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 1SA, United Kingdom; ⁴Division of Neuroscience, University of Edinburgh, Edinburgh, EH8 9JZ, 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.

Supplemental material is available at http://www.genesdev.org.

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 knock the entire fak gene; instead, loxP sites were introduced into the fak gene that allowed for inactivation of the gene by homologous recombination. 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 FAKlox/lox mice with mice expressing a modified estrogen receptor–Cre fusion protein (CreERT²) [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 K14CreERT²/FAKlox/lox mice were viable and dis-

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-ERT²) expressed under control of the keratin-14 promoter (K14CreERT²/FAKlox/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.

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Present address: Chiba-City Kaihin Hospital, 3-31-1 Isobe, Mihama-Ku, Chiba City, Japan.

Corresponding author.

E-MAIL g.mclean@beatson.gla.ac.uk; FAX 44-141-942-6521.

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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 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 initiating/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

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 pMCI-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. [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.

GENES & DEVELOPMENT 2999
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 fak−/− cells (Ilic et al. 1995), not only because the latter are mesenchymal but also because they are deficient in p53 that might influence their behavior (Ilic 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 peripheral 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/FAKflox/flox 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 fak−/− keratinocytes displayed reduced migration rates [by ~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/FAKflox/flox skin to monitor in vivo migration and apoptosis, respectively. K14CreERT2/FAKflox/flox 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(T2)], which targets Cre expression to epidermal keratinocytes, have been described [Li et al. 2000]. To facilitate cell-type-specific FAK ablation, FAK/loxP mice were then mated to K14CreER(T2) transgenic mice. The resulting offspring carrying K14CreER(T2) that were either homozygous for the floxed FAK gene [K14CreER(T2)/FAKlox/lox], carried K14CreER(T2) and two copies of the wild-type FAK allele [K14CreER(T2)/FAK+/-] or the
FAK floxed allele alone (FAKfloxflox), were identified by PCR analysis and used for subsequent experiments. All animals were used across 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'-AGAAGCTTATGAATAGAAG-3' and FAK54, 5'-GTCGTGTTGTTCTGAAATAGGTTG-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 (FAKfloxflox) exhibited a single major band of 1.9 kb, mice that were heterozygous for floxed FAK (FAKwt/flox) contained two major bands at 1.9 and 1.4 kb, and wild-type [wt] mice [FAKwt/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'-ATTGCCC-TGTCAATTACCGGTCT-3' and 5'-ATJCAAGGTTCATTCTTTCCG-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 used for subsequent experiments. All animals were used across onto and maintained on an FVB genetic background.

**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 phosphate-buffered formalin prior to paraffin embedding and histological examination. Tumors were scored as either papillomas or carcinomas by morphological appearance on collection, followed by histological confirmation after H&E staining of paraffin sections. All experiments were carried out in accordance with the United Kingdom Animal Scientific Procedures Act [1986].

**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 (Callbiochem). Detection was by incubation with horseradish peroxidase-conjugated secondary antibody [New England Biolabs], and visualization was by 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 using a two-step immunohistochemical technique. Two-micron-thick FFPE tissue sections mounted onto Superfrost slides [Menzel-Glazer] were dehydrated in HistoClear solution [National Diagnostics] followed by stepped 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 diluted 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 Axioskop 50 microscope and Axiosvision software version 3.1.

**References**


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