Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression

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We have generated mice with a floxed fak allele under the control of keratin-14-driven Cre fused to a modified estrogen receptor (CreER\(^{T2}\)). 4-Hydroxy-tamoxifen treatment induced fak deletion in the epidermis, and suppressed chemically induced skin tumor formation. Loss of fak induced once benign tumors had formed inhibited malignant progression. Although fak deletion was associated with reduced migration of keratinocytes in vitro, we found no effect on wound re-epithelialization in vivo. However, increased keratinocyte cell death was observed after fak deletion in vitro and in vivo. Our work provides the first experimental proof implicating FAK in tumorigenesis, and this is associated with enhanced apoptosis.

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Cellular focal adhesions are complex structures consisting of many interacting proteins that provide both physical adhesion to extracellular matrix, usually via transmembrane integrin receptors, and that also transmit survival and/or growth signals into the cell interior. De-regulation of these adhesions may permit cancer cells to migrate and invade into surrounding tissue during the development of malignant disease, or to survive and grow under normally inappropriate conditions. One major component of integrin adhesions is focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that is pivotal in many signaling pathways (Illic et al. 1997). FAK regulates integrin-mediated adhesion and cell migration, and contributes to proliferation and cell survival (Schaller 2001; Hauck et al. 2002). Despite numerous reports that FAK expression correlates with the development of cancer (Owens et al. 1995, 1996; Agochiya et al. 1999; Jones et al. 2000; McLean et al. 2003), there is no direct evidence whether, and if so how, FAK contributes to cancer development.

Using fak heterozygous (+/−) mice, we previously demonstrated that reduced FAK expression had a negative impact on chemically induced papilloma formation (McLean et al. 2001). However, these benign tumors from fak+/− mice elevated expression of FAK to a level that was indistinguishable from papillomas from wild-type fak+/+ mice, preventing us from addressing the key question of whether FAK expression is causally involved in malignant progression. Here, we address whether FAK plays a causal role in development of the malignant phenotype in vivo, by generating mice in which we had both spatial and temporal control over fak deletion. Specifically, we used gene targeting in embryonic stem (ES) cells to generate mice that are homozygous for a floxed fak allele, and that also express a 4-hydroxy-tamoxifen (4-OHT)-regulated Cre recombinase (Cre-ER\(^{T2}\)) expressed under control of the keratin-14 promoter (K14 CRE-ER\(^{T2}\)/FAK\(^{lox/lox}\)). We could delete fak from the epidermis of these mice upon addition of 4-OHT, but more importantly from benign papilloma tumors once these had developed. Our results provide the first evidence that FAK modulates the efficiency of benign tumor formation and plays a crucial role in malignant conversion.

Results and Discussion

To investigate the role of FAK during the formation and development of skin tumors, we used Cre/loxP technology to target the introduction of conditional mutations into the fak gene in mice. As the fak coding exons are spread over >225 kb, it was not practical to flox the entire fak gene; instead, loxP sites were introduced into the fak gene at positions flanking the exon encoding part of the kinase domain, that is, FAK amino acids 413–444. [The gene-targeting strategy and generation of FAK floxed mice are described in detail in the Supplemental Material.] The targeting vector was designed so that Cre-mediated recombination also introduced a frameshift mutation in the adjacent exon, precluding production of a functional FAK protein. Screening of Cre-expressing ES cells by immunoblotting confirmed that no FAK protein was detectable (Fig. 1A). Using N-terminal antibodies to FAK, no truncated products were visualized [data not shown]. To obtain epidermal-specific fak excision, we crossed homozygous FAK\(^{lox/lox}\) mice with mice expressing a modified estrogen receptor-Cre fusion protein (CreER\(^{T2}\)) [Indra et al. 1999], under the control of the keratin-14 (K14) promoter. This directs 4-OHT-induced excision of floxed fak to epidermal keratinocytes of mouse skin [Indra et al. 1999; Li et al. 2000]. The resulting K14 CreER\(^{T2}/\)FAK\(^{lox/lox}\) mice were viable and dis-
played skin-specific suppression of FAK expression following administration of 4-OHT, while other tissues, such as kidney and spleen, remained unaffected [Fig. 1B]. Although some FAK was also detected in immunobots of epidermal extracts from 4-OHT-treated animals [Fig. 1B, lane 6], this is most likely due to FAK expression in the underlying dermis that contaminates the epidermal preparations, or to the presence of a small number of keratinocytes that had not undergone FAK deletion due to loss of K14 expression through differentiation [Stoler et al. 1988]. Immunohistochemistry revealed reduced FAK expression in both the epidermis and hair follicles of K14CreERT2/FAKlox/lox mice, when compared with underlying dermis [Fig. 1C]. This temporally controlled deletion of fak allowed separation of effects on benign tumor formation from those on malignant conversion.

Skin tumors were induced in 4-OHT-treated and untreated K14CreERT2/FAKlox/lox mice by a two-stage chemical carcinogenesis protocol [Quintanilla et al. 1986; Kemp et al. 1993; Yuspa et al. 1995], as well as in K14CreERT2 and 4-OHT-treated FAKlox/lox mice to rule out possible effects of Cre expression or 4-OHT treatment, respectively. Treatment with the carcinogen 7,12-dimethylbenzanthracene (DMBA) gives rise to activating mutation of the c-Ha-Ras gene [Quintanilla et al. 1986; Pelling et al. 1987], and subsequent treatment for 20 wk with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate [TPA] leads to formation of benign papillomas, a proportion of which progress to form invasive squamous cell carcinomas [SCC] [Burns et al. 1978]. We observed a marked difference in papilloma formation between 4-OHT-treated and untreated K14CreERT2/FAKlox/lox mice. Only 50% of 4-OHT-treated mice formed papillomas after 12 wk, as compared to 100% of untreated mice [Fig. 1D]. More significantly, there was also a substantial reduction in the average number of papillomas formed per 4-OHT-treated K14CreERT2/FAKlox/lox mouse at 22 wk when compared to the untreated control group [Fig. 1E]. Additionally, no difference in benign tumor acquisition was observed in either the K14CreERT2- or the 4-OHT-treated FAKlox/lox mice ruling out any possible effects of Cre expression or 4-OHT treatment, respectively [data not shown]. This indicates that FAK plays a modulatory role in chemically induced, Ras-dependent papilloma formation, with half of the 4-OHT-treated K14CreERT2/FAKlox/lox mice being resistant to DMBA/TPA-induced benign tumor formation. We have reported previously that keratinocytes derived from fak+/– heterozygous mice display impaired signaling to Ras effector pathways [McLean et al. 2001], such as ERK/MAP kinase. Taken with our current findings, the data indicate that FAK is necessary not only for the biochemical effects of Ras in cells in culture, but also for optimal tumor initiation/promoting biological effects of oncogenic Ras in vivo.

Experiments using immortalized fak–/– mouse embryo fibroblasts have implicated FAK in migration and invasion [Ilic et al. 1995], while FAK expression in tumors correlates with invasive potential [Owens et al. 1995]. However, owing to the embryonic lethality of constitutive fak deletion [Ilic et al. 1995], no experiments have been done to test whether FAK has a causal role in the development of malignancy per se. We therefore deleted fak directly from preformed benign papillomas and examined subsequent progression to invasive SCC tumors. K14CreERT2+/Faklox/lox mice were subjected to DMBA and TPA, and subsequently treated with 4-OHT after the majority of papillomas had formed [at 15 wk as indicated in Fig. 2A]. Immunohistochemistry confirmed fak gene excision after 4-OHT treatment, as judged by loss of specific FAK staining in hyperproliferative epidermal regions of papilloma sections [Fig. 2B, right panel]. As judged by visual identification and subsequent histological confirmation of SCC, 4.1% of papillomas
Underwent conversion to carcinoma in untreated mice, whereas malignant conversion of papillomas in 4-OHT-treated K14CreERT2/FAK^lox/lox mice was reduced to 0.6% [Fig. 2C]. An observed conversion frequency of 3.9% in 4-OHT-treated K4CreERT2/FAK^lox/lox mice excluded the possibility of this observation being linked to administration of 4-OHT [Fig. 2C]. The 0.6% conversion frequency represented two SCCs that developed in 4-OHT-treated K14CreERT2/FAK^lox/lox mice (lanes 1 and 2) and 4-OHT-treated K4CreERT2/FAK^lox/lox mice [3]. Conversion frequency is determined by calculating the number of carcinomas recorded per total number of papillomas for each study group [D]. Anti-FAK immunoblot of proteins extracted from SCCs derived from untreated (lanes 1–6) or 4-OHT-treated (lanes 7,8) K14CreERT2/FAK^lox/lox mice.

Figure 2. Deletion of fak from papillomas blocks malignant progression to SCC. [A] Mice either K14CreERT2/FAK^lox/lox [left panel] or FAK^lox/lox (right panel) were treated with vehicle alone or with 4-OHT (+4-OHT) at 15 wk after the majority of papillomas had formed. [B] Immunohistochemical anti-FAK staining of paraffin-embedded sections of papillomas from K14CreERT2/FAK^lox/lox mice treated with vehicle alone (left panel) and with 4-OHT (right panel). [C] Papilloma to carcinoma conversion frequency of 4-OHT-treated and untreated K14CreERT2/FAK^lox/lox mice (1 and 2) and 4-OHT-treated FAK^lox/lox mice (3). Conversion frequency is determined by calculating the number of carcinomas recorded per total number of papillomas for each study group. [D] Anti-FAK immunoblot of proteins extracted from SCCs derived from untreated (lanes 1–6) or 4-OHT-treated (lanes 7,8) K14CreERT2/FAK^lox/lox mice.

Our data thus provide the first direct experimental evidence that FAK causally contributes to the development of malignancy in vivo, specifically during the benign papilloma to SCC transition. Furthermore, this is associated with a requirement for FAK to maintain a normal rate of cell migration, and survival signaling in keratinocytes in vitro. To determine which, if either, of these effects is likely to be responsible for the observed suppression of tumorigenesis upon FAK deletion, we carried out punch biopsy wound repair assays and analyzed caspase-3 activation in 4-OHT-treated K14CreERT2/FAK^lox/lox skin to monitor in vivo migration and apoptosis, respectively. K14CreERT2/FAK^lox/lox mice [either treated or untreated with 4-OHT] were wounded with 3-mm punch biopsies [Fig. 4A, day 0, upper panels], and subsequent wound closure was monitored. No visible difference in vivo wound repair was observed—as judged at 7 d after wounding—when re-epithelialization was essentially complete in both cases [Fig. 4A, day 7, lower panels]. Earlier time points at days 1 and 3 did not indicate any lag in re-epithelialization of the wounded skin [data not shown]. In contrast, we observed a difference in staining of activated caspase-3 both in the skin and in papillomas.
from treated, but not untreated, mice [Fig. 4B]. Activated caspase-3 is accepted as a key marker of apoptotic cells and is now widely considered to be a more reliable indicator of apoptosis in tissue sections than TUNEL staining (Marshman et al. 2001; Duan et al. 2003). In particular, we observed the strongest staining in cells of the hair follicles of 4-OHT-treated mice, where the majority of target cells for DMBA-induced tumorigenesis are thought to reside (Fig. 4B, middle panels; Argyris 1980). Moreover, FAK deletion from preformed benign papillomas caused stronger staining of active caspase-3 [Fig. 4B, lower panels]. Thus, it is FAK’s role in promoting cell survival that is tightly linked to tumor formation and progression in this mouse.

This, together with the increased expression of FAK during acquisition of malignancy [Owens et al. 1995; McLean et al. 2003], is in keeping with recent observations that FAK can promote invasion in vitro by regulating production of matrix metalloproteinases [Shibata et al. 1998; Hauck et al. 2002]. We have now causally implicated FAK as a determinant of malignant behavior in vivo, identifying FAK as an excellent candidate for further study as a potential target to suppress spread of the disease.

Materials and methods

Animals and gene targeting

Design, construction, and generation of mice containing targeted loxP sites in the FAK gene are described in detail in the Supplemental Material. Transgenic mice expressing the modified Cre recombinase–estrogen receptor fusion under control of the keratin-14 promoter [K14CreER<sup>T2</sup>], which targets Cre expression to epidermal keratinocytes, have been described [Li et al. 2000]. To facilitate cell-type-specific FAK ablation, FAK<sup>lox/lox</sup> mice were then mated to K14Cre<sup>ERT2</sup> transgenic mice. The resulting offspring carrying K14Cre<sup>ERT2</sup> that were either homozygous for the floxed FAK gene [K14CreER<sup>T2</sup>/FAK<sup>lox/lox</sup>], carried K14Cre<sup>ERT2</sup> and two copies of the wild-type FAK allele [K14CreER<sup>T2</sup>/FAK<sup>+/-</sup>] or the...
FAK floxed allele alone (FAK flox/flox), were identified by PCR analysis and used for subsequent experiments. All animals used were crossed onto and maintained on an FVB genetic background.

PCR genotyping
Mice that were homozygous for the floxed fak gene were routinely identified by PCR analysis. Tail DNA was prepared by standard protocols and subjected to PCR analysis using the following primers: FAK30, 5'-AGAAGGTATGGAATAGAAG-3' and FAK54, 5'-GTCTGGTGTCTCTGATAAGGTTGGC-3', and the following FAK-specific amplification protocol: 95°C/30 sec [1 cycle]; 94°C/10 sec + 57°C/30 sec + 68°C/3 min [10 cycles]; 94°C/10 sec + 57°C/30 sec + 68°C/3 min [10 sec/cycle] (20 cycles); 68°C/7 min [1 cycle]. Following amplification, PCR DNA products were digested overnight with HindIII and analyzed by agarose gel electrophoresis. Mice that were homozygous for the floxed fak mutation (FAK flox/flox) exhibited a single major band of 1.9 kb, mice that were heterozygous for floxed FAK (FAK flox/wt) contained two major bands at 1.9 and 1.4 kb, and wild-type [wt] mice [FAK wt/wt] contained a single band at 1.4 kb [see Supplemental Material]. Mice that additionally contained the K14CreERT2 transgene were again identified by PCR analysis using the following primers: 5'-TATCCCTTCATTACCGGTCT-3' and 5'-ATCAACGTGTTTCTTGTCCG-3', and standard amplification protocols. Cre-positive mice exhibited a single PCR product of 350 bp.

Preparation and administration of 4-OHT
Preparation and administration of 4-OHT was as described [Indra et al. 1999] except that a reduced dose of 200 µg in 100 µL of sunflower oil was allowed to recover for 10 d before wounding. All mice were anesthetized with halothane, and the dorsum was shaved and cleaned with 70% alcohol. Three-millimeter full-thickness cutaneous punch-biopsy wounds were made maintaining the underlying fascia. The wounded tissue was collected at 0 and 7 d post-injury, bisected, and either fixed in 10% formalin at room temperature for 24 h, or in acid alcohol (96% ethanol/1% acetic acid) at 4°C for paraffin embedding, or snap-frozen in OCT (Thermo Lifesciences) and stored at -80°C for cryo-sectioning.

Chemical carcinogenesis
Chemical carcinogenesis using DMBA and TPA was performed on individual study groups of 20 female 8-wk-old animals as previously described [McLean et al. 2001]. The number of benign and malignant tumors was recorded weekly for 45 wk after DMBA treatment. Benign tumor numbers did not increase after 24 wk. Upon collection, tumor tissue was either flash frozen in liquid nitrogen or fixed overnight in 10% acetic acid at 4°C for paraffin embedding, or snap-frozen in OCT (Thermo Lifesciences) and stored at -80°C for cryo-sectioning.

Time-lapse microscopy
K14CreERT2/FAK flox/flox keratinocytes were harvested as described and plated onto collagen-I-coated six-well tissue culture dishes (Becton Dickinson). Following 4-OHT or vehicle alone treatment, cells were monitored using time-lapse video microscopy with images captured from each well every 20 min over a 10-h period. Quantification of cell motility was achieved by analysis of captured images using cell tracking software (Keletic Imaging Ltd).

Confluent immunofluorescence microscopy
K14CreERT2/FAK flox/flox keratinocytes were grown on glass coverslips, fixed at room temperature for 10 min with 3.7% formaldehyde/PBS, and permealized with 0.5% Triton X-100 in PBS and incubated with anti-FAK mAb (Transduction Laboratories). Antibody detection was via fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson), for 45 min at room temperature. Fluorescence was visualized using a Bio-Rad MRC 600 confocal microscope.

FACS analysis
K14CreERT2/FAK flox/flox keratinocytes were either treated with 4-OHT or vehicle alone, washed 1× in PBS, and fixed in 70% ethanol for 3 h at 4°C. Fixed cells were pelleted at 1000 rpm for 5 min, washed 1× in PBS, and resuspended in propidium iodide solution (1 mg/mL). Labeled cell populations were analyzed by fluorescence activated cell sorting (FACS) analysis using a Becton Dickinson CellQuest FACSscan.

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