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Advances in Brief

Decreased Focal Adhesion Kinase Suppresses Papilloma Formation during Experimental Mouse Skin Carcinogenesis[1]

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Abstract

Although focal adhesion kinase (FAK) is elevated in epithelial cancers, it is not known whether FAK expression influences tumor development *in vivo*. We found that *fak* +/− heterozygous mice display reduced 7,12-dimethylbenz[a]anthracene-induced papilloma formation that correlates with reduced FAK protein expression in the skin. However, the frequency of malignant conversion of papillomas into carcinomas is indistinguishable in *fak* +/− mice and their wild-type *fak* +/+ littermates, most likely because papilloma FAK protein expression is elevated to wild-type levels. We also found that keratinocyte FAK protein expression is important for cellular responses downstream of ras *in vitro* (monitored by extracellular signal-regulated kinase activation after integrin engagement). Because 7,12-dimethylbenz[a]anthracene induces an activating mutation of H-*ras,* this provides one possible explanation for suppression of papilloma formation when FAK protein is limiting.

Introduction

Changes to the composition and function of cell-ECM[5] adhesions can alter the cell's capacity to attach and migrate through surrounding tissue. It is therefore likely that changes in expression and activities of the components of such adhesions could make an important contribution to cancer development (1). At the cellular level, focal adhesion components control various important aspects of oncogenic behavior, including anchorage-independent survival and growth (2), cytoskeletal disorganization (3), and altered adherence to ECM (4).

FAK is a nonreceptor tyrosine kinase, specifically located at cell-matrix adhesions, that acts at a point of convergence in a variety of signaling pathways known to influence cell adhesion and structure, motility, growth, survival, and differentiation (5). Elevated expression of FAK protein is also associated with a wide variety of human epithelial cancers. Work from our laboratory has demonstrated that FAK protein is elevated in cell lines derived from squamous cell carcinomas of the head and neck when these were compared with normal keratinocytes (6) and also during the malignant conversion of colonic adenoma cells to their carcinoma counterparts *in vitro* (7). Other studies have found similar elevated FAK protein expression in cervical carcinoma cell lines (8), prostatic carcinoma tumors (9), and colon and breast tumors and cell lines, where a correlation between FAK protein expression and the invasive potential of tumors has also been described (10). Despite this mounting circumstantial evidence supporting the link between FAK and the malignant phenotype, it has not been established whether FAK plays a direct role during tumorigenesis *in vivo*. In this study, we used a gene targeting approach to examine the effects of modulating the *in vivo* expression levels of FAK, in association with the well-characterized mouse skin carcinogenesis system (11). Specifically, we examined the effect of reduced FAK protein expression in the skin during tumor formation and progression *in vivo*. The mouse skin model of multistage carcinogenesis is well understood with respect to both the phenotypic and genetic changes that occur (12, 13). Because homozygous deletion of the *fak* gene is embryonically lethal (14), we used mice containing a heterozygous *fak* deletion. These mice exhibited a corresponding reduction in FAK protein expression in the epidermis and a significant reduction in the formation of benign skin tumors (papillomas).

Materials and Methods

Animals. Mice carrying a targeted mutation in the *fak* gene were generated by insertion of a neomycin cassette into the first exon of the FAK kinase domain. A 1161-bp cDNA fragment encoding murine *fak* nucleotides 550-1711 (15) was used as a probe to screen a murine 129Sv genomic library (kindly provided by H. Baribault, Burnham Institute, La Jolla, CA). A targeting vector was constructed using a 1.8-kb fragment containing the first exon of the kinase domain (nucleotides 1344–1441; Ref. 15), flanked by 2.1-kb (5′) and 1.8-kb (3′) homology arms. Insertion of the neomycin cassette results in gene disruption and causes a frameshift such that no functional mRNA is produced. The targeting vector was linearized and introduced into embryonic stem cells by electroporation. G418-resistant clones were isolated, and homologous recombination was verified by Southern blotting using internal and external probes. Targeted clones were injected into C57BL/6 blastocysts (The Burnham Institute Transgenic Facility), and resulting chimeric males crossed with C57BL/6 females to obtain germ-line transmission. All mice used were then crossed through four generations into an FVB inbred genetic background; control mice were homozygous for *wt fak* (*fak* +/+) and were littermates taken from the same breeding colony. Mice containing a heterozygous deletion in the *fak* gene were identified by PCR analysis of tail DNA using the following primers: F1 (5′-gcc att gaa caa gat gga ttg cac-3′) and B1 (5′-ttc ccg ctt cag tga caa cgt c-3′).

Chemical Carcinogenesis. Chemical carcinogenesis was performed on 40 8-week-old female mice. Animals were split into two groups, one containing 20 (+/−) *fak* mice and the other containing 20 (+/+) *fak* control animals. Both groups were treated in an identical manner throughout. Tumors were induced *in vivo* by shaving the dorsal skins of the mice, and 2 days later, topically applying a single dose of 25 μg of DMBA in 150-μl acetone. Mice were then treated topically twice a week for 20 weeks with 6.25 μg of TPA in 150-μl acetone. The number of benign and malignant tumors was recorded weekly for 45 weeks after DMBA treatment. Benign tumor numbers did not increase after 24 weeks attributable to the discontinuation of TPA treatment. Tumor tissue was snap frozen in liquid nitrogen and/or fixed overnight in

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[5] The abbreviations used are: ECM, extra cellular matrix; FAK, focal adhesion kinase; *wt,* wild-type; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; mAb, monoclonal antibody; ERK, extracellular signal-regulated kinase.

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phosphate buffered formalin, followed by paraffin embedding. Tumors were scored as papillomas or carcinomas by morphological appearance on collection, followed by histological confirmation after H&E staining of paraffin sections. All animal experiments were performed in accordance with the United Kingdom Animal Scientific Procedures Act (1986).

Generation of Murine Keratinocytes. Primary murine keratinocytes were obtained from dorsal skin sections of 2-day-old mice essentially as described (16). Cells were plated in keratinocyte growth medium [modified MCDB 153 medium (Clonetics, San Diego, CA) containing the following supplements: 0.4% bovine pituitary extract, 10 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 50 ng/ml amphotericin-B, and 50 μg/ml gentamicin]. $CaCl_2$ solution was added to give a final Ca^{2+} concentration of 0.05 mM, and cells were maintained in a humid 37°C/5% CO_2 incubator. Primary cultures were trypsinized, and the trypsin was inactivated in medium containing 20% FCS before plating on fibronectin. For fibronectin plating experiments, dishes were coated by preincubating overnight at 4°C in a solution of 10 μg/ml fibronectin (Stratatec) in PBS before being used for cell plating.

Protein Analysis. For protein analysis, dishes of cells were washed in ice-cold PBS and either used immediately or flash frozen at −70°C. Cell monolayers were lysed in radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EGTA, 0.1% SDS, 1% NP-40, and 1% sodium deoxycholate] with the inclusion of inhibitors (500 μM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 100 μM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml benzamidine, and 10 μg/ml aprotinin; Sigma Chemical Co.). Samples were sonicated and clarified by centrifugation at 21,000 × *g* at 4°C. Alternatively, frozen tumor samples were dissected into small particles before being suspended in radioimmunoprecipitation assay buffer and treated as above. Protein concentrations were measured by Micro bicinchoninic acid protein assay kit (Pierce), and samples were then suspended in sample buffer [50 mM Tris-HCl (pH 6.7), 2% SDS, 700 mM β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue] at 100°C for 2 min followed by analysis by SDS-PAGE. For Western blotting, proteins were transferred to nitrocellulose using a semidry blotting apparatus. Unoccupied binding sites were blocked with 5% dried milk powder in PBS + 0.1% Tween 20, followed by probing with either FAK mAb at 0.5 μg/ml (clone 77; Transduction Laboratories), anti-ERK (1:10,000; Sigma Chemical Co.), or antiphospho-ERK (Promega). Detection was by incubation with horseradish peroxidase-conjugated secondary antibody (New England Biolabs), and visualization was by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Results

Papilloma Formation Is Suppressed in *fak* +/− Mice. Because circumstantial evidence links FAK protein expression with development of malignancy, we addressed whether variation in FAK expression levels *in vivo* would affect tumorigenesis. We first tested whether ablation of one copy of *fak* resulted in a similar reduction in FAK protein expression by comparing epidermal extracts from both *fak* +/+ and *fak* +/− mice using immunoblotting. Dorsal skin samples were collected, and epidermal proteins were prepared and pooled from four mice before Western blotting using an anti-FAK mAb as probe. As expected, and confirming previous reports (17), FAK protein expression was reduced in the epidermis of *fak* +/− mice, when compared with expression levels in the *fak* +/+ littermate controls (Fig. 1*A, top panel*). Comparison with another focal adhesion component that associates with FAK, namely c-Src, showed no detectable difference in expression between the *fak* +/− and *fak* +/+ mice (Fig. 1*A, bottom panel*).

fak +/− mice and *wt* littermates were treated with a single dose of DMBA followed by twice weekly application of TPA for 20 weeks. After the instigation of treatment, an early difference was observed in the benign tumor latency, with visible papillomas appearing in the *fak* +/+ mice at 5 weeks. In contrast, the *fak* +/− mice showed no visible tumor production until week 7 (Fig. 1*B*). In addition, 100% of mice in the control group had developed papillomas by week 11, whereas there was a delay of 5 weeks (until week 16) before 100% of the *fak* +/− mice also produced papillomas (Fig. 1*B*). More significantly, there was also a substantial and consistent reduction in the papilloma number per mouse in the *fak* +/− group, compared with the control *fak* +/+ group throughout the first 24 weeks of observation (Fig. 1*C*). The difference in papilloma numbers was evident as early as week 8, with the papilloma number per mouse at 18 weeks in the *fak* +/− mice showing a 46% reduction compared with the *wt* control group ($P < 0.0001$, by the Bonferroni two-sample *t* test; Fig. 1*C*). It is likely that this is a minimal estimate of suppression of papilloma formation because mice with high benign tumor loads were removed prematurely from the study.

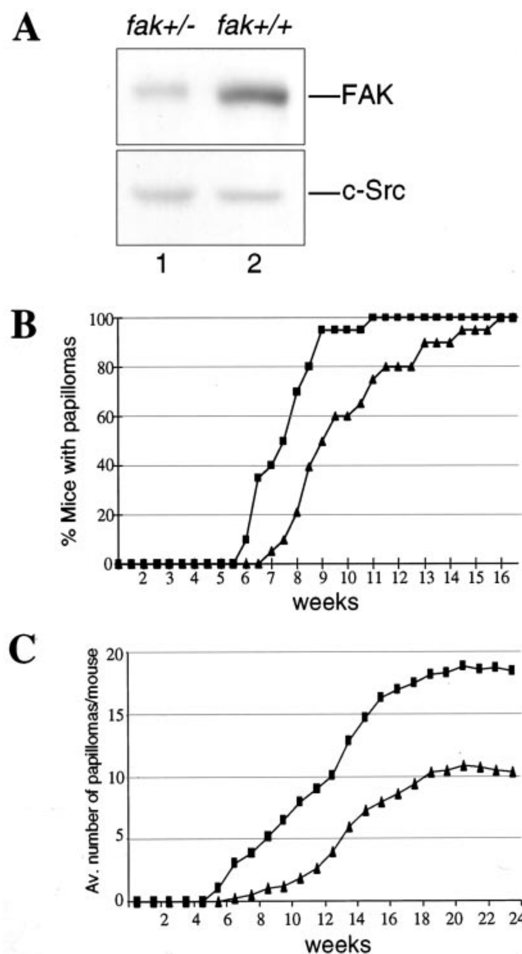

Fig. 1. *A,* FAK protein expression in mouse epidermis. FAK protein expression was detected by Western blotting protein samples obtained from pooled dorsal skin sections, from either *fak* +/− mice (*Lane 1*) or *fak* +/+ mice (*Lane 2*). Proteins (50 μg) were transferred to nitrocellulose and either reacted with FAK-specific mAb, clone 77 (*top panel*), or anti-c-Src antibody 327 (*bottom panel*). Two groups of 20 FVB female mice, 20 × *fak* +/+ and 20 × *fak* +/−, were treated with a single initiating dose of 25 μg of DMBA in 150-μl acetone followed by biweekly topical treatments with 6.25 μg of TPA in 150-μl acetone applied to the dorsal skin. Papilloma numbers were recorded weekly and are expressed as either percentage mice containing at least one papilloma (*Y* axis; *B*) or average tumor number per mouse (*Y* axis; *C*) *versus* weeks post-DMBA treatment (*X* axis); *fak* +/+ = ■; *fak* +/− = ▲.

FAK Heterozygote Mice Show No Difference in Rate of Conversion to Malignant Carcinoma. During chemical carcinogenesis, benign papillomas usually show an ~5% conversion rate to malignant squamous cell carcinomas (18). Mice were observed with regard to progression of papillomas to carcinomas on a weekly basis. Visual diagnosis was confirmed by histological examination of tumor sections (data not shown). Carcinomas were first observed on the *fak* +/+ mice at week 18; there was a delay period of 4 weeks until the first carcinoma was recorded in the *fak* +/− group at 22 weeks.

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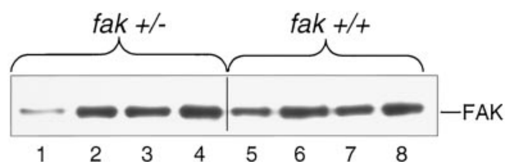

Fig. 2. FAK protein expression is indistinguishable in papillomas derived from *fak* +/− and *fak* +/+ mice. FAK protein expression in benign papillomas was detected by Western blotting. Proteins were extracted from either pooled dorsal skin sections (*Lanes 1* and *5*) or papillomas from six individual mice, specifically from either *fak* +/− (*Lanes 2–4*) or *fak* +/+ mice (*Lanes 6–8*). Proteins (50 μg) were transferred to nitrocellulose and probed with an anti-FAK mAb (clone 77; Transduction Laboratories).

However, despite the difference in papilloma number between the *fak* +/+ and *fak* +/− mice, there was no significant observed difference in the malignant conversion frequency between the two groups. In *fak* +/+, mice gave a conversion frequency of 3.7%, whereas the frequency for the *fak* +/− mice was 4.8% (Table, 1). Therefore, although reduced FAK protein expression levels in the *fak* +/− mice led to a marked reduction in benign papilloma formation, the papillomas that did develop were equally as likely to undergo malignant conversion to carcinomas in the heterozygous mice. There are two possible reasons for this finding. Either reduced FAK protein expression in the *fak* +/− mice did not influence malignant conversion, or FAK protein was up-regulated to compensate for the loss of one *fak* allele.

Papillomas from *fak* +/+ and *fak* +/− Mice Express Similar Amounts of FAK. To distinguish between the possibilities described above, we investigated levels of FAK protein expression in papillomas harvested from both groups of *fak* +/+ and *fak* +/− mice. Proteins were extracted from papillomas and tested for FAK expression by Western blotting using an anti-FAK-specific mAb as probe. No significant difference was evident between FAK protein expression in papillomas harvested from *fak* +/+ mice or *fak* +/− mice (Fig. 2, *Lanes 2–4* and *6–8*), with FAK protein being readily detectable in both and increased over that in normal epidermis. This is in contrast to the lower levels of FAK detected in normal skin of *fak* +/− mice in comparison with *wt* (Fig. 2, *Lanes 1* and *5*), demonstrating that FAK protein is increased in *fak* +/− mice during papilloma formation. Additionally, immunohistochemical analysis was carried out using paraffin-embedded tumor sections taken from *fak* +/+ and *fak* +/− mice, and FAK expression levels and patterns were indistinguishable, with strong superbasal expression observed in the epidermis from both groups of mice (data not shown). This further suggests that there may be selective pressure to raise FAK protein expression to enable papilloma formation.

Signaling Downstream of ras Is Suppressed in Keratinocytes Derived from *fak* +/− Mice. Our results show that reduced FAK protein expression can suppress benign tumor formation. However, it remains unclear how reduction in FAK protein expression might affect papilloma formation.

The mouse skin carcinogenesis protocol is well characterized with regard to the genetic changes that occur after chemical treatment. Tumor initiation by DMBA gives rise to an activating mutation of codon 61 of the H-*ras* gene (19, 20), implicating signaling downstream of ras in papilloma formation. Interestingly, there is considerable evidence suggesting that FAK is able to signal upstream of ras, specifically to the ras-ERK pathway by its ability to bind Grb2 via tyrosine-925 in its COOH terminus (21). To test whether signaling downstream of ras was influenced by reduced FAK protein expression in *fak* +/− mice, we derived primary keratinocyte cultures from the dorsal skin of both *fak* +/− and *fak* +/+ mice. Keratinocytes were plated onto fibronectin and protein extracts subjected to Western blotting using an antiphospho-ERK antibody (Fig. 3, *top panel*) or an anti-ERK antibody to measure total ERK protein (Fig. 3, *middle panel*) to determine whether signaling to ERK was compromised by the reduced FAK protein expression in the *fak* +/− keratinocytes (Fig. 3, *bottom panel*). Plating of keratinocytes from *fak* +/+ mice onto fibronectin caused a strong phosphorylation of ERK, clearly evident after 30 min and stronger after 60 min (Fig. 3, *Lanes 3* and *4*). In contrast, ERK phosphorylation and activation were suppressed and delayed in keratinocytes from *fak* +/−, with no enhanced phosphorylation evident after 30 min and only relatively weak phosphorylation evident after 60 min (Fig. 3, *Lanes 1* and *2*).

The attenuation of signaling to components of the pathway downstream of ras provides one possible explanation for the suppression of papilloma formation during chemically induced carcinogenesis, because activation of ras is a key step in tumor activation. This, together with the apparently elevated FAK protein expression in papillomas that do form, suggests that FAK protein expression must be maintained at a normal level to allow mutated H-*ras* to signal key changes within the cell that are required for tumor initiation.

Discussion

Much circumstantial evidence implicates FAK in the development of human malignancies. One theme linking many reports is the importance of raised FAK protein expression in cancer cells that correlates with invasive potential (22). Despite this, no experiments have been carried out to directly test how modulation of FAK protein *in vivo* affects tumor formation or progression. To address this, we have carried out chemical carcinogenesis experiments using mice that lack one copy of the *fak* gene. We have used skin carcinogenesis because it is well established in providing proof of the cause-effect relationship between carcinogen exposure, genetic alterations, and biological events leading to neoplasia (11).

We have used *fak* +/− mice to show that reduced FAK protein expression in the skin suppresses benign tumor formation (Fig. 1) but not malignant conversion of tumors that do form (Table 1). Specifically, there was a 46% reduction in papilloma formation after chemical treatment, when compared with the control group of mice that express *wt* levels of FAK (Fig. 1). Similar gene dosage effects, particularly of genes such as those encoding $p21^{WAF}$ and p53 that are implicated in cell growth control and survival, have been observed

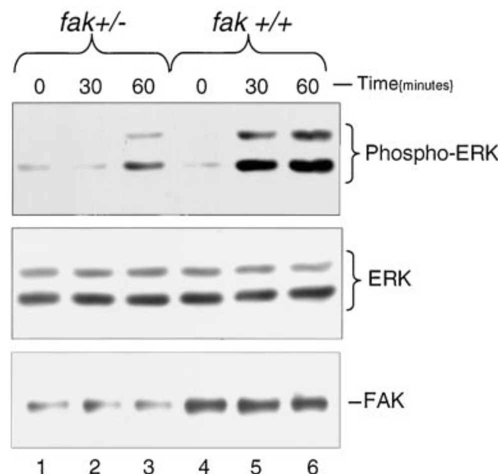

Fig. 3. Integrin-induced ERK activation is compromised in *fak* +/− keratinocytes. Keratinocytes were obtained from the skins of *fak* +/− (*Lanes 1–3*) mice or *fak* +/+ (*Lanes 4–6*). Cells were suspended by trypsinization and replated onto fibronectin-coated dishes for the times indicated. Proteins were extracted, transferred to nitrocellulose, and probed with either an antiphospho-ERK antibody at 2 μg/ml (*top panel*), an anti-ERK antibody at 1 μg/ml (*middle panel*), or an anti-FAK mAb (*bottom panel*).

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Table 1 *FAK heterozygous mice show no difference in malignant conversion frequency*

Mice from both *fak* +/− and *fak* +/+ groups were subjected to chemical carcinogenesis, and total papilloma and carcinoma numbers were recorded. Carcinoma numbers were scored by morphological appearance on collection followed by histological confirmation after H&E staining of paraffin.

Mouse line	No. of mice	First carcinoma appearance (weeks)	No. of carcinomas per no. of papillomas	Malignant conversion frequency
fak +/+	20	18	13/349	3.70%
fak +/−	20	22	10/210	4.80%

previously in heterozygous mice, with *p21*$^{\mathrm{WAF}}$ +/− inducing an increase in papilloma formation, whereas the *p53* +/− mice induced a decrease (13, 23).

A key event in the formation of DMBA-induced papillomas is the introduction of an activating mutation at codon 61 of the H-*ras* gene (13), implying that signaling downstream of ras is a major trigger of tumor initiation. Because we established that the decreased number of papillomas formed in *fak* +/− heterozygous mice also contained DMBA-induced H-*ras* mutations at a mutation frequency similar to *wt* of 90% (data not shown), we addressed whether signaling downstream of ras was normal in keratinocytes derived from *fak* +/− heterozygous mice. We found that integrin-dependent stimulation of ERK/mitogen-activated protein-kinase was compromised in *fak* +/− keratinocytes when compared with *fak* +/+ *wt* keratinocytes after the replating of suspended cells on fibronectin (Fig. 3). This indicates that FAK protein expression may be limiting for integrin-dependent activation of the ras/ERK pathway, at least in keratinocytes. FAK is believed to be upstream of ras in signaling between integrins and the ras/ERK pathway, specifically via integrin-induced phosphorylation of FAK on tyrosine-925 providing a binding site for the SH2 domain of Grb-2, which subsequently recruits mSOS, leading to ras activation (21). Interestingly, it has been shown that activation of ERK by an activated mutant ras is still adhesion dependent (24), providing a possible explanation for the ability of reduced FAK protein expression to influence signaling in the presence of the DMBA-induced activating H-*ras* mutation present in papillomas and a rationale for the suppression of papilloma formation under these circumstances.

A possible role for elevated FAK protein expression in tumor cells is in the acquisition of the invasive phenotype. Cancer cell invasion is a complex process that requires cells to attach and migrate through underlying ECM and into surrounding tissue. Because FAK functions as a key regulator of ECM-dependent cell migration (5, 14, 25, 26) and because endogenous levels of FAK protein expression can limit the rate of cell motility in some cell types (25), it seems likely that elevated FAK protein in tumor cells may release normal constraints on the rate of cell motility and, in turn, facilitate invasion. However, our observation that the papilloma to carcinoma conversion frequency was similar in both the *fak* +/− heterozygous and *fak* +/+ *wt* mice groups seemed to indicate that FAK protein expression was not an important determinant of tumor progression. This interpretation was complicated by the finding that FAK protein expression in papillomas from both *fak* +/− and *fak* +/+ mice was indistinguishable (Fig. 2), indicating that FAK protein expression was elevated during papilloma formation despite only a single operational *fak* allele in *fak* +/− mice. This additionally suggests that there is a selection pressure for elevating FAK protein during tumor initiation, perhaps explaining why there is no constraint on additional progression to carcinoma in the *fak* +/− mice. How and why elevated FAK protein is selected for during papilloma formation remains unclear. One possibility is that raised FAK protein provides an advantage with regard to promoting cell growth or survival after induction of the activating H-*ras* mutation and that the low level of FAK protein in *fak* +/− heterozygous mouse skin is incompatible with tumor cell growth, or survival, during chemically induced transformation. This is compatible with FAK's role in conferring the ability of transformed cells to grow in an anchorage-independent environment (2) and act as a survival factor in adherent cells (27, 28).

In conclusion, the results we present here provide the first direct evidence for a cause and effect relationship between the level of FAK protein expression tumor formation *in vivo*. Whether there is also an *in vivo* role for FAK during malignant conversion remains to be tested by use of conditional *fak* knockout strategies.

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Gordon W. McLean, Ken Brown, Margaret I. Arbuckle, et al.

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