Tumorigenesis and Neoplastic Progression

Mammary Epithelial-Specific Disruption of Focal Adhesion Kinase Retards Tumor Formation and Metastasis in a Transgenic Mouse Model of Human Breast Cancer

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Focal adhesion kinase (FAK) is a central regulator of the focal adhesion, influencing cell proliferation, survival, and migration. Despite evidence demonstrating FAK overexpression in human cancer, its role in tumor initiation and progression is not well understood. Using Cre/LoxP technology to specifically knockout FAK in the mammary epithelium, we showed that FAK is not required for tumor initiation but is required for tumor progression. The mechanistic underpinnings of these results suggested that FAK regulates clinically relevant gene signatures and multiple signaling complexes associated with tumor progression and metastasis, such as Src, ERK, and p130Cas. Furthermore, a systems-level analysis identified FAK as a major regulator of the tumor transcriptome, influencing genes associated with adhesion and growth factor signaling pathways, and their cross talk. Additionally, FAK was shown to down-regulate the expression of clinically relevant proliferation- and metastasis-associated gene signatures, as well as an enriched group of genes associated with the G₂ and G₂/M phases of the cell cycle. Computational analysis of transcription factor-binding sites within ontology-enriched or clustered gene sets suggested that the differentially expressed proliferation- and metastasis-associated genes in FAK-null cells were regulated through a common set of transcription factors, including p53. Therefore, FAK acts as a primary node in the activated

signaling network in transformed motile cells and is a prime candidate for novel therapeutic interventions to treat aggressive human breast cancers. (Am J Pathol 2008; 173:1551–1565; DOI: 10.2353/ajpath.2008.080308)

Transformation of mammary epithelial cells involves a complex set of genetic events that promote proliferation, survival, and invasion.^{1,2} However, recent reports have demonstrated a crucial role for the epithelial microenvironment and in particular the epithelial-extracellular matrix (ECM)/stromal interaction in tumorigenesis, invasion, and metastasis.^{3–7} Using three-dimensional culture models of mammary ductal structure and tumorigenesis, it has been shown that integrins, which can regulate cell-matrix and cell-cell adhesions, play a substantial role in regulating the malignant phenotype and growth of epithelial cells.^{8,9} Moreover, it has recently been demonstrated that targeted deletion of β_1 -integrin in the mammary epithelium results in inhibition of mammary tumorigenesis, and that β_1 -integrin is required for continued tumor cell proliferation.¹⁰ Hence, the cell-ECM interaction, via integrin-mediated adhesion, is now known to directly regulate mammary carcinoma formation and progression, which in turn raises important questions regarding the roles of integrin-associated signaling molecules in regulating mammary carcinoma.

Focal adhesions (FAs) are sites of integrin-clustering and are composed of a large complement of scaffolding and signaling proteins that link the actin cytoskeleton to

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the ECM.¹¹ The primary functions of these complexes are to provide physical attachment to the ECM, transduce force between the cell and ECM, and act as a signaling node from which multiple signaling cascades emanate to regulate cell proliferation, survival, and migration.^{12–16} Furthermore, several growth factor receptors that are known to play a role in carcinoma development and metastasis, including the epidermal growth factor (EGF) and insulin-like growth factor (IGF) family of receptors, co-localize and participate in signaling cross talk with integrins at FAs.^{17–22} Notably, focal adhesion kinase (FAK), a focal adhesion protein and the focus of this study, may be a point of convergence between integrin and growth factor signaling.^{18–21}

FAK is a nonreceptor tyrosine kinase primarily localized to cell-matrix adhesions, that acts as a central regulator of the focal adhesion to influence cell proliferation, survival, and migration.^{16,23} FAK regulates focal adhesion signaling by phosphorylating multiple substrates and by acting as a scaffold for protein-protein interactions, which in turn also regulate downstream signaling cascades.²⁴ For instance, integrin-stimulated phosphorylation of FAK at Y397 creates a high-affinity site that is recognized by several Src homology 2 (SH2) domain-containing proteins such as Src, Shc, PI3K, GRB7, and PLC- γ ,^{25–30} and FAK phosphorylation at Y925 by Src links FAK via Grb2 to the Ras pathway.^{31,32} In addition, FAK is known to modulate the activity of several transcription factors important in human breast cancer, such as NF- κ B, CREB, STAT1, KLF8, Smad1, Prx1, AP-1, and p53.^{33–41} Hence, FAK directly regulates many fundamental adhesion and growth factor signaling pathways and transcription factors central in human cancer, and is therefore well poised to regulate mammary tumorigenesis and metastasis.

Further evidence that FAK may play an important role in carcinoma behavior arises from reports demonstrating FAK overexpression in cancers of the breast, colon, ovary, prostate, and thyroid.^{42–46} Moreover, FAK overexpression correlates with more aggressive and invasive breast carcinomas.^{47,48} One outcome of this overexpression may be increased proliferation because overexpressing FAK in human malignant astrocytoma cells results in increased tumor cell growth in a mouse xenograft model,⁴⁹ and high levels of FAK correlate with high mitotic index in human breast carcinomas.⁴⁸ Correspondingly, epidermis-specific deletion of FAK reduces chemically-induced papilloma formation and blocks malignant conversion.⁵⁰ Consistent with these findings, expression of the FAK inhibitory protein, FAK-related nonkinase (FRNK) in rat mammary adenocarcinoma cells results in decreased tumor volume and significantly decreases the number of lung metastases in a rat xenograft model.⁵¹ Yet, despite these reports the specific role of FAK in mammary carcinoma initiation and progression is not well understood.

Because of strong evidence that FAK may play a significant role in human breast cancer initiation and progression, and the fact that it is not known if FAK plays a direct role in mammary tumorigenesis, we examined the role of FAK in these processes. Until recently, analysis of

FAK in these processes *in vivo* has been precluded because of embryonic lethality of whole animal FAK knockout. Therefore, using a conditional floxed FAK knockout, that was generated using Cre/LoxP technology,⁵² we studied the influence of mammary epithelial-specific deletion of FAK on tumorigenesis and progression in the polyoma middle-T (PyVT) transgenic model of human breast cancer.⁵³ This breast tumor model correlates well with many features of human cancer, recapitulating the multistep progression of human cancer by advancing from hyperplasia to adenoma, and then to early and late carcinoma.^{54,55} Additionally, tumors arising in PyVT mice do so with complete penetrance within 7 to 10 weeks and are reliably invasive and metastatic.^{53–55} Furthermore, it has been shown that Src, a primary FAK signaling partner,¹⁶ is necessary for transformation in PyVT mice.⁵⁶ Yet, ablation of β_1 -integrin in the PyVT model does not alter Src recruitment or activation, but does result in repressed FAK activation even in the presence of paxillin- and FAK-containing FAs, implying that FAK activation may also be necessary for PyVT-induced mammary tumorigenesis.¹⁰

In this study we demonstrate that ablation of FAK in mammary epithelial cells substantially retards tumor formation and growth, and suppresses invasion and metastasis to the lung. Importantly, tumors that do arise in mice homozygous for the FAK^{fllox} allele show substantial Cre-mediated recombination of the allele and the resulting absence of FAK protein in tumor cells. Loss of FAK results in an altered signaling cascade with reduced phosphorylation of p130Cas, Src, and ERK1/2 and a resulting shift in the transcriptome. Microarray analysis of tumor samples demonstrates a significant reduction in adhesion and adhesion-related genes and a compensatory increase in growth factor receptor signaling cascade genes. The result of this shift is a decrease in expression for proliferation and metastasis gene signatures known to represent tumor behavior in human breast cancer. Hence, these observations indicate that FAK is important in mammary tumor initiation and growth, but that FAK is not necessary for transformation because tumors will form and persist in the absence of FAK. However, these tumors do not progress to carcinoma and subsequently do not metastasize.

Materials and Methods

Mice

Mice were maintained and bred at the University of Wisconsin under the approval of the University of Wisconsin Animal Use and Care Committee. MMTV-PyVT and MMTV-Cre were originally obtained from The Jackson Laboratory, Bar Harbor, ME. Generation and characterization of floxed FAK mice have been described elsewhere.⁵² Tumor data were acquired by examining the mice every 2 to 3 days after mice were ~6.5 weeks of age.

Polymerase Chain Reaction (PCR) Analysis of Cre-Mediated Recombination

Genomic DNA was isolated using the Promega SV genomic DNA purification system (Promega, Madison, WI). PCR analysis of genomic DNA was performed using primers described by Beggs and colleagues.⁵² The amplified PCR products consisted of a wild-type (1.4 kb), floxed FAK (1.6 kb), and 327-bp recombined fragment.

Primary Cell Isolation, Three-Dimensional Culture, and Adenovirus Infection

Glands or tumors were finely minced and digested with 2 mg/ml of collagenase and 10 μ g/ml of hyaluronidase under gentle agitation for 1.5 hours at 37°C. Debris was removed by five rounds of centrifugation at 1100 rpm during which cells were washed in Dulbecco's modified Eagle's medium/F12 containing penicillin (100 U), streptomycin (100 μ g), and amphotericin B (0.25 μ g/ml). Cells were then plated onto collagen-coated (30 μ g/ml; BD Biosciences, San Jose, CA) plates in serum-free media (Dulbecco's modified Eagle's medium/F12 with 20 ng/ml of EGF, 10 μ g/ml of insulin, and 0.5 mg/ml of hydrocortisone). Three rounds of differential trypsinization were used to remove nonepithelial cells. After purification, cells were either subjected to adenovirus infection or cultured within three-dimensional collagen gels. For three-dimensional culture, cells were placed into 2.0-mg/ml collagen gels (BD Biosciences) that were neutralized with 2 $\times$ HEPES buffer and culture media containing 2% fetal bovine serum. After 24 hours in three dimensional culture, cells were infected with adenovirus (Ad-GFP or Ad-Cre-GFP⁵² or Ad-FRNK-GFP, which was a gift from Dr. Christopher S. Chen, University of Pennsylvania, Philadelphia, PA) using a multiplicity of infection that resulted in an infection efficiency of at least 70% without showing signs of toxicity.

Mammary Gland Whole Mounts

Mammary whole mounts were prepared by fixing tissues in Carnoy's solution (10% glacial acetic acid/30% chloroform/60% absolute ethanol), followed by rehydration and staining with carmine alum. Tissues were then dehydrated, cleared with xylene, and mounted. Quantitative analysis of the area of hyperplasia was calculated from a common threshold value set with density slicing in ImageJ software (National Institutes of Health, Bethesda, MD).

Histology and Immunofluorescence

For histology, mammary glands and tumors were fixed in formalin followed by paraffin-embedding. Tissue sections were stained with hematoxylin and eosin (H&E). For immunofluorescence, primary cells and frozen sections were fixed in 4% paraformaldehyde in phosphate-buffered saline, or ice-cold acetone, respectively, followed by blocking and permeabilization in 2% donkey serum

plus 5% FAF-BSA or Vector Mouse-On-Mouse blocking agent (Vector Laboratories, Burlingame, CA) and 0.2% Triton X-100, respectively. Primary antibodies (FAK C-20 pAb: Santa Cruz Biotechnology, Santa Cruz, CA; fluorescein isothiocyanate-conjugated pan-cytokeratin mAb: Sigma, St. Louis, MO; Src Y418 pAb: Biosource, Camarillo, CA; ACTIVE ERK1/2 pAb, Promega) were incubated overnight at 4°C. Anti-rabbit tetramethyl-rhodamine isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA) and bisbenzimidazole (Sigma) were used as secondary reagents.

Lung Metastasis

Lungs were harvested at 15 weeks, photographed for examination of surface metastasis, then fixed in formalin and processed for histology. Sections were cut every 100 μ m through the entire tissue and sections stained with H&E. Total lung metastases over all sections were then counted.

Multiphoton Laser-Scanning Microscopy

Multiphoton excitation and second harmonic generation were performed as previously described.⁵⁷

Western Blotting

Samples were lysed in 10 mmol/L Tris, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L Na₄P₂O₇, 2 mmol/L NaVO₄, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mmol/L phenylmethyl sulfonyl fluoride, and protease inhibitor cocktail (Sigma), and Laemmli buffer was then added. Glands and tumors were flash-frozen in LN₂ and ground to powder before addition of lysis buffer. For immunoblotting, membranes were blocked with 5% bovine serum albumin and incubated with anti-FAK C-20 (1:500, Santa Cruz Biotechnology) or 4.47 (1:500; Upstate, Lake Placid, NY), anti-cytokeratin-8 (1:250, Santa Cruz Biotechnology), anti-GAPDH (1:1000, Santa Cruz Biotechnology), anti-p130Cas Y249 (1:500, Biosource), anti-p130Cas (1:1000; Transduction Laboratories, Lexington, KY), anti-Src Y418 (1:500, Biosource), anti-Src (1:1000; Cell Signaling Technology, Beverly, MA), anti-ACTIVE ERK1/2 and anti-ERK1/2 (1:2500, Promega) as appropriate. Horseradish peroxidase-conjugated secondary antibodies (1:5000, The Jackson Laboratories) were used.

RNA Isolation and Microarray

Total RNA was isolated from 24 animals (FAK^{+/+}/Cre, $n = 5$; FAK^{flox/flox}/Cre, $n = 5$; FAK^{+/+}/Cre/PyVT, $n = 7$; FAK^{flox/flox}/Cre/PyVT, $n = 7$). Multiple glands or tumors were pooled from each mouse to increase statistical power.⁵⁸ After harvest, tissues were rinsed and flash-frozen in LN₂. Samples were reduced to frozen powder then lysed with TRIzol reagent (Invitrogen, Carlsbad, CA), separated

with heavy-phase lock gel (Eppendorf, Westbury, NY), and further purified using RNeasy columns with DNase digest (Qiagen, Valencia, CA). Total RNA quantity and purity were analyzed with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). RNA quality was analyzed by acrylamide gel electrophoresis, and quality and quantity confirmed using 2100 BioAnalyzer chips (Agilent, Santa Clara, CA). All RNA was of high quality and appropriate quantity. RNA samples were then prepared for hybridization and hybridized to Affymetrix 430 2.0 mouse arrays (Affymetrix, Santa Clara, CA), in partnership with the UW Biotechnology–Gene Expression Center. Full array datasets are publicly available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo).

Data Analysis and Bioinformatics

GC-RMA normalization, statistical analysis, and principal component analysis (PCA) were performed with ArrayAssist (Stratagene, La Jolla, CA). For PCA, components accounting less than 70/n of the variability were not included.⁵⁹ Clustering data by ontology was performed using the ArrayAssist GO browser. Hierarchical clustering was executed with Cluster and Java-Treeview.⁶⁰ For cluster analysis of human breast tumors, publicly available gene expression data from van de Vijver and colleagues,⁶¹ was used. Toucan (<http://homes.esat.kuleuven.be/~saerts/software/toucan.php>) was used to computationally determine enriched transcription factor binding sites (TFBSs) within select gene lists.⁶² Promoter sequences 1-kb upstream and 0.2-kb downstream of the transcriptional start site were analyzed with Toucan's MotifScanner using the TRANSFAC database (Public 7.0 Vertebrates) to determine TFBSs within the sequences. The mouse 1-kb proximal 1000 ENS-MUSG (3) background model was used with a stringency level of 0.1. Statistically overrepresented binding sites were generated relative to the appropriate expected frequency file for mouse. The complete lists of enriched TFBSs are available on request.

Statistical Analysis

Tumor incidence data were compared using the Logrank test. Two-group data were analyzed with *t*-testing. The Grubb's test for outliers was applied to densitometry data from pERK and pSrc tumor blots and three blot-artifact induced outliers were removed (pSrc: 1 wt, 1 floxed; pERK 1 wt) before subsequent *t*-testing. Analysis of variance followed by the Tukey-Kramer multiple comparison test was used for multigroup data.

Results

Generation of Mammary Epithelial-Specific FAK Knockout Mice

Mice carrying the conditional floxed allele(s) were generated using Cre/LoxP technology. LoxP sites flanking the second kinase domain exon allow Cre-mediated recom-

bination leading to a premature translational stop codon that results in loss of FAK protein expression without up-regulating FRNK or producing any truncated FAK protein.⁵² Mammary-specific deletion of FAK was achieved by introducing the floxed FAK allele into mice expressing Cre recombinase under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. To determine whether FAK is required for mammary tumor initiation and progression *in vivo*, the floxed FAK allele was introduced into mice expressing mammary-specific Cre recombinase and the polyomavirus middle-T oncogene, also under MMTV control. To confirm the functionality of the system, Cre-mediated recombination of the floxed-FAK allele was examined by PCR analysis of mammary gland DNA. Using primers that amplify the wild-type (1.4 kb), floxed-FAK (1.6 kb), and Cre-mediated recombined (327 bp) fragments,⁵² the targeted excision of the allele was confirmed (Figure 1A). Importantly, the addition of LoxP sites into the gene did not alter FAK protein behavior in the FA. Cre-negative FAK^{flox/flox} cells plated on collagen formed normal FAK-positive FAs (Figure 1B), while displaying no obvious difference in phenotype or activated Src localization when compared to cells expressing wild-type FAK (data not shown).

Mammary Epithelial-Specific Ablation of FAK Protein

FAK protein expression was absent in the epithelium of FAK^{flox/flox}/MMTV-Cre mice (Figure 1, C–E). Western blot analysis of whole mammary glands revealed a substantial reduction in FAK protein levels in mutant glands (Figure 1C). Use of antibodies specific for the N- and C-terminal regions of FAK confirmed the absence of truncated FAK products or changes in FRNK expression (data not shown), consistent with previous reports using floxed-FAK mice.^{50,52,63} The remaining presence of FAK protein in the FAK^{flox/flox}/MMTV-Cre tissue was likely attributable to cells in the stromal and vascular compartments that do not express Cre recombinase.^{10,52} Therefore, to confirm that FAK was being excised efficiently in the epithelial population, we purified primary mammary epithelial cells from FAK^{flox/flox}/Cre-negative mice and infected them with an adenovirus expressing Cre recombinase and GFP (ad-Cre-GFP). After an infection efficiency of ~85%, Western blot analysis revealed the ablation of FAK expression 48 hours after infection (Figure 1D). Moreover, to verify epithelial-specific knockout, FAK localization studies in the gland were performed. FAK protein expression was specifically lost in cytokeratin (CK)-positive epithelial cells, in frozen tissue sections from FAK^{flox/flox}/MMTV-Cre mammary glands, whereas cells in the stromal compartment remained FAK-positive (Figure 1E).

The Effects of FAK Loss in the Normal Mouse Mammary Gland

Before examining the influence of FAK on mammary tumor behavior, it was necessary to determine the effects of

Figure 1. Tissue-specific deletion of FAK in the mammary epithelium. **A:** PCR analysis of DNA from FAK^{+/flox} mammary glands confirming Cre-mediated excision of the floxed FAK allele. **B:** Immunofluorescence of primary FAK^{flox/flox} epithelial cells plated on collagen-coated glass (30 μg/ml) for 24 hours showing normal FAKs. **C:** Western blot analysis of FAK expression levels in lysates from FAK^{+/+}/Cre and FAK^{flox/flox}/Cre mammary glands demonstrating FAK knockdown in floxed glands. **D:** Anti-FAK Western blot of protein obtained from purified FAK^{flox/flox} mammary epithelial cells 48 hours after infection with Ad-GFP or Ad-Cre-GFP. **E:** Immunofluorescence analysis of frozen sections from FAK^{+/+}/Cre and FAK^{flox/flox}/Cre mammary glands showing epithelial-specific ablation of FAK protein. Anti-cytokeratin (CK) antibodies (green) demonstrate the epithelial compartment. CK, cytokeratin; Ep, epithelial; St, stromal.

Figure 2. Deletion of FAK delays mammary tumor formation and suppresses growth. **A:** Example of the gross examination tumor burden disparity between FAK^{+/+}/Cre/PyVT and FAK^{flox/flox}/Cre/PyVT mice. **B:** Kaplan-Meier tumor-free survival curves for FAK^{+/+}/Cre/PyVT ($n = 11$), FAK^{+/flox}/Cre/PyVT ($n = 16$), and FAK^{flox/flox}/Cre/PyVT ($n = 16$) animals. Data from FAK^{flox/flox}/Cre/PyVT animals was significantly different from both FAK^{+/+}/Cre/PyVT and FAK^{+/flox}/Cre/PyVT animals ($P < 0.0001$). **C:** Number of glands positive for palpable tumors up to 105 days (counted until eight glands were positive). Significant differences exist between FAK^{flox/flox}/Cre/PyVT and both FAK^{+/+}/Cre/PyVT and FAK^{+/flox}/Cre/PyVT animals for each time point greater than day 54 (P values for each time point ≤ 0.005). Data are mean $\pm$ SEM. **D:** Tumor size designated as small (<0.5 cm), medium (0.5 to 1.0 cm), and large (>1.0 cm in diameter) at week 15. FAK^{flox/flox}/Cre/PyVT mice had significantly ($*P < 0.001$) fewer medium and large tumors than either FAK^{+/+}/Cre/PyVT and FAK^{+/flox}/Cre/PyVT animals. Data are mean $\pm$ SEM. **E:** Combined tumor burden mass per animal at week 15. FAK^{flox/flox}/Cre/PyVT mice had significantly ($*P = 0.001$) lower tumor mass than either FAK^{+/+}/Cre/PyVT and FAK^{+/flox}/Cre/PyVT animals. Data are mean $\pm$ SEM. **F:** Whole mounts of the fourth (inguinal) mammary gland from 10-week-old FAK^{+/+}/Cre/PyVT and FAK^{flox/flox}/Cre/PyVT animals. **G:** Quantification of hyperplastic area in whole mounts of the fourth (inguinal) mammary glands from three pairs of littermates, showing significantly ($*P = 0.032$) less hyperplasia in glands from FAK^{flox/flox}/Cre/PyVT mice. Data are mean $\pm$ SEM.

FAK protein loss on normal gland development and behavior. To examine ductal outgrowth, whole-mount analysis of 4- and 6-week-old virgin animals was performed. Normal ductal outgrowth was observed, with no gross differences seen between glands from control and mutant mice (see Supplementary Figure S1 available at <http://ajp.amjpathol.org>). However, qualitative analysis of more developed glands (week ≥ 6) displayed evidence of decreased branching (Supplementary Figure S1 at <http://ajp.amjpathol.org>), consistent with a recent study reporting a significant decrease in ductal branch points in mammary-specific FAK knockout mice.⁶⁴ Yet the structure of individual ducts in all ages examined (4 to 15 weeks) was normal (Supplementary Figure S1 at <http://ajp.amjpathol.org>). Furthermore, Nagy and co-workers⁶⁴ reported a lactation defect in mammary-specific FAK knockout mice that may be STAT5-dependent. Consistent with this study, microarray analysis (described in detail later in this article) comparing virgin FAK^{+/+}/Cre and FAK^{flox/flox}/Cre revealed a significant down-regulation of genes associated with milk production. Genes for the four major milk caseins (*Csn1s2a*, *Csn1s1*, *Csn2*, *Csn3*; all $P < 0.001$), lactalbumin (*Lalba*, $P = 0.001$), and whey acidic protein (*Wap*, $P = 0.014$) were all significantly down-regulated greater than sixfold. Correspondingly, expression of STAT5b showed a modest (1.2-fold), but significant ($P = 0.046$) down-regulation, supporting the possibility that FAK may contribute to milk production via the JAK-STAT signaling cascade.

Ablation of FAK Expression Delays Mammary Tumor Initiation and Retards Growth

To determine whether FAK is required for mammary tumorigenesis, tumor formation was monitored in FAK^{+/+}/Cre/PyVT, FAK^{+/flox}/Cre/PyVT, and FAK^{flox/flox}/Cre/PyVT mice throughout 15 weeks. Dramatic differences in tumor development were present between mice carrying a wild-type FAK allele when compared to mice carrying the FAK^{flox/flox} allele (Figure 2A). Although 100% of FAK^{+/+} and FAK^{+/flox} mice formed tumors by ~ 70 days, only $\sim 55\%$ of FAK^{flox/flox} formed tumors by day 105, representing a significant difference in tumor formation ($P = 0.0001$, Figure 2B). Consistent with the data shown in Figure 2B, the number of tumor-positive glands was significantly decreased in FAK^{flox/flox}/Cre/PyVT mice (Figure 2C, P values for each time point ≤ 0.005), indicating that even in mutant mice that did form tumors, not all glands underwent transformation within 105 days (also see example in Figure 2A). Furthermore, tumors that did form in FAK^{flox/flox}/Cre/PyVT mice were smaller in volume. Although each set (FAK^{+/+}, FAK^{+/flox}, and FAK^{flox/flox}) possessed small (< 0.5 cm in diameter) tumors, FAK^{+/+} and FAK^{+/flox} mice formed a significantly greater number of medium (0.5 to 1.0 cm)- and large (> 1.0 cm)-sized tumors (Figure 2D, $P < 0.001$ for FAK^{flox/flox} versus both FAK^{+/+} and FAK^{+/flox}). In fact large tumors in FAK^{flox/flox}/Cre/PyVT mice were rare, even when tumors presented at similar time points to wild-type animals (data not shown),

consistent with the observation of significantly lower tumor burden mass in FAK^{flox/flox} mice (Figure 2E, $P = 0.001$). Lastly, quantitative analysis of whole-mount preparations from 10-week-old mice supports the data presented in Figure 2B. At 70 days, substantial transformation and growth had taken place within control animals (Figure 2F, left) whereas mice of the FAK^{flox/flox} background (Figure 2F, right) had significantly less transformation (Figure 2G, $P = 0.032$). Combined these observations strongly suggest that mammary-specific ablation of FAK substantially impairs epithelial tumor progression in the mammary glands of MMTV-PyVT mice, consistent with a recent study by Lahlou and co-workers,⁶⁵ in which the authors report a significant increase in tumor latency after FAK excision.

Mammary-Specific Deletion of FAK Suppresses Metastasis to the Lung

The PyVT breast tumor model correlates well with many features of human cancer and results in reliable pulmonary metastasis.^{53,55} Polyomavirus middle-T transgenic mice develop multifocal metastatic mammary adenocarcinomas in the lungs. Therefore this model system provides a powerful model to study not only tumor initiation but also progression to metastasis. Given the well-described role of FAK in regulating cell migration,¹⁶ we examined the influence of FAK on the development of pulmonary metastasis in PyVT mice. Examination of late-stage (week 15) tumor morphology demonstrated that tumors that did arise in conditional FAK knockout animals were not locally invasive (benign) whereas FAK^{+/+} tumors were highly invasive (Figure 3A). Hence, tumors in FAK^{flox/flox}/Cre/PyVT mice had not continued to progress through malignancy and as a result did not substantially metastasize to the lung (Figure 3, B and C). Lung-surface metastasis was not detected in the FAK^{flox/flox} animals that had developed mammary tumors (Figure 3B), consistent with quantitative data showing a significant reduction in lung metastases in mutant mice (Figure 3C). Furthermore, in FAK^{flox/flox} mice that presented with palpable tumors at time points comparable to the control group ($\leq \sim 70$ days), no significant metastasis was detected (Figure 3D), and other investigators recently report that any floxed tumor cells that do metastasize to the lung remain FAK-positive having not undergone efficient Cre-mediated recombination,⁶⁵ supporting the conclusion that FAK loss suppresses pulmonary metastasis.

Confirmation that Tumors in FAK^{flox/flox} Mice Form in the Absence of FAK

In a recent study examining the role of β_1 -integrin in mammary tumorigenesis, it was shown that tumors that did form in the conditional knockouts were β_1 -integrin-positive because of incomplete Cre-mediated recombination.¹⁰ Because Cre-mediated recombination can be less than 100% efficient, we examined tumor samples from FAK^{flox/flox}/Cre/PyVT mice to determine whether tu-

Figure 3. FAK ablation suppresses metastasis to the lung. **A:** Representative histology images of H&E-stained late-stage mammary tumor sections, showing the invasive phenotype of FAK-positive cells and the noninvasive phenotype of FAK-negative cells. **B:** Representative images of lung surface metastases at week 15. **C:** Total number of metastases within the lungs of FAK^{+/+}/Cre/PyVT ($n = 5$) and FAK^{flox/flox}/Cre/PyVT ($n = 5$) mice at week 15. Data are mean $\pm$ SEM. **D:** Number of metastasis per animal as function of time after palpable tumor detection, presented as day 105 (week 15) minus day of palpable tumor detection. Scale bar = 100 μ m.

mors formed in the presence or absence of FAK protein. PCR analysis of tumor DNA indicated that of the 22 biopsies examined (taken from different tumors arising in 11 FAK^{flox/flox} mice), all showed substantial recombination of the floxed-FAK allele, with three samples still retaining a detectable portion of the floxed (1.6 kb, see Figure 1) allele (representative data shown in Figure 4A). Western blot analysis of separate biopsies from the same tumor pool supported these PCR data by demonstrating that recombination resulted in significant loss of FAK protein (Figure 4, B and C). Much of the residual FAK signal is thought to arise from nonepithelial cells in the tumors as discussed above, but because of the multifocal nature of PyVT tumors the possibility that some portions of the tumor contained FAK-positive epithelial cells that did not undergo efficient recombination could not be excluded. Therefore, frozen tumor sections were examined by immunofluorescent microscopy (Figure 4D). No clusters of FAK-positive cells were identified within populations of cytokeratin-positive epithelial cells. Consequently, FAK was efficiently ablated within our system and as such, subsequent analysis describes changes in transformed epithelial cells that lack FAK protein.

FAK Deletion Suppresses Phosphorylation of p130Cas, Src, and ERK1/2

On adhesion-mediated FAK activation, FAK and Src form a signal complex that results in a conformational change

Figure 4. Epithelial cells in FAK^{flox/flox}/Cre/PyVT tumors are FAK-negative. **A:** PCR analysis of DNA extracted from ~10-mg tumor biopsies taken from tumors that formed in FAK^{+/+}/Cre/PyVT (lane 1), FAK^{+/flox}/Cre/PyVT (lanes 2 to 4), and FAK^{flox/flox}/Cre/PyVT (lanes 5 to 11) mice. Gel image is representative of data from 42 tumor biopsies taken from 19 mice (22 biopsies were from 11 FAK^{flox/flox}/Cre/PyVT mice), showing floxed tumor cells have undergone Cre-mediated recombination. **B:** Western blot analysis of FAK expression levels in lysates of ~10-mg tumor biopsies from FAK^{+/+}/Cre/PyVT, FAK^{+/flox}/Cre/PyVT, and FAK^{flox/flox}/Cre/PyVT mice. Image is representative of data from 42 tumor biopsies taken from 19 mice. **C:** Densitometry quantification of FAK protein levels from blots discussed in **B**. Data are mean $\pm$ SEM. **D:** Immunofluorescence analysis of frozen sections from FAK^{+/flox}/Cre/PyVT and FAK^{flox/flox}/Cre/PyVT mammary tumors showing epithelial-specific ablation of FAK protein in FAK^{flox/flox} animals.

and activation of Src kinase activity.^{16,23} The binding of Src to Y397 phosphorylated FAK promotes Src-mediated phosphorylation of the FAK at Y925, which links FAK via Ras to extracellular signal regulated kinase (ERK).^{14,16,23} Because FAK and Src are heavily cross regulated^{14,16,23} and Src has been shown to be necessary for tumorigen-

Figure 5. Decreased Src, ERK, and p130Cas phosphorylation in FAK-negative epithelial tumor cells. **A** and **B**: Src and ERK1/2 phosphorylation levels in ~10-mg tumor biopsies from FAK^{+/+}/Cre/PyVT and FAK^{flox/flox}/Cre/PyVT mice quantified by densitometry analysis of immunoblots. All data points are shown; horizontal line represents the mean. **C** and **D**: Immunofluorescence analysis of frozen sections from FAK^{+/flox}/Cre/PyVT and FAK^{flox/flox}/Cre/PyVT mammary tumors showing epithelial-specific decreases in phosphorylated Src(Y418) and ERK1/2 in FAK^{flox/flox} animals. **E**: Multiphoton excitation (MPE)/second harmonic generation (SHG) image of live intact tumor from PyVT mice showing interaction between epithelial tumor cells and the collagenous stroma. **F**: Western blot analysis of proteins obtained from purified FAK^{flox/flox} (Cre-negative) epithelial cells cultured within three-dimensional collagen matrices for 72 hours. After 24 hours in three-dimensional culture, cells were infected with either Adeno-GFP or Adeno-Cre-GFP to knockdown FAK, as indicated. Note that tumor numbers 1 through 9 designate nine separate cultures of purified primary cells from nine independent tumors that were then cultured in three-dimensional collagen gels for the experiments. **G**: Western blot analysis of proteins obtained from purified MMTV-PyVT epithelial cells cultured within three-dimensional collagen matrices for 72 hours. After 24 hours in three-dimensional culture, cells were infected with either Adeno-GFP or Adeno-FRKN-GFP. Fold change represents mean $\pm$ SEM; $n = 4$ paired samples. Scale bars = 10 μ m.

esis in PyVT mice,⁵⁶ we examined Src phosphorylation at Y418 in FAK-negative tumor cells. Western blot analysis of tumor biopsies that had significantly reduced FAK levels showed a significant decrease in Src phosphorylation at Y418 (Figure 5A, $P = 0.012$). Given that Src is required for tumor formation in PyVT transgenics⁵⁶ it is not unexpected that Src retains some activity in FAK-null cells that do form tumors. Similarly, phosphorylation of ERK1/2 was reduced. Yet the decrease in ERK phosphorylation within tumor biopsies only showed a statistical trend and did not meet the commonly used 0.05 P value cutoff for significance (Figure 5B, $P = 0.13$). However, the tumor biopsy samples were heterogeneous, and contained cells that did not undergo Cre-mediated recombination, as discussed above. Therefore to clarify the effects of FAK ablation specifically in epithelial cells, frozen tumor sections were analyzed by immunofluorescent microscopy using phospho-specific antibodies to Src and ERK. Examination of tumor sections revealed substantial down-regulation of phosphorylated Src and ERK in epithelial cells, which was distinct from the surrounding stromal cells (Figure 5, C and D).

To confirm the findings that FAK-negative epithelial cells had reduced levels of phosphorylated Src and ERK, as well as determine the state of the adaptor protein p130Cas, which also regulates cell migration and binds to FAK after Src-mediated FAK phosphorylation,^{66–68} we studied purified FAK^{flox/flox} cells cultured within three-dimensional collagen matrices. In the PyVT model, like human breast cancer, epithelial cells interact with the collagen matrix (Figure 5E),⁵⁷ and metastatic cells migrate directly along collagen fibers.^{57,69} Hence, the

reconstituted three-dimensional collagen provides a relevant system for studying tumor behavior within the three-dimensional microenvironment. In this study, primary, cytokeratin-positive (not shown), epithelial cells were purified from nine FAK^{flox/flox}/PyVT (Cre-negative) tumors. After purification, cells were seeded into collagen gels for 24 hours then infected with either ad-GFP (control) or ad-Cre-GFP (to excise FAK) for 48 hours. After the combined 72 hours, Western blot analysis revealed a down-regulation of p130Cas(Y249), Src (Y418), and ERK1/2 phosphorylation (Figure 5F). This is likely attributable to the fact that FAK can directly facilitate phosphorylation of Src, and FAK and Src can promote phosphorylation of p130Cas, which is necessary for formation of the p130Cas-Crk-DOCK180 complex and Rac activation to promote migration.⁷⁰ Further, Src-mediated FAK phosphorylation at Y925 links FAK to ERK1/2,³⁰ and FAK, Src, and ERK can complex at FAs with paxillin to regulate epithelial morphogenesis.⁷¹ The fact that activity of all three of these key regulators are down-regulated by FAK loss further supports the concept of FAK as a central node in the FA signaling network.

As an alternative approach, examination of wild-type PyVT tumor cells (cultured in three-dimensional matrices as discussed above) were infected with ad-FRKN-GFP, which suppressed FAK(Y397) phosphorylation, resulting in a similar suppression in Src and ERK phosphorylation (Figure 5G). Hence, ablation of FAK alters key portions of the FAK signaling cascade that regulate proliferation, invasion, and migration and therefore it seems likely that these pathways might play a role in the observed changes in tumor formation, growth, and metastasis.

Loss of FAK Alters the Tumor Transcriptome

To further study the molecular changes associated with loss of FAK in mammary tumors at the systems level, we analyzed the gene expression profiles of FAK^{+/+}/Cre and FAK^{flox/flox}/Cre glands, and FAK^{+/+}/Cre/PyVT, and FAK^{flox/flox}/Cre/PyVT tumors (which do not appear to significantly differ in stromal cell composition; Supplementary Figure S2 available at <http://ajp.amjpathol.org>). By quantifying shifts in the transcriptome we aimed to help elucidate the mechanisms by which FAK-negative cells transformed, yet demonstrated retarded growth and invasion; and subsequent loss of pulmonary metastatic potential.

As a first-step in our analysis, we examined FAK transcript levels in the glands and tumors from FAK^{+/+} and FAK^{flox/flox} mice. Array analysis revealed a significant ($P = 0.028$) increase in FAK (*Ptk2*) expression (1.5-fold) in FAK^{+/+}/Cre/PyVT tumors when compared to normal FAK^{+/+}/Cre glands that do not carry the polyoma transgene, supporting the use of this system to study human cancers that overexpress FAK. Further, examination of mRNA levels indicated a significant decrease in the FAK transcript in both glands and tumors from floxed animals. In tumors, FAK mRNA levels were reduced 1.65-fold ($P = 0.003$) in FAK^{flox/flox}/Cre/PyVT when compared to FAK^{+/+}/Cre/PyVT controls, consistent with data shown in Figure 4 demonstrating a more than twofold reduction in FAK protein levels. Significantly, levels of the FAK family member Pyk2 are up-regulated in the FAK^{-/-}/p53^{-/-} embryonic fibroblast cell line.^{72,73} In contrast, here we observed no changes in transcript levels of *Ptk2b* (Pyk2) in either glands or tumors from FAK^{flox/flox}/Cre and FAK^{flox/flox}/Cre/PyVT animals, respectively, when compared to control animals wild-type for FAK (not shown); consistent with a previous report of Pyk2 levels in cortical extracts using the same FAK^{flox/flox} mice.⁵² Therefore, in our model, FAK levels are significantly reduced, whereas the primary candidate for compensation, Pyk2, showed no changes.

To reduce the dimensionality of the data sets as well as determine the extent to which loss of FAK influences the pattern of expression, we performed PCA. Examination of the first three principal components (which accounted for >97% of variability in the data) clearly demonstrates separation of the gene expression patterns from glands and tumors, with FAK-negative tissues well distinguished from wild-type glands and tumors (Figure 6Ai). Further examination of the first component (C1), which is likely a measure of the average overall gene expression,⁵⁹ showed a reduction in average expression levels in FAK-negative glands and tumors (Figure 6Aii). Yet, the overall expression levels for FAK^{flox/flox}/Cre/PyVT tumors are significantly higher than levels seen in either FAK^{flox/flox}/Cre or FAK^{+/+}/Cre glands, and the FAK-negative tumors are more closely related to the FAK^{+/+}/Cre/PyVT PC cluster (Figure 6A, i, ii). Thus, although FAK loss influenced the tumor transcriptome, the overall expression program in FAK-negative tumors more closely resembles FAK^{+/+} tumors than either normal or FAK-negative mammary glands, implying that loss of FAK might result in differential expression of a subset of genes that influence cell

behavior, but that many of the changes associated with epithelial transformation are present. Such a behavior would be consistent with our findings that FAK^{flox/flox} animals form delayed tumors that do not follow the progression of tumors in FAK^{+/+} animals.

Epithelial-Specific Deletion of FAK Results in Decreased FA Transcripts

To examine the microarray-generated dataset in a biologically meaningful way, we grouped genes based on their biological processes and molecular functions. Statistically significant genes were input into the ArrayAssist Gene Ontology (GO) Browser and statistically enriched groups of genes were obtained based on like ontology. These groups were examined in conjunction with current literature and final groups formulated.

Assessment of transcripts for proteins associated with classical FAs, and matrix adhesion in general, revealed a significant down-regulation of FA-associated genes in FAK-negative cells (Figure 6B). Genes for the hallmark FA proteins paxillin (*Pxn*) and vinculin (*Vcn*), as well as a number of integrin subunits were down-regulated relative to FAK^{+/+}/Cre/PyVT tumors (also see Supplementary Figure S3 available at <http://ajp.amjpathol.org> for protein-level validation of a subset of differentially expressed genes: paxillin, vinculin, Dock180, Rac1, cyclin B1). The transcript for another protein in this class, zyxin, was also down-regulated (although modestly). Yet the zyxin regulator/binding-partner, LASP-1, was strongly down-regulated. Likewise, mRNA levels for integrin-linked kinase (ILK) were modestly suppressed, whereas two other members of the ILK ternary signaling complex, PINCH (*Lims1*) and α -parvin (*Parva*) were strongly down-regulated. In contrast, mRNA levels for talin (*Tln1*) were up-regulated, whereas levels of filamin B (*Flnb*) were significantly reduced, raising the possibility that talin up-regulation is an attempt to compensate for reduced filamin.⁷⁴

In addition to decreased expression of adhesion-related genes, transcripts for proteins that are known to be regulated by, or to regulate, FA signaling were also differentially expressed. Examination of genes grouped by their association to the Rho family GTPases revealed significantly lower levels of *Rhoa*, *Rac1*, and *Cdc42* in FAK^{flox/flox}/Cre/PyVT tumors when compared to FAK^{+/+}/Cre/PyVT (Figure 6C). Interestingly, expression of Rho protein activators and RhoGEFs were also down-regulated, whereas RhoGAP genes (including those for p190RhoGAP A and B; *Grf1* and *Arhgap5*, respectively) were up-regulated. Combined with the down-regulation of *Rhoa*, these data imply that FAK-negative tumor cells may have decreased Rho activity. Furthermore, the decreased phosphorylation of p130Cas (Figure 5F) combined with the decrease in the Crk, Dock180/ELMO, and Rac transcripts may imply a down-regulation of Rac signaling as well. However, mRNA levels for Trio, a GEF for both Rho and Rac, and a FAK binding partner,⁷⁵ are up-regulated. Although Trio has been traditionally associated with GEF activity for RhoA and Rac1, more recent

Figure 6. Altered gene expression patterns in FAK-negative epithelial cells. **A:** i: PCA of FAK-regulated genes. Data were mean centered then PCA performed from all experimental samples (FAK^{+/+}/Cre, *n* = 5; FAK^{lox/lox}/Cre, *n* = 5; FAK^{+/+}/Cre/PyVT, *n* = 7; FAK^{lox/lox}/Cre/PyVT, *n* = 7). ii: Projection of the first principal component that may be a measure of average expression. **B:** Heat map of enriched transcripts associated with adhesion and FA signaling. **C:** Heat map of enriched transcripts associated with Rho family GTPase signaling. **D:** Heat map of enriched transcripts associated with the cytoskeleton and actin remodeling. **E:** Heat map of enriched growth factor receptor tyrosine kinases. **F:** Heat map of enriched growth factor transcripts. Note for **B–F**, the scale represents the fold-change in Log₂ space of the average expression of FAK^{lox/lox}/Cre/PyVT (*n* = 7 arrays) over FAK^{+/+}/Cre/PyVT (*n* = 7 arrays) tumors, with red indicating an increase and green a decrease in relative transcript abundance.

studies are demonstrating that Trio preferentially activates RhoG, a member of the Rac/Cdc42 subgroup of the Rho GTPase family, which in turn activates Rac1 and Cdc42 and promotes migration via the DOCK180/ELMO complex.^{76,77} Because, Trio cannot bind to FAK in FAK-negative cells to up-regulate RhoA, and Crk, DOCK180, and ELMO are repressed along with decreased p130Cas phosphorylation, it seems reasonable to speculate that the data implies molecular conditions that are conducive to less activated states for both Rho and Rac. In contrast, although *Cdc42* was decreased, *Cdc42* GEFs were up-regulated.

Examination of groups of transcripts for proteins associated with the cytoskeleton indicated a significant set of differentially expressed genes (Figure 6D). Of interest to understanding the altered behaviors of FAK-negative tumor cells observed in this study, genes for a number of cytoskeleton-associated proteins that influence actin dynamics associated with cell motility were differentially expressed. In particular, the changes in *Pak1*, *Limk2*, and *Ssh1* (all of which are part of the Rho family GTPase signaling network) imply the possibility of increased cofilin activity, whereas decreases in the N-WASP interacting protein gene *Wipf1* and a number of Arp2/3 subunits may suggest less actin branching. Further, *Enah* and *Evl* (and their motor protein Myosin-X; *Myo10*) whose pro-

Figure 7. FAK ablation suppresses proliferation-associated gene expression. **A:** Proliferation signature (PS) genes that are up-regulated in wild-type tumors, compared to wild-type mammary glands. **B:** PS genes that are down-regulated by targeted FAK deletion in tumors (top: red font indicates genes associated with the G₂, G₂/M phases of the cell cycle). G₂, G₂/M genes that are down-regulated in FAK^{lox/lox} tumors (bottom). **C:** Proliferation node cluster generated by hierarchically clustering the average expression values of significant genes from FAK^{+/+}/Cre/PyVT and FAK^{lox/lox}/Cre/PyVT tumors, with average expression values from the corresponding glands (FAK^{+/+}/Cre and FAK^{lox/lox}/Cre), and identifying clusters containing enriched groups of PS genes. **D:** Computationally predicted TFBSs that are enriched in G₂, G₂/M genes. Percent indicates the number of genes in the entire set that contain the TFBSs within the promoter region. n is the number of TFBSs found within the set, and p represents the probability of finding more than n occurrences within the sequences.

teins protect elongating actin filaments by antagonizing capping protein (CapZ), are up-regulated. Overall these data suggest actin filament elongation with decreased branching, which could be related to the increase in *Cdc42* GEFs and other molecules that promote filopodia in two-dimensional culture or invadopodia in three-dimensional microenvironments. These findings suggest that FAK-negative cells have misregulated actin dynamics such that they cannot efficiently migrate and metastasize. However, although it is clear that ablation of FAK results in repression of FA-associated transcripts and differential expression to the GTPase and cytoskeleton-associated members of FA signaling network, these data do not provide direct information regarding the binding and activation states of these molecules. As such, more direct conclusions regarding the roles of the GTPase and cytoskeleton-associated signaling families on mammary tumor cell behavior will remain elusive until more reductionist studies can be completed based on this work. Yet,

Figure 8. FAK deletion results in reduced proliferation of transformed mammary epithelial cells. **A:** Immunofluorescence analysis of frozen sections from control (FAK^{+/flox}/Cre/PyVT) and FAK^{flox/flox}/Cre/PyVT mammary tumors showing reduced Ki-67 staining (red), a standard marker of proliferation potential, in cytotkeratin-positive (green) epithelial cells. **B:** Quantification of the Ki-67 proliferation index represented as the percentage of double-labeled Ki-67 (red in **A**) and pan-cytokeratin (green in **A**) cells to the number of pan-cytokeratin-positive cells. Data are mean $\pm$ SEM obtained from >5000 cells per condition in $n = 3$ tumor samples per group (* $P < 0.0001$).

it is apparent that these pathways are misregulated, which in conjunction with their known functions and the observed phenotypic changes in mutant cells, make it reasonable to conclude that part of the mechanisms behind our observations is a disruption of the FA-Rho GTPase family-cytoskeleton-associated-cytoskeleton network.

Epithelial-Specific Deletion of FAK Results in Increased Growth Factor Receptor Transcripts

Because FAs act as nodes from which multiple signaling cascades originate to regulate cell proliferation, survival, and migration, and FAK-negative cells are able to form tumors, albeit at reduced levels, it appeared likely that another growth-promoting stimulus was in place. In fact, ontology-grouped genes showed enrichment for multiple growth factor pathways. In particular, transcripts for multiple growth factor receptor tyrosine kinases were up-regulated in FAK^{flox/flox}/Cre/PyVT tumors when compared to tumors from the FAK^{+/+}/Cre/PyVT background (Figure 6E). Insulin-like growth factor receptor (and IGF-associated adaptor protein Grb2; 1.4-fold, $P = 0.015$) as well as platelet-derived growth factor receptor genes were strongly up-regulated in FAK-null tumor cells. Epidermal growth factor receptor (EGFR) showed a trend toward up-regulation, whereas Erbb3 was not changed. However, when interpreting these results, it should be noted that both EGFR and Erbb3 were down-regulated in FAK^{flox/flox}/Cre glands when compared to FAK^{+/+}/Cre glands. Thus, the tumor cells in fact started from a sig-

Figure 9. FAK deletion disrupts expression of genes associated with local invasion and metastasis. **A:** Heat map of 68 genes down-regulated by tissue-specific FAK knockout (KO) that are known to be up-regulated in an invasive subpopulation of MMTV-PyVT tumor cells (Wang et al., 2007). **B:** Heat map of 30 genes up-regulated by tissue-specific FAK knockout (KO) that are known to be down-regulated in an invasive subpopulation of MMTV-PyVT tumor cells (Wang et al., 2007). **C:** Prognostic value of the 98 metastasis genes in human breast cancer. The 98 genes (shown in **A** and **B**) divide breast cancer patients into two main clusters (cluster analysis provided in Supplementary Figure S4, which can be found at <http://ajp.amjpatbol.org>; blue and orange lines). Kaplan-Meier survival analysis of the two groups shows that the patients differ significantly in metastasis-free time. Average expression in patients with poorer outcome (orange line) matched the expression direction (up- or down-regulated) of Wang and colleagues⁸² for 68% of the metastasis-associated genes, validating these genes, which are oppositely regulated by FAK knockout, as important for metastasis in the human population. **D:** Computationally predicted TFBSs that are enriched in metastasis-associated genes. Percent indicates the number of genes in the entire set that contain the TFBS within the promoter region. n is the number of TFBSs found within the set, and p represents the probability of finding more than n occurrences within the sequences.

nificant deficit in receptor transcript and therefore no change in these cases might in fact indicate elevated signaling to bring expression levels up from this deficit; and in the case of EGFR may represent a substantial increase. A similar pattern can be seen with *Pdgfc* and *Pdgfd* (Figure 6F). Although *Pdgfa* expression was increased, *Pdgfc* and *Pdgfd* show no significant changes, but start out down-regulated in mutant glands. Additionally, *Fgf1* was up-regulated whereas *Tgfa*, *Igf2*, and *Vegfa* were down-regulated in FAK-negative tumor cells. Taken together, these results suggest that increased growth receptor expression might be part of a compensatory mechanism by which FAK^{flox/flox}/Cre/PyVT cells promote transformation and growth in the absence of FAK and subsequent attenuation of FA signaling. However, given our findings that phosphorylation of Src, p130Cas, and ERK (all of which can be activated by growth factor signaling)^{14,70} are suppressed, and genes for the PI3K pathway (also downstream of RTKs, especially IGFR) are down-regulated in floxed cells, it seems likely that increased growth factor signaling is part of the mechanism by which FAK-null cells transform and grow, but that this signaling is suboptimal in the absence of FAK.

FAK Deletion Suppresses Key Proliferation Signature Genes and Disrupts Expression of G₂, G₂/M-Associated Genes in Tumor Cells

Because elevated proliferation is hallmark of cancer² and FAK is known to regulate proliferation,^{15,23} we examined enriched groups of genes associated with the cell cycle. Inspection of genes comprising the breast carcinoma proliferation signature⁷⁸ revealed the presence of the signature in FAK^{+/+}/Cre/PyVT tumors when compared to FAK^{+/+}/Cre glands, further supporting the relevance of PyVT transgenics to model human breast cancer (Figure 7A). Notably, 15 proliferation signature genes were significantly decreased in floxed tumors, with 60% of these genes associated with the G₂ and G₂/M phases of the cell cycle (Figure 7B). Previously, FAK has been linked to regulation of G₁ progression and cyclin D1 expression in fibroblasts⁷⁹ and glioblastoma cells.⁸⁰ In floxed tumors, however, down-regulated groups of genes associated with G₂ and M were enriched (Figure 7) and proliferation significantly decreased (Figure 8), whereas *Ccnd1* (cyclin D1) was not differentially expressed. To generate a more complete list of G₂ and G₂/M genes regulated by FAK, we compared our dataset with a comprehensive list of cell-cycle regulated genes⁸¹ and other bioinformatics databases. This analysis identified 35 G₂, G₂/M genes differentially expressed in FAK-negative tumors (Figure 7B). Furthermore, many of these genes were grouped by hierarchically clustering significant genes (Figure 7C; note that *Plk4*, *Traip*, *Anln*, and *Aurkb* were located within 15 gene spaces in the adjacent cluster as well). Hence, despite decreased ERK activity, it may be possible that up-regulated RTK activity compensate for the effects of FAK loss on the G₁ phase of the cell cycle, but cannot overcome disruption of G₂, G₂/M in FAK-negative cells, which may result from down-regulation of genes associ-

ated with the PI3K pathway, Rho family GTPase signaling, and actin remodeling.

Because genes that cluster together may share common regulatory mechanisms we examined the G₂, G₂/M cluster node and the G₂, G₂/M list generated by aligning to the data from Whitfield and colleagues⁸¹ for enriched TFBSs to gain additional insight into FAK's mechanisms of regulation (Figure 7D). Of interest, a common theme across both lists of top scorers was enrichment for p53, Zic1–3, PAX4, HNF4, Sp1, and Spz1, indicating that FAK or FAK-associated signaling pathways may converge on these TFs to regulate the tumor cell cycle.

FAK Regulation of Migration and Metastasis-Associated Genes

FAK-negative tumor cell metastasis to the lung is suppressed (Figure 4) and FAK is known to regulate cell migration.^{16,23} Therefore, we identified genes for proteins that are known regulators of migration, and aligned our dataset to a published dataset reporting genes associated with invasion/metastasis in PyVT mice⁸² to gain additional insight into FAK's role in suppressing metastasis in floxed animals. Disruption of FAK alone can attenuate cell migration, and as discussed above, it seems likely that disruption of the FA, GTPase, and actin-modifying network (Figure 6), along with decreased activation of migration-regulating molecules such as Src and p130Cas (Figure 5), are primary mechanisms behind potential defects in migration. However, metastasis is a complex process and tumor cells in three-dimensional microenvironments are known to use multiple compensatory mechanisms to facilitate local invasion and metastasis.⁸³ Therefore, we examined the role of FAK in regulating genes that are known to play a role in metastasis. Wang and co-workers⁸² identified ~900 genes that are differentially expressed in a metastatic subpopulation of PyVT tumor cells. Alignment of our FAK^{flox/flox}/FAK^{+/+} (both/Cre/PyVT) dataset to the 900 gene list revealed 98 genes, which were significantly regulated in a contradictory manner by FAK loss (Figure 9, A and B). Sixty-eight genes up-regulated during metastasis were down-regulated in mutant cells (Figure 9A), whereas 30 genes that were down-regulated during metastasis were up-regulated in FAK-negative cells (Figure 9B). To further understand the relevance of this gene set to human disease, we compared their expression in human breast cancer patients, and found that these 98 genes divide patients into two main clusters that differ significantly in metastasis-free outcome (Figure 9C). Computational prediction of enriched TFBSs for both up- and down-regulated genes again revealed enrichment for p53, Zic1 and 3, PAX4, Sp1, and Spz1 binding sites (Figure 9D; note that HNF4 and Zic2 were also enriched in down-regulated genes, ranking 12th and 15th, respectively, and are not shown). Hence, the FAK-regulated signaling network appears to converge on these transcription factors to regulate both proliferation and metastasis, indicating a common regulatory mechanism by which FAK is regulating transcription.

Discussion

Mice with mammary epithelial-specific loss of FAK develop mammary tumors. However, tumor presentation is delayed in the PyVT mouse model and tumor growth is attenuated. This result is consistent with a recent report also showing that transformation can occur in the absence of FAK.⁶⁵ However, Lahlou and colleagues⁶⁵ report that a significant portion of the transformed epithelial cells in their system remain FAK-positive because of incomplete Cre-mediated recombination. In the current study Cre-mediated recombination efficiency was much greater, likely explaining reported differences in tumor latency; a disparity that may be reasonably explained by the use of different conditional FAK knockout and MMTV-Cre strains. However, because FAK was efficiently ablated herein it supports our conclusion that FAK is not required for transformation but that tumor growth and progression to metastasis is suppressed in FAK-null cells.

Late-stage (week 15) tumors that lacked FAK did not show evidence of invasion suggesting that FAK is required for progression to the malignant phenotype. This finding is consistent with data showing that FAK deletion in papillomas blocks malignant conversion to squamous cell carcinomas⁵⁰ and that FAK knockdown inhibits local invasion in mammary 4T1 carcinoma cells.⁸⁴ Furthermore, floxed-FAK epithelial cells did not efficiently metastasize to the lungs with an ~50-fold reduction in lung metastasis and the majority of animals have zero to one lung metastasis, consistent with reported data showing that expression of FRNK reduces lung metastasis.⁵¹ Interestingly, other investigators recently reported that in the FAK^{flox/flox}/Cre/PyVT model any floxed tumor cells that do metastasize to the lung remain FAK-positive having not undergone efficient Cre-mediated recombination.⁶⁵ Hence, it appears likely that *in vivo*, loss of FAK inhibits the progression to pulmonary metastasis, identifying FAK as a potential target to suppress cancer metastasis.

Intriguingly, the FAK-regulated signaling network appears to converge on a conserved set of transcription factors to regulate both proliferation and metastasis. Of particular relevance to cancer biology is the observation of enriched TFBSs for p53 within these gene signatures. p53 is a known tumor suppressor and regulator of both the G₁/S and G₂/M transitions,⁸⁵ as well as a transcriptional repressor of cyclin B.^{85–87} Interestingly, FAK has been recently reported to bind directly to p53 and reduce its transcriptional activity⁴¹ and proliferation of FAK^{−/−} embryonic fibroblasts requires inactivation of p53,⁸⁸ whereas FAK-interacting protein (FIP200) can bind p53 and extend its half-life.⁸⁹ As discussed by Mitra and Schlaepfer,¹⁵ because p53 and FAK may compete for the same binding region on FIP200, conditions of limited FAK levels may promote FIP200 stabilization and enhancement of p53 activity. Furthermore, a recent study by Lim and colleagues,⁹⁰ has elegantly shown that loss of FAK activates p53 and impairs cell proliferation, whereas FAK protein inactivates p53 via Mdm2-dependent p53 ubiquitination and is dependent on nuclear localization of FAK. Combined, these studies suggest that loss of FAK in

mammary epithelial cells may result in increased p53 activity and attenuated proliferation via the G₂/M phases of the cell cycle, including direct transcriptional regulation of G₂/M-associated genes such as cyclin B.

In summary, we demonstrate that FAK is not necessary for transformation but is necessary for tumor progression and metastasis. The underlying mechanism relates to FAK regulation of small GTPase, FA, and actin-associated signaling pathways linked to migration, metastasis, and the G₂, G₂/M portions of the cell cycle. FAK regulates these pathways at the level of posttranslational phosphorylation events as well as the level of transcriptional regulation via a conserved subset of transcription factors, including p53. Yet, the exact role of p53 in regulating G₂, G₂/M, and metastasis-associated genes in relation to FAK function remains to be elucidated. In a similar manner, much work is needed to understand the connection between FAK and the remaining TFs because their relationship is even less clear. Future studies to help elucidate the signaling network and its regulation of transcription in mammary carcinoma should provide great insight into how the cell's interaction with the microenvironment regulates cell behavior.

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