myrFAK/I936E/I998E and myrFAK/Y925F were expressed in MECs and MEFs, and apoptosis was quantified following detachment from ECM. The anoikis suppression afforded by myrFAK in both cell types was abolished when either the paxillin or SH2-domain binding sites were deleted (Fig. 6e). Thus, these data show that functional paxillin and SH2-domain binding sites within the C-terminus of FAK are required for adhesion-dependent survival signalling in both MECs and MEFs.

Paxillin signalling is required for FAK to suppress epithelial anoikis

To confirm that paxillin was required to suppress anoikis, we expressed paxillin in which tyrosine 31 and 118 were substituted with phenylalanine (GFP-PaxY31/118F) (Fig. 7a) in MECs. Previous studies have shown that PaxY31F/Y118F functions as a dominant negative and can block fibroblast migration (Petit et al., 2000). GFP alone, GFP-paxillin, and GFP-PaxY31/118F all expressed to similar levels in MEC (Fig. 7b). Mutation of the both phosphorylation sites did not affect the ability of paxillin to target to focal adhesions in adherent cells, although it did displace endogenous paxillin from these sites, seen by loss of phosphopaxillin immunostaining (Fig. 7c). When we examined survival, adherent MECs expressing wild-type GFP-paxillin showed no increase in apoptosis compared with cells expressing GFP alone (Fig. 7d). By contrast, expression of GFP-PaxY31/118F resulted in a marked and significant increase in apoptosis (P<0.05).

Thus, even though wild-type and mutant paxillin are both recruited to focal adhesions, its phosphorylation on Y31/Y118 is critical for MEC survival.

Multiple signalling complexes recruited to the C-terminus of FAK are required to suppress anoikis

As myrFAK/Y925F could still bind paxillin and myrFAK/I936E/I998E was still phosphorylated on Y925, we asked whether FAK suppressed anoikis via multiple signalling complexes. MyrFAK, myrFAKY397F, myrFAK/I936E/I998E and myrFAK/Y925F were expressed in HEK293T cells. These were detached from ECM and crosslinked with DSS before immunoprecipitating the expressed proteins with an antibody against the V5 epitope tag. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted for FAK and paxillin (Fig. 8a). Immunoblotting with an anti-FAK antibody identified that myrFAK and myrFAKY397F formed two distinct crosslinked complexes, at approximately 200 kDa and 300 kDa (Fig. 8a, lanes 1 and 3, marked with an asterisk). Neither complex was observed without crosslinking (Fig. 8a, lanes 2 and 4) or in cells not expressing myrFAK (lanes 9 and 10). Paxillin was identified in the 200-kDa complexes with myrFAK, myrFAKY397F and myrFAK/Y925F, but not with myrFAK/I998/936E. Conversely, the 300 kDa complex was not observed with myrFAK/Y925F (Fig. 8a, lane 7).
As these data suggested that FAK formed a number of independent signalling complexes, we asked whether co-expression of the paxillin binding and Y925 mutants could complement each other and restore survival signalling. Whereas neither myrFAKI936E/I998E nor myrFAKY925F alone protected MECs from anoikis, co-expression of both suppressed it to a similar level as seen with wild-type myrFAK (Fig. 8b). This suggests that the ability of FAK to suppress anoikis depends upon its ability to bind both paxillin and a second molecule that interacts with Y925, but that these do not form in the same signalling complex on the same FAK molecule.

Western blotting of 293T whole-cell lysates expressing myrFAK constructs identified that both myrFAK and myrFAKY925F maintained paxillin phosphorylation on Tyr31 and Tyr118 in detached cells (Fig. 8c, lanes 1 and 4). Paxillin was not phosphorylated in cells expressing myrFAKY397F, despite the observation that the two interacted (Fig. 8c, lane 2). Thus, wild-type FAK forms two distinct complexes, only one of which (at approximately 200 kDa) contains paxillin. The paxillin interaction and signalling is maintained by myrFAKY925F, but not myrFAKI936E/I998E. The components of the higher complex are at present unknown, and are a current focus of research.

Finally, we were interested to see whether there were any obvious cell-type differences in the signalling components immediately downstream of FAK. A number of the signalling pathways examined showed no obvious difference between MECs and MEFs. However, one notable survival pathway, Akt (protein kinase B) was seen to be adhesion dependent in MECs, whereas it was predominantly growth factor dependent in MEFs (Fig. 8d). To determine whether this could represent a FAK-dependent, cell-type-specific difference, myrFAK-
expressing MEFs and MECs were detached from ECM for 1 hour and immunoblotted for phosphorylated Akt (Fig. 8e). Akt phosphorylation was maintained in MEC that expressed myrFAK following detachment from ECM, but was lost in untransfected cells. By contrast, growth factor-deprived MEFs showed no changes in Akt phosphorylation under the same conditions.

Together, these data indicate that FAK forms multiple signalling complexes. Furthermore, depending on the cellular context, these complexes transmit distinct survival signals.

Discussion

In this paper we have examined the proximal survival signalling mechanisms downstream of FAK in murine cells from distinct lineages. We show that although FAK is a common transducer of ECM-derived survival signals in fibroblasts and epithelia, its activation is interpreted differently in each cell type. Thus, in agreement with previous studies, we find that FAK suppresses anoikis in fibroblasts via its SH3-interacting PR domains but that these are not required in epithelial cells. However, we also find that both paxillin and SH2-domain interactions via tyrosine 925 are also required for adhesion-dependent survival. As we have utilised a model whereby FAK is constitutively activated in cells detached from ECM and maintained on poly-HEMA for 24 hours. Apoptosis was quantified in transfected cells by nuclear morphology. Results are the mean of three independent experiments. Error bars indicate standard error. *, Significant difference from mock-transfected cells (ANOVA with Bonferroni’s test); n/s indicates no significant suppression of anoikis compared with mock-transfected cells.
required for cell survival, rather than apoptosis, in fibroblasts demonstrated that Jnk activation downstream of FAK was actually required (Khwaja and Downward, 1997). Yet another study dispensed for anoikis and instead activation of PI3-kinase was significant.

Although well established in cell migration, the role of paxillin in survival signalling is not well understood. One previous study has directly implicated paxillin as a mediator of survival signals (Subauste et al., 2004). In that study, competition between vinculin and FAK for paxillin binding determined the amount of paxillin available for downstream signalling via ERK. Thus, vinculin-null F9 cells were resistant to apoptosis because more paxillin was available for interacting with and being phosphorylated by FAK. Expressing either the paxillin-binding region of vinculin, or paxillinY31/118F, could reduce resistance to apoptosis. Here, we have shown that in normal cells, paxillin provides a necessary link in the FAK-dependent survival pathway, and that this may be common in multiple cell types. Thus, we clearly demonstrate a role for paxillin in survival signalling.

Paxillin alone is not sufficient for FAK to transmit survival signals, as mutation of tyrosine 925 also blocked survival signalling (Almeida, 2000; Hood et al., 2003; Howe, 2001; Igishi et al., 1999). Mutation of tyrosines 31 and 118 to phenylalanine block Crk-mediated fibroblast migration (Petit et al., 2000). Paxillin also interacts with paxillin kinase linker (PKL), integrin-linked kinase (ILK), α-parvin and PTP-PEST (Cote et al., 1999; Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Turner et al., 1999). The paxillin–PKL interaction is critical for PKA-mediated cell motility. PKA can activate Jnk and ERK pathways and could potentially be involved in FAK/paxillin survival signalling (Almeida, 2000; Hood et al., 2003; Howe, 2001; Igishi et al., 1999).

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Studies on cell migration support our data that cellular context determines via which downstream adaptors FAK will signal. In MDCK cells, paxillin controls cell migration, whereas in other cell types p130Cas is required (Lamorte et al., 2003; Petit et al., 2000; Schlaepfer et al., 1994; Yano et al., 2000). Our data show that p130Cas is not required for FAK-dependent survival in epithelial cells. Instead, interactions with FAK via both paxillin and tyrosine 925 are necessary. Paxillin is an adapter molecule that can link FAK to multiple pathways (Deakin and Turner, 2008). The N-terminus of paxillin contains two phosphorylation sites, tyrosines 31 and 118, which bind Crk and can control Ras and ERK signalling (Dolfi et al., 1998; Igishi et al., 1999). Mutations of tyrosines 31 and 118 to phenylalanine block Crk-mediated fibroblast migration (Petit et al., 2000). Paxillin also interacts with paxillin kinase linker (PKL), integrin-linked kinase (ILK), α-parvin and PTP-PEST (Cote et al., 1999; Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Turner et al., 1999). The paxillin–PKL interaction is critical for FAK-mediated cell motility. PKA can activate Jnk and ERK pathways and could potentially be involved in FAK/paxillin survival signalling (Almeida, 2000; Hood et al., 2003; Howe, 2001; Igishi et al., 1999).

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that FAK forms multiple signalling complexes, each of which functions independently but all of which can contribute to survival signalling. Depending on the cell type, more than one of these pathways is required for ECM-dependent survival.

Our in vitro results show that FAK signals to suppress anoikis using multiple pathways, and that distinct cell types utilise different subsets of these pathways. However, it is important to consider the relevance of this in vivo. Tissue-specific FAK knockouts support a model whereby the role of FAK to suppress anoikis shows cell lineage specificity. For example, FAK deletion in keratinocytes resulted in defects in epidermal thickness and hair growth, but there were no apoptosis defects in vivo. However, keratinocytes isolated from these animals died rapidly in vitro, indicating that FAK was required to support survival under certain conditions (Essayem et al., 2006). By contrast, deletion of FAK in endothelial cells resulted in an increase in apoptosis in vivo, leading to lethality before day 10.5 due to extensive haemorrhaging (Braren et al., 2006). Interestingly, whereas fibroblasts isolated from FAK–/– embryos had defects in migration but not survival, FAK–/– endothelial cells in vivo did not show defects in migration but did undergo apoptosis (Judson et al., 1999; Lark et al., 2003; Lark et al., 2005; Lightfoot et al., 2004; Owens et al., 1995; Owens et al., 1996). Together, these data indicate that how FAK controls cell survival depends very much on cellular origin as well as environmental context. This has implications for how FAK signalling may alter during tumour metastasis, when epithelial cells become more mesenchymal and invade diverse ECM types. Our data suggest that alterations in how FAK signals can affect cell survival in different ECM environments. FAK is upregulated in a range of tumours, including breast, colon, thyroid and ovarian (McLean et al., 2005). Indeed, the FAK locus is amplified in 79% of sporadic breast cancers (Naylor et al., 2005). Deletion of FAK in a mouse model of skin cancer blocked malignant progression (McLean et al., 2004).

In conclusion, our data indicate that FAK can signal via multiple, divergent pathways to suppress apoptosis. These results suggest that even though FAK may represent a common adhesion-dependent kinase activated by a wide range of integrins and extracellular matrices, it shows a complexity of downstream interactions that fine-tune cell-type-specific phenotypes.

Materials and Methods

**Reagents**

Anti-V5 was from Invitrogen. Anti-phospho-paxillin Y31, anti-phospho-paxillin Y118, anti-phospho-FAK Y937, anti-phospho-FAK Y925 and anti-phospho-FAK Y577 were from Biosource (Nivelles, Belgium). The polyclonal anti-FAK was a gift from Andy Ziemiecki (University of Berne, Switzerland). Anti-actin was from Sigma and the anti-mtHsp70 was from Affinity Bioreagents (Golden, CO). Anti-p130Cas, anti-paxillin and anti-phosphotyrosine (PY20) were from BD Transduction Labs. Anti-phospho-p130Cas, anti-phospho-Akt and anti-phospho-paxillin were from Cell Signalling Technology (Danvers, MA). Secondary antibodies (anti-rabbit, anti-goat and anti-mouse peroxidase conjugates) were from Jackson Laboratories. Fluorescent
conjugated secondary antibodies (anti-rabbit FITC and anti-mouse RXR) were from Alexis Corp. (Lausen, Switzerland). Hoechst was from Sigma. Rhodamine-phallidin was from Molecular Probes.

Expression constructs

Constitutively active FAK was created by cloning a viral-Src myristylation tag to the N-terminus of FAK. Oligonucleotides encoding the myristylation sequence were annealed and ligated into pDNA6/V5-His. Full-length wild-type FAK was then cloned in frame 3' to the myristylation sequence. The oligonucleotides encoding the myristylation tag were:

\[ CAGCGCCGGCGCCATATGTGGTAC-3' \]

Specific mutations in myristylated FAK were created using site-directed PCR (QuikChange, Stratagene) and mutagenic oligonucleotides. The mutated bases are CGGCGCT GGCTGGGGTCCTTAGGCTTGCTCTTGCTGCTCCCCATG.

For apoptosis assays, cell death was quantified by nuclear morphology as previously described. The p130Cas expression vectors were generous gifts from Dusko Ilic and Vasken Ohanian for comments on the manuscript. Deposited in PMC for release after 6 months.

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