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Focal Adhesion Kinase: a Prominent Determinant in Breast Cancer Initiation, Progression and Metastasis

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Abstract

Focal adhesion kinase (FAK) is an intracellular non-receptor tyrosine kinase. In addition to its role as a major mediator of signal transduction by integrins, FAK also participates in signaling by a wide range of extracellular stimuli including growth factors, G-protein coupled receptor agonists, cytokines, and other inflammatory mediators. The link between FAK and breast cancers is strongly suggested by a number of reports showing that FAK gene is amplified and overexpressed in a large fraction of breast cancer specimens. In addition, increased FAK expression and activity frequently correlate with metastatic disease and poor prognosis. Since its discovery in early 1990s, numerous studies have shown a role for FAK in the regulation of cell spreading, adhesion, migration, survival, proliferation, differentiation, and angiogenesis. Many of these studies in cultured cells provided strong evidence to connect FAK expression/activation to the promotion of cancer. Recently, a prominent role of FAK in promoting mammary tumorigenesis, progression and metastasis has been unveiled by different animal models of human breast cancer, including xenograft models in immunodeficient rodents and spontaneous tumor models in transgenic mice that have specific deletion of FAK in the mammary epithelial cells during embryonic or postnatal development. These *in vivo* studies established FAK as a prominent determinant in mammary cancer initiation, progression and metastasis. Furthermore, a novel function of FAK in maintaining mammary cancer stem/progenitor cells *in vivo* has been recently reported, which may provide a novel cellular mechanism of FAK in promoting breast cancer initiation and progression. The wealth of knowledge accumulated over almost two decades of research on FAK should help to design potentially novel therapies for breast cancer.

1. Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States and worldwide. In America, approximately one in every ten women will develop the disease in their lifetime, and it is the second leading cause of cancer-related death in women (from the National Cancer Institute, at <http://www.cancer.gov>). Breast cancer treatment is particularly

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None Declared

difficult when metastasis, the spread of breast cancer to other locations in the body, occurs. Although the past several decades have seen a significant progress in the understanding of the molecular and cellular mechanisms of breast cancer and the development of new diagnostic, prognostic and therapeutic strategies, the survival rate for breast cancer patients with metastatic disease has not changed significantly [1]. The fundamental problem of conventional cancer therapies highlights the urgent necessity of finding novel treatment strategies to target metastatic cancer cells to eradicate various cancers including breast cancer.

The process of metastasis and invasion of tumor cells requires these cells to alter their ability to adhere to both surrounding cells and the extracellular matrix (ECM). Cellular interactions with ECM through integrins play crucial roles in many aspects of tumor initiation and progression [2,3]. Focal adhesion kinase (FAK), an intracellular tyrosine kinase recruited to the sites of integrin clustering or focal adhesions, functions as a major mediator of signal transduction by cell surface receptors including integrins, growth factor and cytokine receptors [4]. FAK has been shown to play key roles in the regulation of cell spreading[5–7], adhesion [8–12], migration[13–17], invasion[18–21], survival[22–29], proliferation[30–33], differentiation[34], and angiogenesis[29,35,36]: processes that are all involved in the development of cancer. These functional characteristics suggest that FAK may play key roles in promoting tumorigenesis and metastasis, and it may serve as a critical target in the eradication of various cancers including those of the mammary gland. The purpose of this review is to provide current knowledge of FAK in tumor initiation, progression, and metastasis in the context of breast cancer, and offer perspectives for this protein in the molecular and cellular mechanisms of breast cancer.

2. Mechanisms of FAK activation and action

FAK is a 125-kDa non-receptor protein tyrosine kinase identified in early 1990s as a protein associated with focal adhesions and phosphorylated in an integrin-dependent manner and in response to v-Src-mediated transformation [37–39]. As outlined in Fig.1, FAK is structurally distinct from other nonreceptor tyrosine kinases in its lack of Src homology 2 (SH2) and SH3 domains. FAK protein is highly conserved across different species, and is composed of an N-terminal FERM (band four point one, ezrin, radixin, and moesin) domain, a central kinase domain, and a C-terminal domain that includes two proline-rich motifs and a focal adhesion targeting (FAT) sequence responsible for its localization to focal adhesions. Both the N-terminal and C-terminal domains of FAK have been shown to mediate its interaction with a variety of other proteins, which are critical for the activation of FAK by integrins or other cell surface receptors and for its regulation of different cellular functions.

FAK is phosphorylated and activated not only by integrin-ECM engagement, but also by diverse cellular responses to a wide variety of extracellular stimuli, including growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) [40], and hepatocyte growth factor (HGF)[41], cytokines [42], G protein-coupled receptor agonists [43], as well as phospholipids and lipid mediators [44,45]. Upon activation, FAK is first autophosphorylated at Y397 to create a binding site for many SH2 domain containing molecules including the upstream Src kinase itself [46,47], PI3K[48,49], Grb7 [50,51], Shc [52], and PLC γ [53,54]. The association of Src tyrosine kinase with FAK leads to conformational change and activation of Src, which subsequently phosphorylates other sites of FAK, including Y407, Y576, Y577 in the kinase domain to maximize FAK kinase activity [55], Y861 in the C-terminal domain to promote association of FAK with the α v β 5 integrins in response to vascular endothelial growth factor (VEGF) stimulation [56], and Y925 to promote binding of growth-factor-receptor bound protein 2 (Grb2) to mediate activation of Ras-MAPK signaling [57].

In addition to its function as a tyrosine kinase, FAK also serves as a scaffolding protein. A major scaffolding function of FAK upon its autophosphorylation at Y397 and association with Src at this site is to allow efficient Src phosphorylation of several signaling molecules bound to other sites of FAK. The C-terminal region of FAK contains a number of protein-protein interacting sites, including two proline-rich regions, which serve as binding sites for a variety of SH3 domain-containing proteins including the adaptor protein p130Cas [58], the RhoA specific GTPase activating protein Grb (GTPase regulator associated with FAK) [59], ASAP1 (ARF-GAP containing SH3, ANK repeats, and PH domain) [60], and endophilin A2 [61]. The diverse interactions of FAK with different signaling molecules trigger diverse downstream signaling pathways to regulate cell spreading, adhesion, migration, invasion, survival, and proliferation.

The role of FAK as a central node in mediating diverse signal transduction events suggests that aberrant FAK signaling may contribute to the process of cell transformation and cancer development. Indeed, numerous studies have shown that, in addition to v-Src-mediated transformation, many other forms of oncogenic transformation induce or require FAK activity and its phosphorylation in different tyrosine residues. For example, constitutive tyrosine phosphorylation and kinase activation of FAK was induced by endogenous expression of BCR-ABL oncoprotein in Philadelphia chromosome positive leukemia cell lines and by exogenous transfection of BCR-ABL in murine interleukin-3 (IL-3)-dependent NFS/N1.H7 cells [62]. V-Crk, an oncogenic product of avian sarcoma virus CT10, has been reported to constitutively activate PI3K/AKT pathway to efficiently transform chicken embryo fibroblasts by inducing the phosphorylation of FAK at Y397 and subsequent activation of the Src family tyrosine kinase, while v-Crk was unable to activate PI3K/AKT pathway in FAK-null cells [63]. FAK is essential for oncogenic transformation and cell invasion induced by ErbB-2/ErbB-3 receptor signaling, which promotes FAK activation and phosphorylation at Y397, Y861 and Y925 [64]. In both constitutively H-Ras-transformed and H-Ras-inducible NIH3T3 fibroblasts, FAK phosphorylation at Y861 was increased and this phosphorylation was crucial for Ras-mediated fibroblast transformation [65]. The activation and phosphorylation of FAK stimulated by integrins as well as many forms of oncogenic transformation provide a plausible mechanism for anchorage-independent growth of cancer cells.

3. The link of FAK to breast cancer in humans

A large body of studies has correlated FAK expression with a wide variety of cancer types in humans [66,67]. The potential link of FAK to breast cancer was first established by Northern and Western blot analyses of tissue homogenates showing that the levels of FAK mRNA and protein were significantly elevated in invasive and metastatic breast tumor specimens in comparison to paired normal tissues, suggesting a role of FAK in promoting breast cancer invasion and metastasis [68]. Subsequent analysis using immunohistochemistry showed that FAK expression was minimal in benign breast epithelium but was strongly positive in ductal carcinoma in situ (DCIS), suggesting that FAK overexpression is not restricted to the invasive phenotype, but rather appears to be an early event in breast tumorigenesis [69,70]. The elevated levels of FAK expression in a large fraction of breast cancers were further conformed by several different studies [71–74] and increased dosage and amplification of the *fak* gene in human breast cancer cells [75] and tumor tissues [74] were also documented. These studies further established that increased FAK expression and activity in breast cancer specimens are frequently associated with indicators of poor prognosis [71,73] and correlate with progression to metastasis [74].

Although elevated FAK expression/activity has been correlated with tumor invasiveness and metastasis for breast cancer and many other tissue malignancies, it should be noted that there are several exceptions. In one study of breast cancer samples, the activation of FAK as detected

by Y397 phosphorylation was found to correlate with malignant transformation, but not with development of invasive tumor properties [76]. Such exception was also reported in other tumor types. For instance, FAK expression was found to be reduced in liver metastases of colorectal adenocarcinoma as compared to the matched primary tumors [77]. Similarly, in cervical cancer, weak expression of FAK has been found to correlate with nodal metastasis, recurrent disease, and poor disease outcome [78]. These studies implicated that FAK may play an important role in malignant transformation of primary tumors, but not necessarily in metastasis. Such specific role of FAK in malignant transformation is also suggested by its strong expression in DCIS [69,70], the early stage of breast cancer.

The molecular mechanisms responsible for the increased FAK expression in breast cancer remain largely unknown. In addition to breast cancers, the fak gene amplification or copy number increase has been observed in other cancer types [79]. However the degree of malignancy is not always correlated with increased FAK expression in all cancers, suggesting the presence of tumor type-dependent mechanisms. Recent analysis of human fak gene promoter has identified p53 and NF κ B as potentially direct repressor and activator, respectively of the promoter [80,81], suggesting an intriguing possibility that aberrant expression or mutation of p53 and/or NF κ B could play a role in increased FAK expression in breast cancer. Indeed, a recent study has shown that, in breast cancer tumors, mutations in p53 resulted in an increase in FAK mRNA and protein expression [82].

4. Mechanisms of FAK promotion of mammary tumorigenesis

A potential role of FAK in breast tumorigenesis was suggested by its overexpression in noninvasive DCIS. As described earlier, FAK is activated following integrin-ECM engagement in normal cells and many forms of oncogenic transformation enhance FAK phosphorylation and activation. The activation of FAK in transformed cells in the absence of adhesion signal was proposed to initiate anchorage-independent growth of cancer cells: the hallmark of cancer. In general, abnormal FAK expression and/or activation contribute to breast tumorigenesis through its regulation of cell survival and/or proliferation.

4.1. FAK in cancer cell survival

The link between FAK and cell survival was first revealed by Frisch *et al* showing that an activated, membrane-targeted FAK construct called CD2FAK in MDCK cells prevents anoikis, a form of apoptosis induced by cell detachment from the ECM [25]. Increased FAK expression in HL-60 leukemia cells has also been shown to suppress apoptosis, which is associated with the phosphorylation of FAK at Y397 and Y925, as well as its kinase activity [83]. Conversely, FAK inhibition, via antibody injection [26,84], antisense oligonucleotides [85], siRNA[28], or expression of FAK dominant negative mutants [23,26,27,86], all lead to the induction of apoptosis in various cancer cells.

In BT474 and MCF-7 breast cancer cells, the expression of FAK carboxy-terminal domain (FAK-CD), which serves as a FAK dominant negative mutant, led to endogenous FAK dephosphorylation and degradation, loss of cell adhesion, as well as induction of Fas-associated death domain protein (FADD) and caspase-8 driven apoptosis [27]. Moreover, breast tumor cells that were viable without matrix attachment also underwent apoptosis following interruption of FAK function [27], suggesting that FAK is a survival factor in breast cancer cells in the absence of matrix signaling. Interestingly, FAK-CD had no effect on the viability of normal human mammary epithelial cells [27], raising the intriguing possibility that inhibiting FAK expression or activity may serve as a therapeutic strategy that kill only malignant but not normal cells. The identification of FADD and caspase-8 in both anchorage-dependent and anchorage-independent apoptotic signaling of breast cancer cells upon interruption of FAK function suggests that FAK is involved in the inhibition of apoptosis by inhibiting components

of death-receptor complex. Indeed, a subsequent study by the same group unveiled that FAK suppresses apoptosis in breast cancer cells by directly sequestering and inhibiting the death domain of receptor-interacting protein RIP [87].

As mentioned earlier, FAK functions as a scaffolding protein to mediate signal transduction from a plethora of signaling proteins. It was reported that FAK interacts with several receptor tyrosine kinases including EGF receptor HER-1 [40,88], HER-2 [89], HGF receptor c-Met [89], PDGF receptor [40], and vascular endothelial growth factor receptor 3 (VEGFR-3) [90]. In breast cancer cells, FAK inhibition has been shown to have synergistic effect with inhibition of EGF receptor signaling as dual inhibition of FAK and HER-1 signaling cooperatively enhances apoptosis [88]. In addition to HER-1, FAK also physically interacts with VEGFR-3 through a small region in the FAT domain, and such interaction suppresses apoptosis [90].

Several studies have revealed that both the catalytic activity and the autophosphorylation at Y397 of FAK are required for protecting cells from apoptosis [25,27,83,91]. Activation of FAK results in the autophosphorylation of FAK at Y397 that creates a binding site for many SH2 domain containing proteins such as Src and PI3K. The FAK-Src complex is one of the important complexes to convey survival signals in breast cancer cells. For example, a recent study has shown that transforming growth factor β (TGF- β) induces clustering of HER2 and $\alpha 6$, $\beta 1$ and $\beta 4$ integrins in HER2-overexpressing mammary epithelial cells by activating FAK and Src, and that inhibition of FAK-Src complex reverses TGF- β -induced resistance to the therapeutic HER2 inhibitor trastuzumab in HER2-overexpressing breast cancer cells [92]. This study suggests that TGF- β integrates HER2/ErbB2 receptor and integrin signaling by activating FAK-Src complex to promote breast cancer cell survival.

In addition to FAK-Src complex, FAK can bind p85 subunit of PI3K and triggers PI3K mediated cell survival pathway [93]. Phospholipid production stimulated by FAK association and activation of PI3K has been shown to activate downstream Akt kinase, which inhibits apoptosis by regulating various cell death machinery proteins [94,95]. FAK mediated PI3K/Akt signaling has been reported to protect cells from apoptosis induced by UV irradiation [96]. In human leukemic HL-60 cells, FAK activation of PI3K/Akt pathway induced the expression of inhibitor of apoptosis proteins (IAPs) through NF κ B and protected the cells from oxidative-stress induced apoptosis [83].

Several recent studies also suggested that FAK can promote cell survival via its interaction and inhibition of p53 function [97,98]. This interaction is mediated by the FERM domain of FAK in a kinase-independent manner and suppresses transcriptional activation of a number of p53 target genes including p21, Mdm2 and Bax [98]. In addition, FAK/p53 complex is also detected in the nucleus where FAK promotes ubiquitin-proteasome mediated degradation of p53 by Mdm2 through interactions with both proteins [98]. Therefore, FAK may promote cell survival by multiple pathways through both kinase-dependent and kinase independent mechanisms.

4.2. FAK in cell proliferation and tumor growth

In addition to a positive role for cell survival, FAK signaling pathways can promote tumor development through stimulation of cell cycle progression. Our laboratory has shown that FAK serves as a mediator of cell cycle regulation by integrins through FAK/Src complex formation in the focal contacts, which promotes ERK activation, cyclin D1 upregulation, and decreased p21 expression [31]. Signaling through FAK leads to transcriptional activation of cyclin D1 promoter [33], and transcription factors including EtsB [33] and KLF8 [99] have been identified as downstream proteins of FAK-mediated cyclin D1 expression and cell cycle progression. Studies from others have also showed that integrin-mediated activation of FAK

and downstream signaling to Jun NH2-terminal kinase is required for cell cycle progression from the G1 to S phase [32].

Several studies have indicated a direct role of FAK in promoting tumor growth. Inhibition of FAK signaling by FRNK has been shown to suppress the proliferation of Hep3 human carcinoma cells and leads to tumor dormancy *in vivo*, and this dormancy can be reversed by the expression of an active mutant of MEK1 (MAPK/ERK kinase 1) [100], suggesting FAK signaling through ERK-MAPK pathway is required to maintain tumor cell growth. Recently, using polyoma middle T oncoprotein (PyMT) induced breast cancer model established in FAK floxed and mammary conditional knockout (KO) mice, several groups, including us, have further revealed the role of FAK in promoting mammary tumor growth *in vivo* [74,101,102]. In these studies, specific ablation of FAK in mammary epithelial cells (MaECs) significantly increased mammary tumor latency and suppressed tumor multiplicity, growth, and progression. Subsequent mechanistic studies indicated that FAK deletion in mammary tumor cells reduced the expression/phosphorylation of p130cas, Src, ERK1/2 and cyclin D1, which may contribute to the tumor dormancy *in vivo* and growth arrest in culture [74,101,102].

5. Mechanisms of FAK to promote breast cancer progression and metastasis

Tumor progression and metastasis are complex disease processes requiring multiple changes in tumor cells as well as their microenvironments. The formation of cancer metastasis is composed of several distinct steps, including cancer cell detachment, migration, invasion, extravasation, and proliferation in distal sites of the body. The role of FAK signaling in promoting cell survival and proliferation contributes to metastasis by enabling tumor cells to survive in different environments and to colonize in distal organs. Ample evidence has also indicated that FAK signaling pathways can stimulate tumor progression and metastasis through their regulation of cell migration, invasion, and angiogenesis.

5.1. FAK and cell migration

Cell migration requires coordinated and dynamic regulation of the integrin-mediated focal adhesions and the cytoskeleton networks they connect with. FAK serves as a key protein in the regulation of focal adhesion dynamics as cells devoid of FAK exhibit impaired migration and have large peripheral focal adhesion structures [16]. Further studies monitoring focal adhesion dynamics in cells deficient of FAK and Src implicated FAK and Src as critical mediators of integrin adhesion turnover that promote cell migration [103].

Upon activation by integrin-mediated cell adhesion, FAK undergoes autophosphorylation at Y397, which serves as a binding site for Src and PI3K. Both Src and PI3K have been shown to regulate focal adhesion dynamics and cell migration mediated by FAK in normal and cancer cells [93,103–106]. However, it has been shown that the expression of FAK, but not its kinase activity, is required for EGF- and PDGF-stimulated cell motility [40], suggesting an important role of FAK protein scaffolding function in mediating its regulation in cell migration. This study also showed that kinase-inactive FAK was transphosphorylated at the indispensable Src-kinase-binding site, Y397, after EGF stimulation [40], suggesting that the phosphorylation of FAK at Y397 is critical for its scaffolding function. The scaffold role of FAK in regulation of cell motility is further supported by the findings that FAK associates with other adaptor proteins such as p130^{Cas} to promote cell migration via a downstream signaling route including Crk, Dock180, and Rac [107,108]. In addition to Rac, FAK also mediates the activation of ERK 2 and ERK5 to promote cell migration. For example, the adaptor function of FAK promotes the assembly of a functional complex composed of FAK, Src, calpain-2 and ERK2, which is important for focal adhesion turnover and cell motility [22,109]. In metastatic MDA-MB-231 breast cancer cells, integrin-mediated ERK5 activation via FAK signaling plays a pivotal role in cell adhesion and haptotactic motility on vitronectin [110].

The role of FAK in promoting cell migration is beyond its association with two major signaling molecules Src and PI3K. In this regard, FAK has been shown to directly interact and phosphorylate an adaptor protein Grb7 to promote cell migration [50,51,111]. FAK has also been shown to promote cell migration through direct modulation of key proteins involving in the remodeling of the actin cytoskeleton, including the Rho subfamily of small GTPases [20, 112,113], N-WASP [114], and the Arp2/3 complex [115]. The detailed molecular mechanisms involving FAK in the regulation of cell migration have been recently reviewed by several different groups [67,106,116,117].

5.2. FAK and cell invasion

FAK has been shown to promote invasion of AU-565 breast cancer cells through an endothelial monolayer, as inhibition of FAK expression using siRNA or disruption of FAK localization using FRNK causes delayed transendothelial migration [118], while the phosphorylation of FAK at Y397 promotes extravasation of AU-565 cells [119]. In circulating cytokeratin-positive micrometastatic cancer cells isolated from patients with breast cancer, phosphorylation of FAK, PI-3K, and impaired actin organization have been documented [120], providing strong evidence that micrometastatic cancer cells have activated FAK signaling which may contribute to their malignant and metastatic nature.

A number of studies have shown that activation of FAK is associated with alterations in endothelial barrier property as many inflammatory mediators, cytokines, and growth factors are able to activate FAK through tyrosine phosphorylation to promote endothelial hyperpermeability [121–125]. Elevated FAK expression and/or activity in cancer cells may promote cancer cells to migrate efficiently through tissue barriers and acquire autonomous motile and invasive properties. The requirement of FAK signaling in promoting invasiveness of cancer cells has been demonstrated in several studies. In one study, it was shown that v-Src mediated transformation of FAK-null fibroblasts could promote the migration of these cells, but FAK expression and activity were essential for the generation of an invasive phenotype [20]. Similarly, inhibition of FAK activity by FRNK reduced v-Src stimulated cell invasion and blocked experimental metastasis in nude mice without significant change in migration of these cells [19]. FAK was required for ErbB-2/3 signaling-mediated cell transformation and invasion [64], and inhibition of Src-FAK signaling in ErbB2-positive breast cancer cells by Herceptin selectively modulated focal adhesion turnover, leading to enhanced number and size of peripherally localized adhesions and inhibition of cell invasion [126].

The ability of tumor cells to invade surrounding tissue structures and metastasize to distal sites also requires capacity for matrix degradation. Tumor cells are known to form cellular structures called invadopodia, which provides sites for the degradation of the surrounding ECM and facilitate tumor invasion into the neighboring stroma [127,128]. FAK-deficient breast cancer cells were recently found to sprout extra invadopodia and also large focal adhesions that were particularly sticky [129]. Interestingly, in the absence of FAK, tyrosine phosphorylated proteins including Src headed to the invadopodia but not focal adhesions [129], suggesting that FAK is required for the coordinated regulation of invadopodia and focal adhesion dynamics to promote breast cancer cell invasion. In addition to a role in regulation of invadopodia and focal adhesion composition and dynamics, FAK has been reported to promote tumor cell invasive capacity through its regulation on matrix metalloproteinases (MMPs). In v-Src transformed cells, FAK was found to promote the formation of the v-Src-Cas-Crk-Dock180 complex which activates Rac1 and JNK, leading to increased expression of MMP2 and MMP9 [20]. Inhibition of FAK has also been shown to reduce secretion of MMP9 in carcinoma cells [130]. Recently, our laboratory has shown that FAK interacts with endophilin A2 and mediate its phosphorylation by FAK/Src complex [61]. Tyrosine phosphorylation of endophilin A2 reduces its interaction with dynamin and decreases endocytosis of MT1-MMP, leading to

increased accumulation of MT1-MMP on tumor cell surface [61]. This study provides a novel mechanism of FAK in promotion of increased invasiveness of cancer cells.

Lastly, FAK has been reported to mediate cancer cell invasion through its ability to promote epithelial-to-mesenchymal transition (EMT). FAK is able to cooperate with Src to disrupt E-cadherin-based intercellular adherin junctions and promote E-cadherin internalization during cancer progression [131,132], thus facilitating EMT and enhancing tumor cell motility and invasiveness. Expression of FAK mutants resistant to Src phosphorylation significantly decreased Src-mediated disruption of E-cadherin-based cell contacts in colon cancer cells [133]. It has been shown that TGF- β -induced EMT requires Src or integrin-dependent FAK activation which leads to E-cadherin downregulation in mouse epithelial cells or hepatocytes [134–138]. Examination of patient specimens demonstrated that increased expression of FAK was correlated with the loss of E-cadherin in nodal metastases of laryngeal tumors [139].

5.3. FAK and tumor angiogenesis

The ability of FAK to promote cell migration, proliferation and invasion suggests a potential role of FAK in endothelial cell sprouting and tumor angiogenesis, which is an integral part of cancer progression and metastasis. In malignant astrocytic tumors, FAK was found to express in the angiogenic blood vessels of high grade tumor specimens and promote capillary tube formation of brain microvascular endothelial cells [140]. Further support for a role of FAK in tumor angiogenesis comes from studies of integrins, the major upstream activators of FAK in the process. It has been shown that blockade of integrins $\alpha v \beta 3$ with monoclonal antibodies or small molecule inhibitors inhibited tumor angiogenesis in a variety of animal models [141–143]. A recent report further showed that the formation of a signaling complex containing FAK and integrin $\alpha v \beta 5$ in a Src-dependent manner is essential for VEGF stimulated angiogenesis in a mouse model [56].

A direct demonstration of FAK in tumor angiogenesis in mouse models is still lacking due to the embryonic lethality of the endothelial cell (EC) conditional knockout (KO) mice. However, in a syngeneic mouse tumor xenograft model, inhibition of FAK activity in murine 4T1 breast carcinoma cells via stable expression of dominant-negative FAK, FRNK, did not affect cell proliferation or anchorage-independent cell survival *in vitro*. However, FRNK-expressing 4T1 cells secreted less VEGF and formed small and less vascularized tumors in comparison to controls, suggesting a role of FAK activity in breast cancer by promoting tumor angiogenesis [144].

The role of FAK and potential mechanisms of FAK in promoting tumor angiogenesis are also explored in our laboratory. Recently, we developed a tumor angiogenesis assay by injecting Matrigel plugs that containing tumor cells and adenovirus expressing Cre (Ad-Cre) into FAK floxed mice. Through this model system, we showed that deletion of FAK by Ad-Cre in ECs migrating into the Matrigel plugs reduced tumor angiogenesis and tumor growth *in vivo*, which could be restored by re-expression of wild-type FAK but not S732A mutant. This study suggested a role of FAK in promoting EC proliferation and tumor angiogenesis in a S732 phosphorylation-dependent manner [145].

6. Role of FAK in breast cancer revealed by animal models

The functional features of FAK in promoting cell adhesion, migration, invasion, survival, proliferation, and angiogenesis have strongly implicated a causative role of FAK in the formation, progression and metastasis of various cancers including those of the breast. Such a causative role of FAK in mammary tumorigenesis and metastasis has been further unveiled by different animal models of human breast cancer, including xenograft models in immunodeficient rodents and spontaneous tumor models in genetically engineered mice.

6.1. Xenograft models

A causative role of FAK in promoting tumorigenesis was initially revealed by the study showing that membrane-bound constitutively activated FAK (CD2-FAK) led to malignant transformation of MDCK cells and promote subcutaneous tumor formation in nude mice [25]. In a rat xenograft breast cancer model transplanted with MTLn3 rat mammary adenocarcinoma cells harboring an inducible dominant-negative FAK (FRNK), continuous expression of FRNK decreased the primary tumor growth in the mammary fat pad by 60% without induction of apoptosis, indicating a role of FAK in promoting mammary tumor growth [146]. Interestingly, lung metastasis formation in this model was found to be prevented when FRNK was induced 1 day before tumor cell injection, whereas induction of FRNK expression 11 days after injection did not affect lung metastasis, suggesting that FAK is required for the early phase of metastasis formation [146]. In a syngeneic mouse tumor model, inhibition of FAK activity or expression in murine 4T1 breast carcinoma cells via dominant negative FRNK or anti-FAK short hairpin RNA (shRNA) suppressed spontaneous metastasis to the lung and peritoneal cavity, and resulted in markedly decreased lethality [147]. Moreover, transient re-expression of wild type but not kinase-dead FAK in FAK knockdown 4T1 cells promoted urokinase plasminogen activator (uPA) production and lung metastasis within 7 days, suggesting that intrinsic FAK activity can control orthotopic breast carcinoma metastasis through the regulation of uPA expression [147]. In addition to inhibit metastasis, suppressed FAK activity in 4T1 cells has also been shown to reduce FAK Y925 phosphorylation, Grb2 adaptor protein binding, and signaling to mitogen-activated protein kinase (MAPK) [144]. Interestingly, this compromised FAK-Grb2-MAPK linkage did not affect 4T1 cell proliferation or survival in culture, but reduced VEGF expression and resulted in small and avascular tumors in mice, suggesting that intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic response in mammary tumors [144].

The overexpression of ErbB tyrosine kinase receptors including ErbB-2/3 has been associated with breast cancer progression. FAK is required for ErbB-2/3 induced tumor progression and invasion for transformed WT, but not FAK-null, mouse embryonic fibroblasts (MEFs) in SCID mice, whereas the restoration of FAK in FAK-null MEFs rescued the deficiency in tumor progression and invasion [64]. Such dependence of FAK for tumor progression also applied to highly invasive MDA-231-M2 breast cancer cells, as stable expression of FAK siRNA drastically reduced lung metastatic capacity of these cells when implanted into the mammary fat pad [64]. This study clearly indicated a role of FAK signaling for ErbB-2/3 induced tumor malignancy and tumor progression.

In addition to an independent role in breast cancer initiation progression, FAK has recently been reported to function cooperatively with its homologous FAK related proline-rich tyrosine kinase 2 (Pyk2) to promote breast cancer cell tumorigenesis and invasiveness, and down-regulation of both Pyk2 and FAK resulted in a potent inhibition of breast cancer progression in a preclinical invasive breast cancer model in SCID mice [148].

6.2. Genetically engineered mouse models

Genetically engineered mouse models offer powerful tools to analyze the molecular and cellular mechanisms of breast cancer induction and progression. A number of genetically engineered mouse models of human breast cancer have been developed [149]. Of these, the PyMT transgenic model (PymT oncogene driven by MMTV-LTR promoter) has been well characterized, and shown many morphological, histological and molecular biomarker similarities to those of human breast cancers that are associated with poor prognosis, including the loss of estrogen and progesterone receptors and the persistent expression of HER-2/neu and Cyclin D1 [150]. Recently, several groups, including ours, have established PyMT mouse models of human breast cancer using different genetically engineered mouse lines harboring

FAK floxed (FAK^{f/f}) or mammary conditional KO (FAK^{f/f}, MMTV-Cre) alleles. These studies, as summarized in the following section, provided direct *in vivo* evidence for the role of FAK in mammary tumorigenesis, progression and metastasis, although some discrepancies regarding the role of FAK in tumorigenesis were revealed [74,101,102,151].

In one report, Lahlou et al have shown that mammary epithelial-specific FAK disruption using the well established Cre-LoxP recombination system blocks mammary tumor progression [151]. In this study, the efficiency of Cre-mediated FAK deletion in MaECs was estimated at 64.3%. Under this relatively low excision efficiency, mammary carcinomas developed in the FAK conditional KO mice all express FAK, while FAK-deleted MaECs, although produce PyMT-induced premalignant mammary hyperplasia, failed to progress to advanced carcinomas and subsequent metastases, suggesting a critical role of FAK in promoting mammary tumor progression. This study also showed that PyMT-induced mammary hyperplasia formation is independent of FAK, suggesting that FAK may not be necessary for the early step of mammary tumorigenesis. However, since hyperplastic lesions convert to neoplastic lesions at a very low frequency [150,152], it is not known whether these FAK-deficient mammary hyperplastic lesions could eventually develop into any clinically significant tumor without the selective pressure of the fast growing tumors where FAK deletion is escaped. Thus, under the animal model that Lahlou et al developed, the role of FAK in mammary tumorigenesis, especially the late stage of tumorigenesis, can not be fully evaluated.

Using the MMTV-Cre transgenic mouse line D that expresses Cre from postnatal day 22 and generates estimated deletion efficiency of 96.4%, Pylayeva et al reported that deletion of FAK in MaECs significantly suppressed PyMT-induced mammary tumorigenesis as the FAK targeted mice (MMTV-PyMT; MMTV-Cre; FAK^{f/f}) developed much less palpable tumors with significantly increased tumor latency compared to the control mice (MMTV-PyMT; FAK^{f/f}) [74]. Remarkably, this study also indicated that virtually all the primary and lung metastatic tumor lesions found in the FAK targeted mice expressed FAK, suggesting that they had originated from the minority of MaECs that had not undergone Cre-mediated deletion of FAK. Interestingly, by monitoring FAK expression/activation in different stages of mammary tumorigenesis, Pylayeva et al found that FAK expression/activation was much higher in mammary intraepithelial neoplasia (MIN) and advanced carcinoma lesions compared to the mammary hyperplastic lesions. Subsequent analysis of Cre expression in MIN (and carcinoma) lesions suggests that they were devoid of Cre activity, indicating that FAK was not deleted in the first morphologically identifiable neoplastic lesions in this model. These observations suggested that FAK was required for mammary tumorigenesis, which is in apparent contrast to the conclusions reached by Lahlou et al (see discussion above). To further elucidated potential mechanisms of FAK in promoting tumorigenesis and progression, the authors analyzed tumor cells derived from control mice after treatment with an adenovirus expressing Cre recombinase. These studies revealed that FAK-deficient PyMT-transformed cells displayed both growth arrest and apoptosis, as well as diminished invasive and metastatic capacity [74]. In addition, using adenovirus-mediated re-expression of WT FAK and a variety of FAK mutants in FAK-deficient PyMT-transformed cells, the role of FAK to promote and sustain tumorigenesis was found to be dependent of its C-terminal proline-rich motif that binds p130^{cas} [74].

Although the above study clearly indicated an important role of FAK signaling in PyMT-induced mammary tumorigenesis and progression, the fact that FAK is expressed in all malignant primary tumors and metastatic nodules derived in the FAK conditional KO mice can not exclude a possibility that tumors originated from FAK undeleted MaECs (termed escapees) simply outgrow those derived from FAK targeted cells, due to the role of FAK in promoting cell proliferation and survival. In such a case, a direct role of FAK in mammary tumorigenesis and metastasis are less clear. In addition, the expression of FAK in tumors of

FAK targeted mice compromises further study to use these cells to explore the molecular and cellular mechanisms of FAK in breast cancer. Recently, using a different line of FAK floxed mice interbred into a MMTV-Cre transgenic mouse line (Line F) that confers Cre-mediated recombination at early embryonic stage, we observed 100% of FAK deletion in the MaECs of our FAK^{f/f}; MMTV-Cre mice [34]. The high excision efficiency of Cre was confirmed in MMTV-Cre; *Rosa26* mice, as we showed that -galactosidase was expressed in almost 100% of MaECs including luminal and myoepithelial cells [102]. After crossing our FAK floxed and mammary conditional KO mice with a transgenic mouse line expressing MMTV-PyMT, we found that targeted deletion of FAK in the mouse mammary epithelium significantly suppressed PyMT-induced mammary tumor formation, growth and metastasis [102]. Interestingly, through Western blotting and immunohistochemical analyses, we found that tumors eventually developed in FAK targeted mice, although with decreased multiplicity and growth retardation, did not express FAK. Similar results and the absence of FAK in PyMT-induced mammary tumors of FAK conditional targeted mice was also reported by another group [101]. These studies suggest that FAK plays a prominent role in promoting PyMT-induced mammary tumorigenesis and tumor progression. However, the ultimate formation of mammary tumors in FAK-null condition indicates that FAK is not required for PyMT-induced tumorigenesis, although we can not rule out the possibility that certain unknown proteins (not PYK2, as PYK2 is not elevated in FAK-null primary tumors) may be up-regulated to compensate FAK function.

7. Role of FAK signaling in breast cancer stem/progenitor cells

Recent identification of a small fraction of highly tumorigenic cells with properties of stem cells from human breast cancers [153] and a wide variety of other tissue malignancies [154–162] has created a whole new conceptual paradigm of how cancers form, progress and relapse. As the cancer stem cells retain the hallmarks of normal organ stem cells, including being rare in the bulk of tumor and entering the cell cycle infrequently, they may constitute a cell population refractory to conventional cancer therapies that are designed to kill rapidly cycling cells [163]. Thus, efficiently targeting these refractory cancer driving cells may hold the key for the eradication of various cancers including those of the breast.

Several lines of evidence suggest that FAK may play a role in breast cancer through its regulation of mammary stem cells (MaSCs) and mammary cancer stem cells (MaCSCs). Firstly, MaSCs, progenitor cells and MaCSCs have all been shown to form mammospheres in serum-free suspension culture [164–167]. The property of these cells to form mammospheres suggests that they possess ability to evade anoikis and survive in an anchorage-independent manner. As the expression of constitutively activated FAK in MDCK cells rendered them resistance to anoikis [25], thus, activation of FAK in MaSCs and MaCSCs may be important for their self-renewal/survival under anchorage-independent conditions *in vitro* and possibly *in vivo*. Secondly, in mouse, MaSCs has been prospectively isolated from the mammary epithelial cells based on high levels of 6 and 1 integrin expression [168,169]. In addition, high level of 1 integrin has also been shown as markers of MaCSCs [170–172]. These studies suggest that FAK, the major mediator of integrin signaling, may play important roles in MaSCs and MaCSCs. Lastly, using a unique conditional knockout model that specifically ablates FAK in MaECs during early embryonic stage, we have recently shown that FAK ablation in our PyMT breast cancer model have a severe impact on mammary cancer stem/progenitor cells, as the ablation of FAK reduced the pool of cancer stem/progenitor cells in primary tumors developed in FAK targeted mice, decreased their self-renewal and migration *in vitro*, and compromised their tumorigenicity and maintenance *in vivo* [102]. These studies raised the possibility that the defects in mammary cancer stem/progenitor cells observed in our FAK targeted mice may contribute at least in part to the suppressed mammary tumorigenesis and progression in these mice. Given the widely recognized role of various cancer stem cells in cancer initiation,

progression and relapse, these studies provide a novel cellular mechanism of FAK in promoting breast cancer initiation and progression by maintaining the breast cancer stem/progenitor cell population.

8. Conclusions and future directions

The established role of FAK in mammary tumor formation, progression and metastasis and a newly identified role of FAK in maintaining mammary cancer stem/progenitor cell population have clearly suggested that FAK signaling plays a very prominent role in breast cancer initiation, progression, and probably relapse. The role of FAK in mammary cancer stem/progenitor cells further suggests that FAK may serve as a potential target to eliminate breast cancer stem cells, leading to a cure of breast cancer. Indeed, several small molecule inhibitors of FAK that inhibit FAK kinase activity and autophosphorylation have been developed by different pharmaceutical companies [67,173], and some of them have already moved to phase I clinical trials and potential efficacy responses in tumor regression and disease stabilization have been observed [174]. Future studies need to confirm if FAK signaling plays a role in human breast cancer stem cells, and to elucidate the molecular mechanisms and downstream effectors of FAK signaling in the maintenance of breast cancer stem cells. Although mammary cancer stem/progenitor cells isolated from primary tumors of FAK targeted mice have severely impaired tumorigenicity in relative to those isolated from primary tumors of control mice, the deletion of FAK did not completely eliminate their tumor regenerative potential [102]. Several developmental signaling pathways including Notch, Wnt, and hedgehog signaling have been demonstrated to play critical roles in the regulation of various normal and cancer stem cells. Abnormal functions and regulations of these signaling pathways are often associated with tumorigenesis and metastasis of various cancers. It would be interesting to determine the relative contributions and potential cross-talk of integrin-FAK signaling and other signaling pathways in breast cancer. These studies will suggest that if the use of a combination of inhibitors for FAK and other signaling pathways may serve as more effective treatment strategies than blockade of a single pathway in targeting and eradicating breast cancer stem cells.

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Fig. 1.

Structural features of FAK and its residues involved in interaction with other proteins. FAK is composed of a central kinase domain flanked by a N-terminal FERM domain and a C-terminal region containing two proline-rich (PR1 and PR2) motifs and a FAT domain. Several known phosphorylation sites as well as residues or regions of FAK mediating association with some of its interacting-proteins are indicated in the diagram.