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

Gordon W. McLean,1,8 Noboru H. Komiyama,3,4 Bryan Serrels,1 Hidefumi Asano,4,7 Louise Reynolds,5 Francesco Conti,5 Kairabaan Hodivala-Dilke,5 Daniel Metzger,6 Pierre Chambon,6 Seth G.N. Grant,3,4 and Margaret C. Frame1,2

1The Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow, G61 1BD, United Kingdom; 2Institute of Biological and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom; 3Wellcome Trust Sanger Institute, Cambridge, CB10 1XA, United Kingdom; 4Institute of Neuroscience, University of Edinburgh, Edinburgh EH9 3JZ, United Kingdom; 5The London Queen Mary’s School of Medicine and Dentistry, John Vane Science Centre, London, EC1M 6BQ, United Kingdom; 6Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, 67404 Illkirch Cedex, Strasbourg, France

We have generated mice with a floxed fak allele under the control of keratin-14-driven Cre fused to a modified estrogen receptor (CreERT2). 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.

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 FAKfloxllox mice with mice expressing a modified estrogen receptor–Cre fusion protein (CreER<sup>T2</sup>) [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 K14CreER<sup>T2</sup>/FAK<sup>flox/flox</sup> mice were viable and dis-

Keywords: Focal adhesion kinase; conditional; cancer; keratinocyte; apoptosis

7Present address: Chiba-City Kaihin Hospital, 3-31-1 Isobe, Mihama-Ku, Chiba City, Japan.
8Corresponding author.
E-MAIL g.mclean@beatson.gla.ac.uk; FAX 44-141-942-6521.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.316304.
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 immunoblots 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 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 initiating/promoting biological effects of oncopgenic 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 pre-formed 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

Figure 1. Deletion of FAK from mouse skin blocks papilloma formation. [A] fak excision in ES cells following introduction of Cre recombinase. Cre was introduced by electroporation of a pMC1-Cre plasmid, and protein extracts were harvested and subjected to Western blotting with an FAK mAb. [Lanes 1–3] No Cre recombinase. [Lanes 4–6] Cre recombinase. [Lanes 4–6] Cre recombinase. [Lanes 4–6] Cre recombinase. [Lanes 4–6] Cre recombinase. [B] Proteins were harvested from spleen, kidney, and skin of K14CreERT2/FAKlox/lox mice either treated with 4-OHT or with vehicle alone. Extracts were blotted onto nitrocellulose and probed with an anti-FAK mAb [top panel] or an anti-tubulin mAb [lower panel]. [C] Immunohistochemical staining of paraffin-embedded skin sections from K14CreERT2/FAKlox/lox mice treated with either 4-OHT [right panel] or vehicle alone [left panel]. [E] Epidermis, HF hair follicles. Deletion of fak in epidermal keratinocytes in vivo inhibits papilloma formation during chemical carcinogenesis. Mice were either treated with 4-OHT (●) or vehicle alone (□) and subjected to DMBA and TPA treatment as described. Graphs indicate percent of mice acquiring papillomas over time [D] and average numbers of papillomas recorded per mouse over treatment time [E]. Graphs represent K14CreERT2/FAKlox/lox mice.
The normal epithelial cells that are the targets for tumorigenesis in the DMBA/TPA skin carcinogenesis model. This provides important advantages over the commonly used \( \text{fak}^{-/-} \) cells (Illic et al. 1995), not only because the latter are mesenchymal but also because they are deficient in p53 that might influence their behavior (Illic et al. 1995). Peripheral FAK staining was visible in untreated keratinocytes, presumably at integrin adhesion sites that represent keratinocyte focal adhesions, whereas 4-OHT treatment caused loss of periphera FAK staining in the majority of cells [Fig. 3A]. The small number of cells that continue to express FAK may be due to suppression of K14 expression that may occur as keratinocytes differentiate [Fig. 3A, right panel, arrow; Stoler et al. 1988]. We first carried out wound repair migration assays, and found that 4-OHT-treated K14CreERT2/FAKlox/lox keratinocytes were unable to repopulate the denuded areas of wounded monolayers when compared to control cultures [Fig. 3B]. To address whether this could be explained by defective cell migration, we tracked individual cells using time-lapse microscopy and found that \( \text{fak}^{-/-} \) keratinocytes displayed reduced migration rates [by \( \sim 50\% \)], but they were still visibly motile [Fig. 3C]. However, we noticed that there were fewer cells present even in the confluent regions at the wound edges after 48 h [Fig. 3B, arrows], suggesting that failure to repair the wound in vitro may be associated with detachment and/or cell death. We collected adherent and detached cells following treatment with 4-OHT and determined the proportion of cells with sub-2n DNA content by FACS analysis. We found that loss of FAK was associated with loss of normal cell cycle profiles and a substantial amount of cell death when compared with untreated primary keratinocytes [Fig. 3D,E].

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/FAKlox/lox skin to monitor in vivo migration and apoptosis, respectively. K14CreERT2/FAKlox/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.

Figure 3. Deletion of FAK from keratinocytes causes reduced migration and increased cell death. (A) Confocal images show anti-FAK staining of peripheral structures in untreated (−4-OHT) or treated (+4-OHT) keratinocytes derived from K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice. Arrows indicate FAK staining in a minority of treated keratinocytes. Images were taken following 48 h of 4-OHT treatment. (B) Phase-contrast images show in vitro wound-repair assays of untreated (−4-OHT) or treated (+4-OHT) keratinocytes derived from K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice at 0 h and 48 h after the wound was made in the monolayer. Arrows indicate areas of the monolayer at the edges of the wound after 48 h. (C) Migration rates of keratinocytes derived from FAK<sup>flox/flox</sup> mice (1) and K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice (2), treated with 4-OHT for 48 h, were determined by time-lapse cell tracking and migration rate software analysis expressed as average speed in microns per second. (D,E) Cell death of untreated (−4-OHT) or treated (+4-OHT) keratinocytes derived from K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice was judged by the percentage of cells with sub-2n DNA content by FACS analysis; 24 h and 48 h refers to the time of sample collection after wounding.

Figure 4. (A) Sections of wounded skin from 4-OHT-treated (+4-OHT) or untreated (−4-OHT) K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice were stained with H&E to examine re-epithelialization at day 0 (upper panels) and day 7 (lower panels) post-injury. Arrows indicate the leading edges of migrating epidermis. (B) Immunohistochemical anti-activated caspase-3 staining of sections of either 4-OHT-treated (+4-OHT) or untreated (−4-OHT) skin (upper and middle panels) or papillomas (lower panels) from K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup> mice. Images taken at either low (upper panels) or high (middle and lower panels) magnification. [HF] Hair follicles.

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 [K14CreERT<sup>2</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 K14CreERT<sup>2</sup> transgenic mice. The resulting offspring carrying K14CreERT<sup>2</sup> that were either homozygous for the floxed FAK gene [K14CreERT<sup>2</sup>/FAK<sup>flox/flox</sup>], carried K14CreERT<sup>2</sup> and two copies of the wild-type FAK allele [K14CreERT<sup>2</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 were used across 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’-AGAAGCTTATGGAATGAG-3’ and FAK54, 5’-GTCCTGTTGTCTCGAATAGTGGTT-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 + 20 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 wt/flox) 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 identified by PCR analysis using the following primers: 5’-ATTGCCCTCTGATTACCCGTCT-3’ and 5’-ATCAACGTTTCTTCTTCTCGG-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 administered. 4-OHT was administered to 8-wk-old female FVB mice as described [Indra et al. 1999], with animals being left for 8–10 d before the initiation of any further experimental procedures. 4-OHT for in vitro experiments was dissolved in ethanol, stored at -20 °C, and diluted before use to a final working concentration of 10 nM.

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 at 0 and 48 h after 4-OHT removal. Tumors were scored as either papillomas or carcinomas by morphological appearance on collection, followed by histological examination. Tumors were collected at 0 and 7 d post-injury, bisected, and either fixed in 10% phosphate-buffered formalin prior to paraffin embedding and histological examination or maintained the underlying fascia. The wounded tissue was subjected to PCR analysis using the following primers: 5’-ATCAACGTTTCTTCTTCTCGG-3’, and standard amplification protocols. Cre-positive mice exhibited a single PCR product of 165 bp.

Protein analysis
Protein extracts from tissue culture cells or frozen tissue samples were prepared and blotted onto nitrocellulose as previously described [McLean et al. 2001]. Membranes were probed with either FAK mAb at 0.5 µg/mL (clone 77, Transduction Laboratories), or anti-tubulin mAb (Calbiochem). Detection was by incubation with horseradish peroxidase-conjugated secondary antibody [New England Biolabs], and visualization was enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Immunohistochemistry
Rabbit polyclonal antibodies against either FAK (Sigma) or activated caspase-3 (Cell Signalling) were used to stain Formalin Fixed Paraffin Embedded (FFPE) tissue section using a two-step immunohistochemical technique. Two-micron-thick FFPE tissue sections mounted onto Superfrost Plus slides [Menzel-Glazer] were dewaxed in HistoClear solution [National Diagnostics] followed by stepted rehydration via a series of graded alcohols to water. A negative control was compared in parallel to the investigated sections by omitting the primary antibody step. Antibody was detected in 0.1 g of bovine serum albumin [BDHI], 0.01 g of Sodium Azide [BDHI] in 0.01 M Tris-buffered saline (pH 7.5) and incubated for 2 h at room temperature, followed by visualization with a Cytomation EnVision kit [DAKO] as per manufacturers’ instructions. All staining was performed using a Sequenza (Thermoshandon) semiautomated staining facility. Resulting sections were analyzed and images were captured digitally using a Zeiss Axioplan 2 microscope and Axiovision software version 3.1.

Generation of murine keratinocytes
Primary murine keratinocytes were prepared and propagated from dorsal skin sections of 1- to 2-d-old mice as previously described [McLean et al. 2001]. Harvested cells were then plated onto collagen-I precoated tissue culture dishes [Rector Dickinson], and maintained in a humid 37 °C/3% CO2 incubator in keratinocyte growth medium [KGM; Clonetics]. For experiments involving fak deletion, keratinocytes were pretreated with either 4-OHT (10 nM) in ethanol or vehicle alone, for 48 h with one change of medium and drug at 24 h. This concentration of tamoxifen was identified by titration experiments to be a dose that induced loss of FAK without adverse effects on control cultures.

Wound healing assay
For in vitro wound healing experiments, K14CreERT2/FAKFlox/flox keratinocytes were grown to confluence in 60-mm collagen-I coated tissue culture dishes [Rector Dickinson] in KGM. Monolayers were wounded using a micropipette tip, and the cells were rinsed with KGM before visualization using a phase-contrast microscope. Cells were treated with either 10 nM 4-OHT or vehicle alone for 48 h before wounding. Wound closure was recorded at 0 and 48 h after 4-OHT removal.

For in vivo wound healing experiments, K14CreERT2/FAKFlox/flox mice were injected intraperitoneally with 4-OHT as described. Mice were 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.

Time-lapse microscopy
K14CreERT2/FAKFlox/flox keratinocytes were harvested as described and plated onto collagen-I-coated six-well tissue culture dishes [Rector 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 (Keine Imaging Ltd).

Confocal immunofluorescence microscopy
K14CreERT2/FAKFlox/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/FAKFlox/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 FACScan.

Acknowledgments
We thank Rebecca Porter for advice and John Wyke and Malcolm Hodgins for critical reading and comments. We also thank Colin Nixon for immunohistochemical analysis. This work was supported by Cancer Research UK at the Beatson Institute [G.W.M., B.S., M.C.F.] and by Collège de France, Association pour la Recherche sur le Cancer, and Foundation pour le Recherche Médicale (D.M. and P.C.).

References

3002 GENES & DEVELOPMENT

McLean et al.
Focal adhesion kinase and malignant progression


