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Commentary

Focal adhesion kinase: A potential target in cancer therapy

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Abbreviations:
ASAP1, Arf-GAP containing SH3 domain, ankyrin repeats and pleckstrin homology domain
Bad, Bcl-2 associated death protein
CAS, Crk associated substrate
Cdk, cyclin dependent kinase
cPLA2, cytoplasmic phospholipase A2
ECM, extracellular matrix
EGF(R), epidermal growth factor (receptor)
ERK, extracellular signal-regulated kinase
FAK, focal adhesion kinase
FERM, erythrocyte band four.1-ezrin-radixin-moesin
FKHR, forkhead transcription factor
FRNK, FAK related non-kinase
FAT, focal adhesion targeting
GRAF, GTPase regulator associated with FAK
Grb, growth factor binding protein
GSK3, glycogen synthase kinase 3
JNK, Jun N-terminal kinase
LD, paxillin leucine aspartate repeat

ABSTRACT

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays an important role in signal transduction pathways that are initiated at sites of integrin-mediated cell adhesions and by growth factor receptors. FAK is a key regulator of survival, proliferation, migration and invasion: processes that are all involved in the development and progression of cancer. FAK is also linked to oncogenes at both a biochemical and functional level. Moreover, overexpression and/or increased activity of FAK is common in a wide variety of human cancers, implicating a role for FAK in carcinogenesis. Given the important role of FAK in a large number of processes involved in tumorigenesis, metastasis and survival signalling FAK should be regarded as a potential target in the development of anti-cancer drugs. Therefore, selective inhibitors of FAK need to be developed. Combination of these selective FAK inhibitors with cytotoxic agents could be a very promising anti-cancer therapy.

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MAPK, mitogen-activated protein kinase
MMP, matrix metalloproteinase
NF-kappa B, nuclear factor kappa B
PDGF(R), platelet derived growth factor (receptor)
PDK1, Phosphoinositide-dependent protein kinase-1
PI-3 kinase, phosphatidylinositol 3-kinase
PIP2, phosphatidylinositol 3,4 biphosphate
PIP3, phosphatidylinositol 3,4,5 triphosphate
PKB, protein kinase B
PKC, protein kinase C
PP1, Phospho-protein phosphatase 1
PTEN, phosphatase and tensin homolog deleted on chromosome 10
PYK2, proline rich tyrosine kinase 2
SH, Src homology
Shc, Src homology containing protein
Sos, Son of sevenless
uPA, urokinase plasminogen activator

1. Introduction

The non-receptor protein tyrosine kinase focal adhesion kinase (FAK) was discovered almost 15 years ago by identification of an increased phosphorylated protein after v-Src transformation of chicken embryo cells [1]. FAK is expressed in virtually all tissues and cell types. After years of extensive study, it is now well-established that FAK plays a crucial role in mediating signal transduction pathways initiated either at the sites of cell attachment or at growth factor receptors. Activation of FAK leads to a number of cell biological processes, including cell attachment, migration, invasion, proliferation and survival. These processes are important in cell, tissue and organ structuring, functioning and remodelling after injury. For example, FAK mRNA and protein levels increase from embryonic day 7.5 during mouse development, indicating that FAK is important during the early stages of embryogenesis. Likewise, FAK is essential in embryonic development, since FAK-null embryos die at day 8.5 [2]. Examination of these embryos revealed that FAK is crucial for vascular development, endothelial cell migration and tube formation, which requires a tightly regulated onset of apoptosis, in part controlled by FAK. Conversely, enhanced FAK signalling may result in uncontrolled proliferation, survival or migration of cells, as observed in cancer development and progression. A genetic basis for cancer is provided by oncogenes, including (non) receptor tyrosine kinases (RPTKs). FAK interacts with several RPTKs and is in many cases required for the full activation of these receptors, implicating a role for FAK in oncogenic transformation. Given the role of FAK in processes important in tumorigenesis and metastasis and the link to prominent oncogenes, FAK might be a promising target in the ongoing search for an anti-cancer drug. In this commentary we summarize the evidence of the importance of FAK in cancer and we discuss the potential of FAK as an anti-cancer drug target.

2. FAK structure

FAK is a ubiquitously expressed kinase for which the amino acid sequence is more than 90% homologous between human, chicken, mouse and frog. FAK mediates signalling by phosphorylation and/or localization of its downstream effectors. The regions required for this are the centrally located kinase domain flanked by a large N-terminal domain containing the FERM region and a C-terminal domain harbouring the focal adhesion targeting (FAT) sequence (Fig. 1).

The kinase domain of FAK shares a high degree of sequence similarity with other protein tyrosine kinases. Clustering of the integrins facilitates the autophosphorylation of tyrosine 397, which increases the catalytic activity of FAK (Fig. 1). The motif surrounding tyrosine 397 facilitates the binding of SH2 (Src homology 2) domain containing proteins. The most important

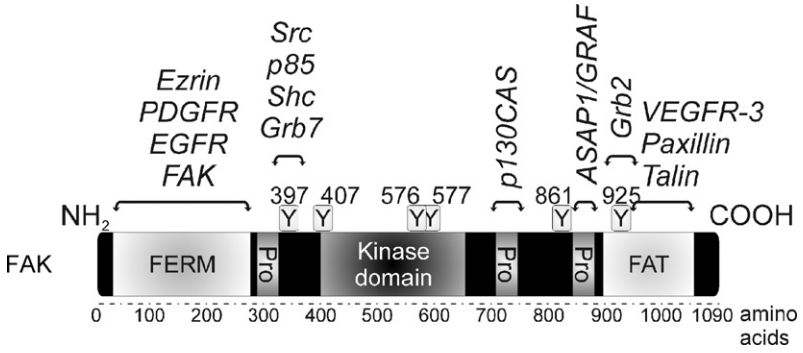

Fig. 1 – Focal adhesion kinase structural features and binding partners. The kinase domain of Focal adhesion kinase is flanked by the N-terminus that harbours the FERM domain, and by the C-terminus that consists, in addition to proline rich domains, of the FAT domain. The FERM domain interacts with growth factor receptors, the autophosphorylation site of FAK (tyrosine 397) is required for its activity and is a binding partner for Src, p85, Shc and Grb7. The kinase domain contains two important tyrosines, 576 and 577, whose phosphorylation is necessary for full kinase activity. The C-terminal domain harbours 2 proline rich domains that interact with SH3 domain containing proteins like p130CAS, Graf and ASAP1. Furthermore it contains an important tyrosine, 925, which, after phosphorylation, interacts with Grb2 and a FAT sequence that localizes FAK to the integrins, both directly and indirectly via the scaffolding proteins paxillin, talin and vinculin.

SH2 containing protein that interacts with FAK-tyrosine 397 is Src. The catalytic loop harbours two tyrosines, Y576 and Y577, whose phosphorylation is induced in response to binding of Src to FAK-tyrosine 397 and is necessary for the full adhesion-induced activation of the kinase domain [3,4]. In addition to autophosphorylation, the kinase domain is implicated in the phosphorylation of several focal adhesion associated proteins, for example paxillin, Grb2 and p130CAS [5].

The N-terminus consists of an autophosphorylation site, tyrosine 397, and a FERM domain (erythrocyte band four.1-ezrin-radixin-moesin), both mediating protein–protein interactions. The most well-known interaction partner of the FAK-FERM domain is the cytoplasmic tail of β-integrins. In addition to protein binding, there are indications that the FERM domain functions as a regulator of FAK activity. In suspension, interaction of the FERM domain with the kinase domain prevents autophosphorylation of tyrosine 397, necessary for the activation of FAK. During attachment of cells, the integrins cluster, enabling the binding of the FERM domain to the cytoplasmic tail of the β-integrin. This results in the unfolding of FAK, thereby releasing its autoinhibition and allowing its autophosphorylation and activation [6]. In addition to this intramolecular interaction between the FERM and the kinase domain, an intermolecular interaction between the FERM domain and full length FAK was shown [7]. This intermolecular interaction is required for optimal kinase activity. Thus, the FERM domain can function both as a negative and positive regulator of FAK.

The C-terminal domain can be divided into FAT sequence and the region between the FAT sequence and the kinase domain. As suggested by the name, the FAT sequence is required for localization at the focal adhesions. Fusion of this 15.5 kDa fragment to other proteins is sufficient for the localization at focal adhesions [8]. Using protein NMR microscopy it was shown that the FAT domain consists of four helices that form a right-turn, elongated bundle, maintained by hydrophobic interactions [9]. Although the FAT domain is able to directly interact with the cytoplasmic tails of integrins, increasing evidence supports an indirect interaction with the integrins via integrin associated proteins like talin and paxillin [10]. The adapter protein paxillin, important in scaffolding a large number of proteins to the focal adhesions, interacts with its LD2 domain to helix 1 and with its LD4 domain to helix 3 of the FAT domain [11]. Between the FAT domain and the kinase domain, two proline rich domains are recognized, which mediate interactions with SH3 domain containing proteins. Some of the FAK-proline rich domain interaction partners are p130CAS, GRAF (GTPase regulator associated with FAK) and ASAP1 (Arf-GAP containing SH3 domain, ankyrin repeats, and PH domain).

Due to alternative splicing and alternative promoters, several isoforms of FAK exist (Fig. 2). The most well-known was isolated from chicken fibroblasts and consists of the C-terminal part of the protein. Since it lacks a catalytic domain, it is called FAK related non-kinase (FRNK) [12]. FRNK competes with FAK for the localization at focal adhesions, but since it lacks a kinase domain, it is often used as an inhibitor of FAK. Other alternatively spliced isoforms of FAK that are predominantly expressed in brain tissue are termed FAK^{+}, FAK-box6, -box7 and -box28 [13]. The only other family member of FAK is proline-rich tyrosine kinase 2 (PYK2) (also called cell adhesion kinase β (CAKβ), related adhesion focal tyrosine kinase (RAFTK) or calcium-dependent protein-tyrosine kinase (CADTK)). PYK2 is slightly smaller than FAK (112 kDa) and the highest expression levels can be found in cells of the nervous system, but it is also expressed in cells of haematopoietic origin as well as several other cell types. Not much is known about the role of PYK2 in cancer cells. In this commentary, we focus solely on the role of FAK.

3. FAK and cancer: clinical evidence

A large number of reports describe the expression and activity of FAK in primary and metastatic human tumor tissue. Most studies show an enhanced expression of FAK mRNA and/or

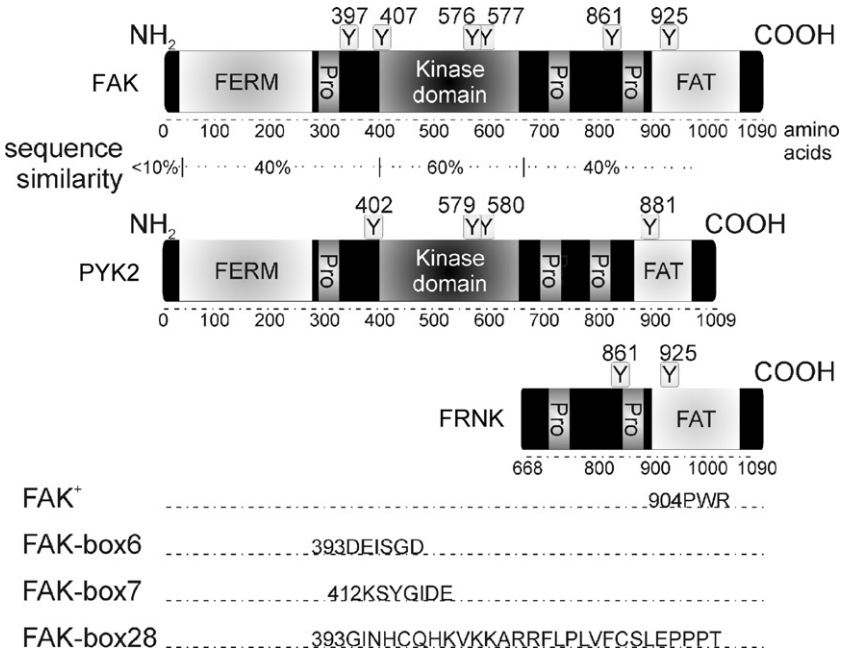

Fig. 2 – FAK, PYK2 and FRNK. Family member PYK2 shows high sequence similarity to FAK, especially in the kinase domain. Important phosphorylation sites of PYK2 are tyrosines 402, 579, 580 and 881. FAK related non-kinase (FRNK) is a negative acting splice variant of FAK, consisting of amino acids 668–1090. It lacks both the N-terminal and the kinase domain, but is still able to localize at the focal adhesion due to its FAT domain.

protein in a variety of human cancers, including squamous cell carcinoma of the larynx [14], invasive colon and breast tumors [15], metastatic prostate carcinoma [16] and malignant melanoma [17]. To more precisely determine the role of FAK in the different steps of tumor progression, matched primary and secondary tumors are informative. In colon adenocarcinoma tissue, the expression of FAK is increased compared to normal colonic mucosa; FAK expression however did not correlate with tumor histological grade or survival and therefore could not be considered as a prognostic marker [18]. In a study of 55 matched pairs of breast cancer and corresponding normal tissue, using both Western blot analysis and immunohistochemistry, a significantly increased expression of FAK at the protein level was found in the tumor tissue. At the mRNA level, no difference was observed [19]. Analysis of matched normal tissue and primary human colorectal adenocarcinomas revealed higher levels of FAK in tumors, whereas reduced expression of FAK was found in liver metastases compared with the matched primary tumors [20]. These data provide evidence for a role of FAK in the pre-metastasis phenotype. In agreement with this study, weak expression of FAK in patients with cervical cancer is specifically correlated with pelvic lymph node metastasis and recurrent disease, resulting in a poor disease outcome [21]. Yet FAK overexpression in oesophageal squamous cell carcinoma is linked with tumor invasiveness and lymph node metastasis [22]. Altogether, these studies suggest that FAK may possess alternative roles in different tumors and/or in different stages of tumor progression. Despite these differences, all studies support an important role for FAK in tumor formation and metastasis.

Several studies attempted to link the expression of FAK to disease prognosis. Expression of tyrosine 397 phosphorylated FAK was associated with early death and shorter survival rate in a cohort of 60 acute myeloid leukaemia patients [23]. In pancreatic carcinoma tissue, no correlation between FAK expression and tumor histological grade, metastasis and overall survival could be found. However, there was a strong correlation between FAK expression and tumor size, implicating a role for FAK in proliferation of these carcinomas [24]. FAK overexpression was also shown to mark the malignant transformation of both squamous cells of the uterine cervix and epithelial cells of breast ducts [25]. In breast carcinoma, activation of both FAK and c-Src correlated with malignant transformation but not with invasive properties [26]. In addition to studies that show increased expression of FAK in tumor tissue at mRNA and protein level, cell lines derived from invasive epithelial tumors have increased dosage and amplification of the FAK gene [27]. Importantly, a recent study showed a close correlation between FAK mRNA levels and protein levels, whereas an increase in gene copy number could not be coupled to an increased expression [28]. Not many studies provide insight in the mechanism of the increased expression of FAK. Unravelling of the promoter region of FAK revealed binding sites for NF-kappa B and p53 [29]; NF-kappa B induces and p53 inhibits transcription of FAK. Since FAK is implicated in a large number of processes involved in tumor progression and metastasis, it is not surprising that all of these studies link the expression and/or activity of FAK to different properties of the cancer cells. Most probably, spatial and temporal FAK activity is required in the different steps of tumorigenesis and metastasis. Therefore, immunohistological data on the expression of tyrosine 397 (and other tyrosines) phosphorylated FAK in different tumor types and at different tumor stages are necessary. These data could provide

additional prognostic insight and mechanistic information on tumor progression.

4. Focal adhesion formation and mechanisms of FAK activation

The first event in the formation of a focal adhesion is binding of a cell to the extracellular matrix (ECM). Cell adhesion to the ECM is directly mediated by anchor-like proteins: integrins. Integrins are composed of α and β transmembrane subunits. There are 16 different α and 8 different β subunits, heterodimerizing into at least 25 different receptors, each being specific for a unique set of ligands. Most of the integrins bind to components of the extracellular matrix (for example, fibronectin, collagen and vitronectin); some bind to soluble ligands (e.g. fibrinogen). Upon binding of the integrins to the extracellular matrix components, the integrins cluster and their cytoplasmic tails provide binding sites for cytoskeletal and signalling molecules. Different domains of FAK are involved in the binding of FAK to the integrins and in the recruitment of other proteins to the focal adhesions. Both the N-terminal FERM domain and the C-terminal FAT domain are sufficient for the localization at the focal adhesions. *In vitro* studies show direct interaction of the FERM domain with the cytoplasmic tail of the β integrin subunit, resulting in a rapid autophosphorylation of tyrosine 397 [30]. Phosphorylation of tyrosine 397 can be an intra- or intermolecular event [31]. Deletion mutants of FAK that only consist of the N-terminal domain localize at focal adhesions and therefore, by competing for localization, act as FAK inhibitors [32]. In addition to the direct interaction of FAK and the integrins, the C-terminal domain of FAK is indirectly linked to the integrins via the cytoskeletal protein talin [33]. Expression of deletion mutants of FAK revealed a 159 amino acids sequence in the C-terminal domain, called the focal adhesion targeting domain, which is sufficient for localization of FAK at the focal adhesions [8]. Further, it is hypothesized that paxillin recruits FAK to the focal adhesions [34]. Paxillin is an adaptor protein that consists of different interaction domains, such as four LD motifs which interact with FAK and four LIM domains which are important in the recruitment of paxillin to the focal adhesions [35]. Likely, both the interactions of the FERM domain with the β integrin subunit and of the FAT domain with paxillin and talin are required to stably localize and autophosphorylate FAK at the focal adhesions. Close proximity to the focal adhesions and autophosphorylation of FAK at tyrosine 397 are critical for the induction of paxillin phosphorylation [10] and thus for the kinase activity of FAK.

As briefly mentioned, FAK is a downstream target of several growth factor receptors, for example epidermal growth factor receptors (EGFR) and platelet derived growth factor receptors (PDGFR). Overexpression or constitutive activation of these receptors is coupled to oncogenic transformation of cells. Therefore, FAK might be an important player in the transduction of oncogenic signalling. To interact with the intracellular domain of the EGFR, FAK requires an intact N-terminal domain. In addition, EGF induced migration also requires an intact C-terminal domain, indicating that targeting of FAK to both the integrins and the EGFR combines these signalling pathways [36]. Binding of Src to tyrosine 397 phosphorylated FAK results in the unfolding of Src and its enzymatically activation. Src kinase can also be activated in response to binding to growth factor receptors. FAK tyrosines 407, 576 and 577 become phosphorylated in response to binding to Src [3]. Tyrosines 576 and 577 lie in the kinase domain and their phosphorylation results in increased kinase activity of FAK. Formation of the FAK–Src complex also leads to phosphorylation of tyrosine 925, which creates a Grb2 binding site and thus links FAK to the MAP kinase pathway [37]. Tyrosine phosphorylated, kinase active FAK binds and phosphorylates a large number of proteins, including paxillin, p130CAS, Grb7 [3] resulting in the formation and turnover of focal adhesion complexes. These complexes mediate a number of downstream signalling pathways related to cell survival, proliferation and migration (Fig. 3) that are described in more detail in the next paragraphs (Table 1).

4.1. *FAK-mediated survival signalling*

In order to survive and grow, most normal cells require, in addition to growth factors, adhesion to the ECM. Lack of cell–ECM contacts results in a specific form of apoptosis:anoikis [38]. Only the stimulus, lost contact with the extracellular matrix, discriminates anoikis from apoptosis. Hallmark of cancer cells is resistance to anoikis. Overexpression of constitutively active FAK rescues epithelial cell lines from anoikis [39], thereby confirming the involvement of FAK signalling in cancer cells. Inhibition of FAK by antisense techniques [40] or antibody-methods [41] results in the onset of apoptosis and numerous studies have clearly confirmed a protective role for FAK in apoptosis [42–45]. The survival signalling mediated by FAK most likely operates via activation of the PI-3 kinase route (Fig. 3) [42]. Phosphorylated tyrosine 397 interacts with and activates PI-3 kinase. Activation of PI-3 kinase results in generation of the second messengers PI(3,4,5)P3 and PI(3,4)P2. These phospholipids recruit protein kinase B (PKB or Akt) to the plasma membrane, and subsequently PKB is phosphorylated by PDK. Activated PKB provides survival signalling by inactivation of a series of pro-apoptotic proteins, for example p21WAF, FKHR, Bad and GSK3 [46]. This PI-3 kinase/PKB survival route can be inhibited by the tumor suppressor gene PTEN, which dephosphorylates both PIP3 lipids as well as the protein kinases Shc and FAK [47]. Since PKB, via PI-3 kinase, is a direct downstream target of both FAK and the epidermal growth factor receptor, in the presence of growth factors it is hard to distinguish FAK and EGFR as upstream activator. In breast cancer BT474 cells EGFR-mediated downstream activation of PKB only partly inhibits apoptosis induced by the dominant negative-acting pro-apoptotic C-terminal domain of FAK [48]. Given the direct interaction between FAK and the EGFR, a possible synergistic action between FAK and growth factor receptors may be required for full PKB activity.

As just mentioned, expression of the C-terminal domain of FAK can result in apoptosis. During apoptosis, caspases become activated. Executioner caspases cleave pro-apoptotic proteins to abrogate survival signalling and eventually induce apoptosis. Interestingly, FAK is cleaved by caspases during apoptosis [49] to generate a FRNK-like polypeptide [50]. Since

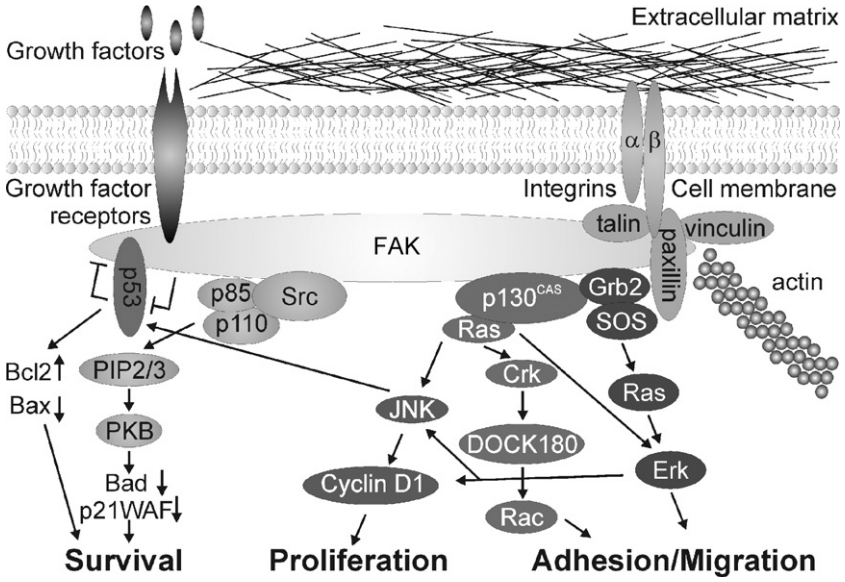

Fig. 3 – FAK-mediated signalling pathways. FAK is via its N-terminal domain targeted to growth factor receptors (GFRs), and via its C-terminal domain to adhesion-mediated clustered integrins. Paxillin, vinculin and talin are important for the localization of FAK to the integrins. Interaction of FAK with GFRs and/or clustered integrins results in the autophosphorylation of tyrosine 397. This provides a binding site for Src kinase and formation of this FAK-Src complex leads to the phosphorylation of additional tyrosines of FAK, resulting in its full kinase activity and in the recruitment of other focal adhesion proteins. By the interaction and/or phosphorylation of a large number of targets, FAK is a mediator of survival, proliferation, adhesion and migration signalling. Binding and phosphorylation of the p85 subunit of PI-3 kinase results, via the formation of PIP2/3 phospholipids and activation of PKB, in survival signalling. Interaction of the N-terminal domain of FAK inhibits the transcriptional activity of p53, thereby also enhancing survival signalling. Binding of Grb2 to phosphorylated tyrosine 925 results in the activation of the Grb2/Sos/Ras/ERK pathway, which is implicated in proliferation and migration and via the activation of JNK in survival signalling. Interaction of p130CAS to a proline rich domain in the C-terminus is mainly implicated in motility. But via the activation of JNK, p130CAS is also involved in mediating proliferation and survival signalling.

FRNK contains the focal adhesion targeting domain, it competes with full length FAK for localization at the focal adhesions, thereby further inhibiting FAK-mediated survival signalling. In addition to activation of the PI-3 kinase/PKB survival signalling route, FAK also transduces survival signals from the ECM via inhibition of the tumor suppressor p53. In the absence of FAK or the correct ECM, apoptosis is induced via the cPLA2, PKCλ/ι, p53 pathway [51]. Other papers show a direct link between the N-terminal domain of FAK and the N-terminal domain of p53. Interaction of these proteins results in reduced transcriptional activity of p53, thereby preventing its pro-apoptotic signalling [52]. A third ECM-FAK-mediated

Table 1 – Overview of the proteins (including references) that are involved in FAK-mediated processes mentioned in this review

Biological process	Proteins of the FAK-mediated signalling cascade	References
Survival	PI-3 kinase → PKB → Bad → GSK3	[42,46]
	p53	[51,52]
	(Src) → p130CAS → Ras → → JNK	[53]
Proliferation	Grb2 → MAPK → Cyclin D1	[57]
	PKC/PI-3 kinase → Rb → Cyclin D3	[60]
Migration	Calpains	[67]
	Src/p130CAS → MAPK	[68]
	Src/p130CAS/PI-3 kinase → MAPK	[69]
	GSK3/PP1	[71]
Angiogenesis	MEK → ERK → VEGF	[83]
Invasion	MMPs	[72]
	ErbB2/3	[73]

survival pathway starts with the recruitment of the SH3 domain of p130CAS to a proline rich region of FAK. After phosphorylation of p130CAS, either by FAK or by Src, Ras is activated. Ras activation can initiate multiple signalling pathways, including ERK, JNK and p38 MAP kinase pathways, but only the Rac1/Pak1/MKK4/JNK route is required for the transduction of the ECM-FAK-p130CAS survival signals [53]. Again, FAK signalling can be linked to p53, since JNK is implicated in the regulation of p53 protein levels [54]. The ECM-FAK-p130CAS survival pathway is activated in the absence of growth factors, whereas for the ECM-FAK-PKB survival pathway additional growth factors are required. Importantly, most of the described experiments that attempt to unravel ECM-FAK-mediated survival pathways have been performed in non-cancerous cells like fibroblasts and endothelial cells. However, the mechanism by which FAK induces survival signalling seems highly dependent on the stimulus and the presence of growth factors. In the context of tumor cells, this means that depending on the mechanism of tumorigenesis, different survival signalling pathways are likely to be activated. Regardless, FAK plays a pivotal role in several of these survival signalling cascades and therefore, under these different conditions FAK might be targeted to inhibit the survival signalling in tumor cells.

4.2. *Proliferation*

Cell cycle transition from G1 to S phase is regulated by a number of cyclin-dependent kinases, which in turn are regulated by phosphorylations, cyclins and cdk inhibitors as reviewed by Draetta [55]. Important for the G1 to S transition are cyclin D, E and A. Cyclin D1 assembles with cdk4/6 in the early G1 phase and is rate-limiting in cellular proliferation induced by a number of stimuli. In accordance, overexpression of cyclin D1 is observed in several tumors and overexpression in mice results in tumor formation [56]. Expression of cyclin D1 is largely dependent on transcription, which in turn is regulated by the Ras/Erk signalling cascade. The first link between FAK and proliferation was suggested by Schlaepfer et al., by connecting tyrosine 925 phosphorylation of FAK, via binding to Grb2, to activation of the MAP kinase pathway [57]. Overexpression of wtFAK accelerates G1 to S phase transition, whereas expression of a FAK dominant negative-acting deletion mutant inhibits cell cycle progression via changes in the expression of cyclin D1 and the cdk inhibitor p21 [58]. Overexpression of cyclin D1 rescues this mutant FAK from cell cycle arrest. FAK regulates cyclin D1 expression upon cell adhesion, most likely through activation of the Erk signalling pathway (Fig. 3) [59]. In addition to cyclin D1, FAK is also implicated in the upregulation of cyclin D3, via the PKC and PI-3 kinase pathways, resulting in retinoblastoma protein phosphorylation, thereby enhancing cell proliferation [60]. We showed that inhibition of FAK by expression of FRNK in MTLn3 cells results in decreased proliferation *in vitro* as well as decreased primary tumor growth *in vivo* [61]. However, conditional expression of FRNK in already existing primary tumors or experimental micro-metastases in the lungs did not affect further tumor and metastasis progression (van Nimwegen et al., manuscript in preparation). Most likely in the primary tumor multiple proliferation signalling cascades can be activated in the absence of active FAK. Thus, although FAK is involved in cell cycle progression under *in vitro* circumstances, it is not clear whether these properties of FAK are relevant for *in vivo* tumor growth. ABI microarray analysis of our tetFRNK-MTLn3 cells in an *in vitro* situation showed that expression of FRNK does not alter the expression of cyclin D1 or other cyclins (unpublished data). Most likely multiple proliferation signalling cascades can be activated in the absence of active FAK dependent on the tumor cell and oncogenic context. Thus, although FAK is under certain conditions involved in cell cycle progression, the cyclin regulating properties of FAK do not seem to be crucial in all tumor cells.

4.3. *Spreading, migration and invasion*

In addition to survival and proliferation, FAK signalling is linked to spreading and migration processes. FAK plays a functional role in integrin signalling leading to cell adhesion [62] and migration [63], requiring its autophosphorylation and complex formation with paxillin (adhesion) and Src (migration/invasion). Fibroblasts isolated from FAK-null embryos appear more rounded and have reduced spreading than FAKwt cells. Surprisingly, since FAK is thought to be required for focal adhesion formation, spreaded FAK-null cells displayed an increased number of focal adhesions, but they showed an impaired migratory phenotype [2]. Live cell imaging of Y397F-FAK revealed that tyrosine 397 phosphorylation is crucial in the disassembly of focal adhesions [64]. Thus in the FAK-null fibroblasts, large focal adhesions are formed, but due to the lack of FAK they are not broken down efficiently supporting impaired migration. In order to migrate, at the leading edge of the cell peripheral adhesions have to be formed, whereas at the rear of the cell focal adhesions must be broken down. FAK is required for the organization of the leading edge in migrating cells [65]. Focal adhesion disassembly at the rear of the cell is (at least partly) mediated by cleavage of focal adhesion proteins, for example FAK, talin and paxillin, by calpains (reviewed in [66]). Increased calcium levels at the rear of the cell [67] and FAK dependent phosphorylation of calpains trigger their activation. Since FAK is both an activator and a substrate of calpains, the interplay between FAK and calpains is important in the disassembly of the focal adhesions at the rear of the cell and thus for migration.

There are several FAK-mediated signalling pathways leading to migration (Fig. 3). One implicates p130CAS as a mediator [68], whereas another indicates that in addition to the FAK-Src-p130CAS complex PI-3 kinase is required [69]. Localization of Src at the focal adhesions is crucial in migration, whereas both mutation of the docking site on FAK (Y397F) and mutation of the SH2 domain of Src results in the loss of motility. Motility can be restored by localization of Src to the focal adhesions by fusion of a focal adhesion targeting domain to Src [70]. A third FAK-mediated signalling pathway leading to migration involves serine 722 phosphorylation through the competing actions of the serine/threonine kinase GSK3 and the phosphatase PP1 during cell spreading and migration [71]. Migration of cells is often studied in chemotaxis assays using EGF. As mentioned before, FAK associates with activated growth-factor receptors through its N-terminal domain and is important in promoting PDGF and

EGF-stimulated cell migration [36]. Downregulation of FAK in human adenocarcinoma cells results in inhibition of EGF-stimulated chemotaxis [72]. This could be attributed to decreased EGF-stimulated MAP kinase activation and inhibition of secretion of the matrix metalloproteinase MMP-9. MMPs are involved in the degradation of matrix components and play an important role in the invasion of tumor cells through basement membrane barriers. Thus regulating MMPs, in part by FAK, could be considered as an important step in metastasis formation. Strikingly, downregulation of FAK hampered chemotaxis and invasion in ErbB2/3 transformed fibroblasts [73]: the dominant oncogenes ErbB2/3 cooperate with FAK in tumorigenesis processes. So, FAK is able to enhance downstream signalling that results in the invasion processes. FAK activity and/or expression is not solely regulated by oncogenes, but also by tumor suppressors. For example, the tumor suppressor NF2/merlin inhibits invasiveness by attenuation of FAK tyrosine 397 phosphorylation, thereby disrupting the interaction of FAK with Src and p85 [74]. Another example is the tumor suppressor phosphatase PTEN, which directly inhibits FAK signalling by dephosphorylation of tyrosine 397 and indirectly by opposing the action of PI-3 kinase by dephosphorylating the signalling lipid PIP3, as reviewed by Yamada et al. [36]. Inhibition of the FAK/p130CAS pathway by expression of PTEN reduces cell motility [75]. So, the expression and activity of FAK can be controlled by oncogenes and tumor suppressors, whereas the activation of FAK is coupled to several migration and invasion pathways.

4.4. *FAK in tumorigenesis/metastasis: animal models*

A large body of evidence indicates that FAK is involved in the different steps of metastasis: proliferation, migration, survival and invasion of cells. In addition to studies unravelling the role of FAK in these separate processes, a number of studies have been dedicated to identify FAK as a mediator of tumorigenesis and/or metastasis in *in vivo* situations. Several studies suggest a direct role for FAK in tumor formation and progression. Chemical (DMBA) induction of skin cancer is suppressed in *fak* heterozygous mice compared to wild type mice. Since a key event in DMBA-induced papillomas is mutation of H-*ras*, and the mutation frequency in the heterozygous and wild type mice is equal, FAK might be limiting for integrin-dependent activation of the ras/ERK pathway. In contrast to papilloma formation, no difference in the rate of conversion to malignant carcinoma was seen. However, close examination of the malignant papillomas showed that the amounts of FAK were similar in the *fak*+/− and *fak*+/+ mice. This suggests that, despite the presence of only a single operational *fak* allele in the *fak* heterozygous mice, FAK protein expression was elevated during tumor progression [76]. Skin-specific deletion of *fak* after formation of papillomas revealed that FAK is directly required for the malignant conversion *in vivo* [77]. The underlying mechanism of this decreased progression is increased apoptosis after deletion of FAK. By using these conditional models, the role of FAK in the different steps of tumorigenesis/metastasis can be determined.

Since FAK mediates multiple signalling pathways by phosphorylation on one hand and by scaffolding on the other hand, it is important to identify which domains of FAK are crucial in tumorigenesis/metastasis. Expression of CD2-FAK in Madin-Darby canine kidney cells enables subcutaneous tumor formation in nude mice [39]. Similarly, overexpression of FAK in U-215MG human malignant astrocytoma cells results in increased tumor cell proliferation in a xenograft model [78]. These studies clearly point to a FAK-mediated increase in proliferation. Oppositely, inhibition of FAK by expression of FRNK in human epidermoid carcinoma cells reduces cellular outgrowth when these cells are inoculated onto chorioallontoic membranes of chicken embryos [79]. This reduced outgrowth is not coupled to an increase in apoptosis but to tumor cell dormancy. Stable expression of FRNK in NIH-3T3 fibroblasts transformed with the dominant oncogene v-Src inhibits cell invasion, but not motility *in vitro* and reduces experimental metastasis, but not tumor growth, in nude mice [80]. On protein level, FRNK expression in this model results in the prevention of Src-mediated ERK2 and JNK activation and a reduction in MMP-2, indicating a role for Src-FAK cooperation in invasion. Constitutive expression of FRNK in B16-F10 melanoma resulted in a 50% reduction in the number of lung metastases [81]. Major concern regarding these tumor formation studies that use stable expression of FRNK, is that a major survival signalling pathway is continuously disrupted. Therefore, it cannot be excluded that the reduced tumor formation is a direct consequence of reduced survival signalling. Since expression of FRNK often leads to cell death, cells that survive stable expression of FRNK probably represent a selection of the founder cell line. Such a selection may affect the characteristics and thereby the *in vivo* behaviour of the tumor cells. To circumvent this problem, we have used a conditional model and showed that expression of HA-tagged FRNK reduces primary tumor growth and inhibits experimental lung metastasis. By expressing FRNK only during the first days after injection of the cells into the tail vein of the rats, we showed that FAK signalling is essential during the initial steps of metastasis, most likely by mediating tumor cell invasion/migration processes [61]. More mechanistic insights into the FAK controlled metastasis was revealed by using FAK shRNA cells in a syngeneic tumor-metastasis model. Mitra et al. showed that the catalytic activity of FAK is able to facilitate metastatic breast cancer progression by the regulation of uPA expression [82]. There are multiple pathways downstream of FAK responsible for the transcription of uPA in this model: reduction of uPA can be induced by inhibitors of Src-family PTKs, JNK and MEK1-ERK, whereas uPA expression in the FAK shRNA cells can be rescued by constitutively active MEK1 or WT JNK1. Microarray data of our tetFRNK-MTLn3 cells that require FAK in the early stages of metastasis showed that uPA is not changed in response to FRNK expression. Another important requirement for the formation of tumors is angiogenesis. Recently, Mitra et al. showed that expression of FRNK in breast carcinoma cells results in dephosphorylation of Y925FAK, disruption of Grb2 binding and inhibition of ERK2 activity and VEGF expression. This results in the formation of tumors that are hardly vascularized. Addition of recombinant VEGF restored angiogenesis, so FAK promotes tumor angiogenesis, at least partly, through the regulation of VEGF expression in this model [83]. Again, no differences in VEGF-A, B, C and D could be detected using microarray analysis of our tetFRNK-MTLn3 cells. Thus, despite the piling

evidence of the importance of FAK in metastasis, there are multiple downstream signalling pathways that are active in a tumor cell type dependent manner.

4.5. *Cytostatics and FAK*

The above provides substantial evidence that FAK plays a prominent role in tumorigenesis, metastasis and in the control of tumor cell survival in general. Therefore, inhibition or modulation of FAK seems to be a potential way to treat cancer. Since FAK is a strong mediator of survival signalling, tumor cells with high levels of FAK could be more resistant against classic anti-cancer therapy. Likewise, a potential novel therapeutic approach could be modulation of FAK in combination with currently used chemotherapeutics. Etoposide-induced DNA strand breakage of tumor-derived endothelial cells is suppressed when cells are attached to fibronectin, indicating a protective role for integrin activation in cytostatic-induced cell death [84]. Since cancer cells have to survive anchorage independently in order to metastasize, integrin-mediated survival pathways are thought to be constitutively activated. This may decrease the effectiveness of cytostatic-induced apoptosis, possible via FAK signalling. Another study showed that paclitaxel-induced apoptosis in breast cancer cells is significantly inhibited by β1 integrin signalling [85]. Integrin-mediated cell attachment protects these breast cancer cells from cytochrome *c* release, depending on the PI-3 kinase survival pathway. Recently, we showed in MTLn3 cells that phosphorylation of FAK on tyrosine 397 decreases during the onset of doxorubicin-induced apoptosis, in a Bcl-2 and caspase-independent manner [86]. Doxorubicin also causes an early activation of protein kinase B (PKB). Expression of FRNK sensitizes MTLn3 cells to apoptosis caused by doxorubicin and inhibits the doxorubicin-induced activation of PKB. Inhibition of PI-3 kinase with wortmannin as well as transient overexpression of PTEN enhances doxorubicin-induced cell death, indicating that FAK and PKB cooperate to suppress doxorubicin-induced cell death. Also complete knock-down of FAK by siRNA techniques results in an increased sensitivity of ovarian cancer cell lines to docetaxel [87]. In response to docetaxel, caspases 8 and 3 are activated and FAK is cleaved. Cell lines resistant to docetaxel do not show FAK cleavage, whereas reduced expression of FAK does sensitize these cells to docetaxel. This indicates that indeed FAK protects against cytostatic-induced apoptosis, although the precise mechanism is still unclear. A possible mechanism could be the FAK-induced downregulation of caspase-8. This is shown in FAK overexpressing HL-60 cells, in which FAK-induced downregulation of procaspase-8 inhibits downstream apoptosis pathways [88].

The first *in vivo* evidence for FAK-induced resistance to cytostatics is provided by Duxbury et al. Inhibition of FAK using siRNA in nude mice enhances gemcitabine-induced cytotoxicity of pancreatic adenocarcinoma *in vivo*, whereas *in vitro* analysis again provides a link to the PI-3 kinase pathway [89]. Whether this effect is directly related to downregulation of FAK in the tumor cells is unclear, since knock down of FAK in for example the endothelial cells can ultimately also be the cause of the enhanced cytotoxicity. Using our syngeneic breast cancer tumor/metastasis model, that allows conditional expression of HA-tagged FRNK in primary tumors and experimental metastases, we find a strong FRNK-induced sensitization of these tumors/metastases towards doxorubicin treatment (manuscript in preparation). In these studies, treatment with doxorubicin alone did not affect tumor growth. Sensitizing tumors towards cytostatics by inhibition of FAK would be a great advance in the treatment of cancer, since in the clinic a large number of tumors are highly resistant towards currently used cytostatics.

5. Future studies

In conclusion, a large number of studies point into the same direction: FAK is an important player in both tumor development and tumor metastasis. The mechanisms by which FAK mediates the different processes involved in tumorigenesis and metastasis are likely to be numerous. In the development of tumors, overexpression of FAK seems to be important for the transduction of signals initiated at sites of cell attachment and by receptor tyrosine kinases, resulting predominantly in survival and proliferation. Therefore, the kinase domain of FAK seems to be important in tumorigenesis. The formation of metastases consists of several steps in which FAK is important (i.e. migration, invasion, survival and proliferation). The FAK-mediated signalling depends on the interaction of the cell with the environment; this may be highly dependent on the type and the localization of the tumor. Therefore, it is hard to identify a universal signalling pathway for FAK that applies to any tumor cell type. Likewise, since activation of FAK mediates a large number of downstream signalling cascades, the pathways and partners involved in the FAK-dependent metastasis are likely to be tumor cell type dependent.

The mounting evidence that FAK is a central protein in processes that are involved in tumorigenesis and metastasis, pinpoints FAK as a potential target in the development of new anti-cancer drugs. Because of its major role as kinase upstream of a number of signalling pathways involved in all the processes of tumor/metastasis formation, manipulation of FAK is likely to be more effective than manipulation of single downstream targets. Nevertheless, given the ubiquitous expression of FAK throughout all tissues, a possible problem could be the toxicity induced by the systemic inhibition of FAK. Of course one of the primary functions of FAK is to provide survival signalling: therefore systemic inhibition of FAK could cause severe side-effects. However, in contrast to tumor cells that overexpress FAK, inhibition of FAK in several normal cells turns out to cause neither cell detachment nor cell death [40]. This can be explained by the fact that survival signalling pathways in tumor cells are disturbed, and therefore these cells probably rely to a greater extent on FAK-mediated survival signalling. Also, with regard to the expression pattern of FAK during embryogenesis and aging, the highest levels of FAK are present during the initial stages of embryogenesis, whereas during aging the expression is further decreased [90]. This indicates that in normal cells FAK is especially important during embryonic development, thus (temporal) pharmacological inhibition of FAK in adults might not cause too severe side effects. This is confirmed in a study by Duxbury et al.: systemic administration of FAK siRNA

in mice did not result in severe side effects [89]. By using conditional tissue specific Cre-mediated knock-out of FAK in mice, the possible effects of inhibition of FAK on normal tissue functioning should be explored. Until now, these systems are mostly used to identify the role of FAK in the development of the specific tissues. For example, while targeted deletion of FAK in neurons interferes with normal development of the forebrain, it does not result in apoptosis of these neurons [91]. For the targeting of FAK as anti-cancer drug, it is probably not necessary to completely eradicate FAK, as deletion of one *fak* allele already diminishes tumorigenesis. Several studies make use of *fak* heterozygous mice; these mice have no phenotype, even though lower expression levels of FAK were detected in all tissues examined [92]. Another promising strategy could be modulation of FAK in combination with cytotoxic agents. Inhibition of the survival signals generated by FAK could sensitize the tumor cells towards the cytotoxic agents and thereby improve currently used anti-cancer therapies. The potential toxicity of the inhibition of FAK is a very important issue and more extensive research is necessary to explore these safety issues.

For the development of drugs that interfere with FAK function, two major possibilities exist. The first one would be inhibition of its kinase domain, thereby preventing the activation of downstream signalling cascades. One problem using this approach is already highlighted in a review by Siesser and Hanks [93]: FAK kinase activity itself may not be absolutely essential for its signalling functions. Another problem in this case would be the specificity of the kinase inhibitor, since kinase domains of a range of different proteins show a high degree of amino acid conservation in the catalytic domains. However, some selective inhibitors of tyrosine kinases have already been developed, for example inhibitors of the EGFR (Iressa), HER2 (Trastuzamab, Lapatinib) and c-Kit (Imatinib) (reviewed by Baselga [94]).

The second possibility for the manipulation of FAK is blocking the adapter function of FAK, by for example preventing binding of proteins to one or multiple tyrosines, to the proline rich domains or by preventing localization of FAK to the focal adhesions. An advantage of this approach is that prevention of the association of FAK to certain binding partners will lead to blockade of specific downstream signalling pathways. However, the question remains whether it is possible to achieve this specificity *in vivo*, since signalling cascades consisting of other kinases could take over the function of FAK, still resulting in the activation of the intended signalling pathways. The first peptide that prevents the interaction of FAK to a binding partner was recently developed by Garces et al.: using a phage library, a peptide was identified that prevents the interaction of FAK with VEGFR-3. This peptide caused cell detachment and apoptosis in breast cancer cell lines, but not in normal breast cells [95]. In addition to the problems with regard to specificity, the main problem is: which protein-interactions should be targeted? So far the development of small peptides preventing the localization of FAK at the focal adhesion seems to be a promising approach for development of a clinical FAK-inhibitor, since in the described experimental models the best effects on tumor and metastasis suppression are achieved by expression of the splice variant of FAK, FRNK. FRNK interferes with the localization of FAK at the focal adhesions, thereby preventing its active role in focal adhesion signalling and focal adhesion turnover. Although difficult, development of FAK adapter function blocking compounds will enhance the efficacy of the current chemotherapeutic treatment of a variety of cancers. More importantly, it will offer possible alternative therapeutic approaches for the currently difficult to treat distant metastases.

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