Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression

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Elevated expression and activation of the focal adhesion kinase (FAK) occurs in a large proportion of human breast cancers. Although several studies have implicated FAK as an important signaling molecule in cell culture systems, evidence supporting a role for FAK in mammary tumor progression is lacking. To directly assess the role of FAK in this process, we have used the Cre/loxP recombination system to disrupt FAK function in the mammary epithelium of a transgenic model of breast cancer. Using this approach, we demonstrate that FAK expression is required for the transition of premalignant hyperplasias to carcinomas and their subsequent metastases. This dramatic block in tumor progression was further correlated with impaired mammary epithelial proliferation. These observations provide direct evidence that FAK plays a critical role in mammary tumor progression.

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Targeted Disruption of FAK in Mammary Epithelium Impairs Mammary Tumor Development in the MMTV-PyVmt Mouse Tumor Model. We next investigated whether mammary-specific disruption of FAK would impact mammary tumor development. To accomplish this, we interbred the conditional floxed fak allele with either one or two copies of the floxed fak allele (FAK^fl/fox/MMTV-Cre/PyVmt and FAK^fl/fox/fox/MMTV-Cre/PyVmt mice, respectively). Cohorts of female mice bearing these different genotypic combinations were subsequently monitored for mammary tumor development by physical palpation. Compared with MMTV-Cre/PyVmt mice bearing wild-type fak

significant reduction in FAK protein levels (SI Fig. 8B). Moreover, excision of one floxed fak allele is sufficient to cause a reduction in FAK protein expression. Residual FAK expression reflects the stromal contribution to FAK, given that the mammary epithelial constitutes 20% of virgin gland. To confirm that the loss of expression was restricted to the mammary epithelium, we performed immunohistofluorescence analyses on mammary gland paraffin sections using both Cre and FAK-specific antisera (SI Fig. 8C). In FAK^fl/fox/MMTV-Cre mice, mammary epithelial cells that were positive for Cre expression completely lacked detectable FAK expression. As expected, mice heterozygous for the floxed fak allele still retained some FAK protein staining. Careful quantification of the number of ductal cells positive for Cre and lacking FAK expression in FAK^fl/fox/MMTV-Cre mice revealed that 64.3 ± 15.6% of the luminal epithelial cells had undergone functional excision. Consistent with FAK immunostaining, introduction of the GTRosa26 Cre-responsive β-galactosidase allele (19, 20) in FAK^fl/fox/MMTV-Cre mice revealed that the majority of the mammary epithelium in mice homozygous for the conditional floxed fak allele had undergone functional excision as evidenced by robust X-Gal staining (SI Fig. 9). Taken together, these observations suggest that FAK expression is dispensable for the initial stages of normal mammary ductal outgrowth.

Fig. 1. Ablation of FAK expression increases the latency of mammary tumor formation and results in fewer hyperplastic lesions in MMTV-PyVmt mice. (A) Kinetics of mammary tumor onset in FAK^wt/wt, FAK^fl/fox, and FAK^fl/fox MMTV-Cre/PyVmt mice. The age indicated is that at which a mammary tumor is first palpable in each transgenic strain. T50 denotes the age at which a tumor is palpated in 50% of the mice. ∗, P < 0.001 vs. FAK^wt/wt mice, Student’s t test. (B) Representative mammary gland whole mounts from 10-week-old virgin FAK^wt/wt (Left), FAK^fl/fox (Center), and FAK^fl/fox (Right) MMTV-Cre/PyVmt mice. (Scale bar: 5 mm.) Average mass (±SEM) of inguinal mammary glands from six, eight, and seven mice, respectively, for each strain is included. ∗, P < 0.01 vs. FAK^wt/wt mice, Student’s t test. (C) Quantification of the area occupied by hyperplastic lesions expressed as a percentage (±SEM) of the total mammary gland surface. Quantitative image analysis of the mammary gland whole mounts was performed by using the Aperio ImageScope software. ∗, P < 0.001 vs. FAK^wt/wt mice, Student’s t test.

Fig. 2. FAK is not required for oncogenic transformation of the mammary epithelium in the MMTV-PyVmt mouse model. (A) Mammary gland whole mount from a 10-week-old FAK^fl/fox/MMTV-Cre/PyVmt/GTRosa26 mouse (representative of n = 4 animals) X-Gal-stained in situ for Cre-mediated β-galactosidase activity. Cre-expressing cells (blue) are present in untransformed ductal structures (arrows). (Scale bar: 5 mm.) (B) A higher magnification of mammary ducts showing hyperplastic nodules both expressing a functional Cre (arrowhead) or not (arrow). (Scale bar: 500 μm.) Representative sections of X-Gal-stained mammary glands from 10-week-old FAK^fl/fox/MMTV-Cre/PyVmt/GTRosa26 (n = 3) (C) and FAK^fl/fox/MMTV-Cre/PyVmt/GTRosa26 (n = 4) (D) mice. Solid nests of tumor cells (asterisks) and hyperplastic regions of the epithelium (arrowheads) are indicated. (Scale bar: 5 μm.) (E and F) Representative X-Gal-stained cryosections of tumors from 12-week-old FAK^fl/fox/MMTV-Cre/PyVmt/GTRosa26 mice (n = 5). Unstained tumoral structures are indicated by asterisks. Stained nontransformed ducts (arrows, E) and a group of stained preneoplastic structures (arrowhead, F) are also indicated. (Scale bars: 10 μm.)

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alleles, initial development of mammary tumors appeared to be marginally delayed in MMTV-Cre/PyVmT mice homozygous for the floxed fak allele by ~1 week (Fig. 1A). Mice heterozygous for the conditional fak allele also developed tumors with delayed onset, albeit at an intermediate point between control and homozygous floxed fak carriers (Fig. 1A). Consistent with these observations, whole-mount analyses of mammary glands derived from these cohorts at 6 and 10 weeks of age revealed that excision of both floxed fak alleles resulted in a dramatic reduction in the number of PyVmT-induced mammary hyperplastic lesions (SI Fig. 10A and Fig. 1B). Quantitative measurements revealed that, compared with the FAK+/+ MMTV-Cre/PyVmT control animals, there was a dramatic reduction both in the mammary gland average mass (SI Fig. 10A and Fig. 1B) and in the percentage of the total mammary gland area occupied by hyperplastic nodules in the FAKflox/flox/MMTV-Cre/PyVmT cohort. Consistent with these observations, examination of mammary-gland sections obtained from 12-week-old FAKflox/flox/MMTV-Cre/PyVmT/GT Rosa26 females (4- to 6-week tumor-bearing animals) showed that, whereas late-stage tumors were uniformly β-galactosidase-negative (Fig. 2E and F, asterisks), only untransformed ductal structures (Fig. 2E, arrows) or hyperplastic lesions (Fig. 2F, arrowhead) exhibited evidence of homozygous deletion of FAK. Thus, these observations suggest that emergence of the early hyperplastic lesions can occur in the absence of FAK. However, given the lack of β-galactosidase expression in the carcinoma regions, these results suggest that FAK

![Image](https://example.com/image.png)
expression is required for the conversion of these early lesions into a fully malignant phenotype.

**FAK-Deleted Cells Do Not Contribute to Either Mammary Carcinoma Formation or Lung Metastasis.** To confirm that FAK expression was confined to hyperplastic lesions, we performed immunohistochemistry (SI Fig. 11) and immunofluorescence analyses on sections of transformed mammary gland from mice wild-type, heterozygous, or homozygous for the floxed fak allele using both FAK- and Cre-specific antisera. Consistent with the X-Gal staining pattern of sequential sections for FAK expression (SI Fig. 11) revealed that samples, these solid tumoral areas retain both Cre and FAK expression although with a reduced level of FAK expression in the FAK<sup>lox/lox</sup> mice (Fig. 3A and SI Fig. 11 A and B). These observations indicate that mammary hyperplastic cells lacking functional copies of the fak allele could not contribute to carcinoma formation.

An important characteristic of PyVmT-induced mammary tumors is that they frequently metastasize to the lung (21). Moreover, FAK expression and/or activity have been associated with increased invasion and metastatic potential (6, 7, 22–24). To investigate whether mammary-specific ablation of FAK can affect the metastasis of PyVmT-induced mammary tumors, we first evaluated the number of metastatic lesions per lung in MMTV-Cre/-PyVmT mice homozygous for either the wild-type or floxed fak allele. The percentage of 8-week tumor-bearing mice that developed pulmonary metastases as well as the number of metastatic lesions per lung in MMTV-Cre/-PyVmT mice was the same (8.3 ± 2.8 and 7.5 ± 2.3, respectively). To assess whether these metastatic lesions could derive from primary hyperplastic lesions lacking FAK, we next examined both Cre and FAK expression patterns using X-Gal and immunohistochemical staining of lungs harvested from wild-type, heterozygous, or homozygous floxed fak PyVmT mice. β-Galactosidase assay on lung lobes from FAK<sup>wt/wt</sup>/MMTV-Cre/-PyVmT and SI Fig. 11 A). Graphical representation of the immunostaining shown in A(C) and B(D).

**Fig. 5.** FAK-negative mammary epithelial neoplastic cells show a reduced proliferative capacity but do not exhibit any increase in apoptosis. Paraffin sections of mammary gland from 6-week-old FAK<sup>wt/wt</sup>, FAK<sup>flox/wt</sup>, and FAK<sup>flox/flox</sup>MMTV-Cre-PyVmT mice were submitted to immunofluorescence analysis of Cre and Ki67 (A) or activated caspase-3 (B) expression. [Scale bars: 50 μm (A), 20 μm (B).]
PyVmt/GTRosa26 mice revealed that these lung metastatic lesions readily stained blue in the presence of X-Gal (Fig. 4 Left). Indeed, 62.5 ± 28.6% (n = 5) of the lung lesions from control animals expressed β-galactosidase. Consistent with this finding, immunohistochemical staining of lung sections with specific antibodies revealed both Cre and FAK expression among the metastatic lesions in the wild-type animals (SI Fig. 12 A and B Left). By contrast, none of the lung metastases from mice bearing both conditional floxed fak alleles (n = 8) exhibited either X-Gal staining or Cre antibody labeling. Consistently, the metastatic lesions lacking Cre expression retained robust FAK antibody labeling (Fig. 4 Right and SI Fig. 12 A and B Right). However, lung lesions from mice bearing a single conditional floxed fak allele displayed both Cre and FAK immunostaining (SI Fig. 12 A and B Center). Thus, just as the PyVmt-induced mammary carcinoma development depended on the presence of FAK, the formation of lung metastases in mice homozygous for the floxed fak allele also requires the retention of functional FAK alleles.

**Mammary-Specific Disruption of FAK Results in a Dramatic Reduction in the Proliferative Potential of PyVmt Hyperplastic Cells.** To investigate the observed defect in mammary tumor progression in the FAK-deficient strains, we measured the proliferative potential of hyperplastic mammary mammary cells by performing immunofluorescence staining of mammary gland sections using antibodies against both Ki67, a conventional proliferation marker, and Cre. To ensure that the mammary glands examined correspond to the early phases of oncogenic transformation, we used only nontumor-bearing mice in this analysis. We first assessed whether lack of FAK expression had an impact on the proliferative potential of the MMTV-PyVmt hyperplastic cells. The results showed that >50% of the Cre-positive hyperplastic cells arising in wild-type mammary glands exhibited robust Ki67 immunostaining (Fig. 5 A Top and C). However, the number of Cre and Ki67 double-positive cells was significantly lower in FAKflox/wt/MMTV-Cre/-PyVmT mammary glands (Fig. 5 A Middle and C). Moreover, staining of sections prepared from “FAK-deficient” glands exhibited a dramatic reduction (i.e., 16%) in the number of Cre-positive cells that also expressed Ki67 (Fig. 5 A Bottom and C).

Using an identical approach, we looked at the apoptotic status of these mammary glands by performing activated caspase-3 immunostaining analyses. Unlike the strong defect in Ki67 staining, the Cre-positive hyperplastic cells observed on FAKflox/wt-/- and FAKflox/flox/MMTV-Cre/-PyVmT mammary glands (Fig. 5 B and D). Taken together, these observations suggest that the dramatic defect in FAKflox/wt/MMTV-Cre/-PyVmT tumor progression is caused by a lack of FAK expression leading to a reduced proliferative capacity of PyVmt tumor cells.

**Discussion**

Activation of FAK has been associated with breast cancer progression. Indeed, a large proportion of primary human breast cancers possess elevated FAK expression that is further correlated with malignant transformation (11, 12, 24) and poor clinical outcome (22, 23). We demonstrate that targeted disruption of FAK in mammary tumor cells can block progression of hyperplasias to full carcinomas. We further show that this block in tumor progression is correlated with a dramatic reduction in the ability of the mammary tumor cells to proliferate. These observations provide compelling evidence that activation of FAK plays a critical role in mammary tumorigenesis.

Whereas targeted ablation of FAK negatively impacts on malignant progression, mammary epithelial-specific loss of FAK did not affect the initial stages of mammary gland outgrowth. This latter observation contrasts a recent study where a similar mammary gland FAK conditional knockout model was used to show that fak deletion delays mammary ductal elongation and induces lactation defects (25). This divergence is plausibly due to the different background strains (C57BL/6) and MMTV-Cre transgenic mice (line F) used. In our model, we have shown that the lack of an obvious mammary ductal outgrowth defect was due to absence of efficient and functional excision of the conditional floxed fak alleles, as evidenced by PCR analysis, expression of β-galactosidase, and, finally, immunohistochemical analyses showing the presence of Cre and the lack of FAK expression in the mammary epithelium. Consistent with these observations, mammary-specific ablation of β1-integrin, which functions upstream of FAK, has also been shown to have little phenotypic impact on the initial stages of ductal outgrowth (5). However, mammary-specific ablation of β1-integrin during the later stages of differentiation resulted in impairment of lactation due to an alteration of mammary epithelial proliferation (26, 27). Although FAK ablation does not affect the ability of mice to lactate, the impact of FAK disruption on the proliferative potential of differentiated mammary epithelial remains to be addressed.

Mice lacking a functional FAK develop mammary tumors, yet there is a dramatic attenuation in the number of hyperplastic lesions. Significantly, those homozygous floxed fak late-stage tumors and their derived lung metastases arise from cells that do not express Cre recombinase and thus have failed to undergo functional excision of the floxed fak alleles. This suggests that FAK function is critical for mammary tumor progression in the PyVmT transgenic mouse model. Consistent with these observations, we have demonstrated that targeted disruption of β1-integrin results in a similar block in tumor progression (5). However, unlike the β1-integrin-deficient strains, mammary-specific deletion of FAK did not hinder the emergence of preneoplastic adenomas. These observations are consistent with the previous findings showing that conditional deletion of fak in the epidermis interferes with the malignant conversion of benign papillomas to carcinomas in a mouse skin carcinogenesis model (10). One key difference between the skin carcinogenesis model and PyVmT mammary tumor model is that the inability of mammary hyperplasias to progress to carcinomas was associated with a decrease in epithelial proliferation, whereas the defect in skin tumorigenesis was associated with enhanced apoptosis (10). Whether the disparity in the response of these mouse tumor models to loss of FAK reflects intrinsic tissue specificity or differences in the nature of the carcinogenic factor used (chemical vs. oncogene) remains to be elucidated. However, our data are consistent with results obtained using MTLn3 rat mammary adenocarcinoma cells expressing FAK-related nonkinase (FRNK), a dominant-negative splice variant of FAK. Indeed, in syngeneic tumors obtained after injection of these cells into the mammary fat pad, van Nimwegen et al. (23) have shown that FRNK-induced expression decreased the primary tumor growth without causing apoptosis. It would also be interesting to assess whether the observed decrease in proliferation could lead to apoptotic cell death at later time points, because apoptosis was assayed on mammary glands from 6-week-old animals.

The finding that mammary-specific deletion of fak impacts on the proliferative capacity of the PyVmT hyperplastic cells is consistent with previous observations showing that mammary-specific ablation of β1-integrin resulted in a proliferative arrest (5). Given the phenotypic similarities between β1-integrin- and FAK-deficient mammary tissues in the PyVmT mouse model, activation of FAK appears to be one of the critical signaling events immediately downstream of β1-integrin. Indeed, loss of β1-integrin is associated with dramatic down-regulation of tyrosine phosphorylation of FAK (5). However, given that the β1-integrin-deficient PyVmT mice failed to develop mammary epithelial hyperplasias, whereas FAK-deficient cells are still capable of forming hyperplastic lesions, there must be other FAK-independent β1-integrin-coupled signaling pathways that are also involved in mammary tumor formation. For instance, the ILK pathway seems to be a very good candidate (2). We have actually shown, using a mammary-specific ILK conditional
knockout mouse model, that ILK deletion has significant effects on PyVmt-induced mammary tumor progression without affecting FAK activity (unpublished data).

Another interesting observation is that FAK-deficient hyperplasias are still detectable as distinct entities in late-stage tumor samples. Remarkably, this resembles the phenomenon of tumor dormancy observed in T-HeP3 human carcinoma cells after inhibition of FAK signaling (28).

The observation that FAK plays a critical role in tumor progression in the MMTV-PyVmt mouse tumor model may have important therapeutic implications for the treatment of human breast cancer. Given the large proportion of human breast cancers that express elevated levels of FAK (11, 22), inhibition of FAK catalytic activity with small-molecule inhibitors (29, 30) may be an important strategy in the management of the disease. In this regard, there have been several reports suggesting that inhibition of FAK can have a profound effect on tumor growth and invasion (23, 24, 29, 31–33). The future development of therapeutic reagents directed against FAK and its coupled signaling pathways has excellent potential for the treatment of primary breast cancers.

**Methods**

**Transgenic Mice.** MMTV-PyVmt, MMTV-Cre, and FAKlox mice were generated and characterized as described (10, 16, 21). GTRosa26 mice were purchased from The Jackson Laboratory and are described in refs. S and 19. The CAG-CAT-EGFP reporter mice are described in refs. 17 and 18. To preclude the possible involvement of genetic background variability, all transgenic mice used were derived from the inbred FVB/N strain. Mammary tumor formation was monitored in virgin mice by biweekly palpation. All experiments involving animals were conducted in accordance with McGill University animal care guidelines.

**Antibodies.** Primary antibodies used in these experiments include rabbit polyclonal anti-FAK (05–543, Upstate Biotechnology), anti-cre recombinase (PRB-106C, Covance), anti-cleaved caspase-3 (9661, Cell Signaling), anti-cytokeratin 14 (PRB-155P, Covance), and mouse monoclonal anti-Ki67 (NCL-L-Ki67-MM1, Novo-casta), anti-FAK (05–537, Upstate Biotechnology), anti-cre (MMS-106P, Covance) and anti-β-actin (AC-15, Sigma). Guinea pig polyclonal anti-cytokeratin 8/18 (BD-PR0GP11) was purchased from Fitzgerald. The Alexa Fluor goat anti-guinea pig 555 (A-21435), goat anti-mouse 555 (A-21422), 633 (A-21050), goat anti-rabbit 555 (A-21428), and 633 (A-21070) antibodies were from Molecular Probes (see **SI Text**).

**Histological and Immunohistochemical Analysis of Tissue Sections.** Thoracic mammary glands or lung lobes were fixed in 10% neutral buffered formalin (Surgipath) and transferred to 70% ethanol the next day. Samples were then paraffin-embedded and sectioned at 5 μm. Endogenous peroxidase activity was blocked with 3% peroxide hydrogen in methanol. Antigen retrieval was accomplished in citrate buffer and by using a pressure cooker (Cuisinart). Sections were then blocked with Power Block Universal Blocking Agent (Biogenex) and incubated in primary antibody as described (5). Lungs were harvested from mice bearing tumors for 8 weeks. Pulmonary metastases were identified by microscopic analyses of hematoxylin/eosin-stained lung step sections. For immunofluorescence analyses, sections were incubated with secondary antibodies for 1 h at room temperature followed by incubation with DAPI (Sigma) for 10 min. They were visualized by using a Zeiss LSM 510 META confocal microscope.

**Mammary Gland Whole Mounts and β-Galactosidase Assay.** Whole mounts were prepared from the no. 4 inguinal mammary glands as described (5). For in situ β-galactosidase assay, no. 3 thoracic and no. 4 inguinal glands were processed as described (5). For X-Gal staining of tumor crossections, small pieces of tumors were placed in plastic molds and frozen in Tissue-Tek OCT (Surgipath) by using liquid nitrogen. Samples were sectioned at 8 μm using a HM 500 OM microscope cryostat (Micron). Sections were dried for 1 h at room temperature, fixed in PBS/0.2% glutaraldehyde (Sigma) for 10 min, rinsed in PBS, and incubated in X-Gal staining solution [2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, and 1 mg/ml X-Gal (Invitrogen) in PBS (pH 7.4)] overnight at 37°C. Sections were fixed again, rinsed, dehydrated through a graded series of ethanol baths and xylene, and mounted with Immumount (Shandon). X-Gal-stained paraffin sections or crossections were counterstained with Nuclear Fast Red.

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