Adhesion signaling in mammary gland development, tumorgenesis and progression

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Chapter 3

FAK is involved in progression of spontaneous mammary gland tumors induced by conditional p53 deletion in the mammary gland

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Abstract

Alterations in the tumor suppressor gene p53 have been implicated in over 50% of human breast cancers. In addition to alterations in p53, focal adhesion kinase (FAK) expression is elevated in numerous cancers including colon-, cervix- and breast cancer. P53 binds to the *FAK* promoter to inhibit its transcription. Due to loss of wild type p53, an event that often occurs in tumors with p53 alterations, p53 is no longer able to inhibit the transcription of FAK, leading to increased FAK expression. In this study we used WapCre-mediated conditional deletion of both the *Fak* and *p53* gene to study the role of FAK in p53-induced spontaneous mammary gland formation. First onset of mammary gland tumors was at 44 weeks regardless of the FAK status. However, the number of mammary gland tumors was clearly reduced in *Fak* deleted mice. Interestingly, all tumors that arose in the Fak null animals showed reduced E-cadherin levels, suggesting the requirement for an additional molecular switch for mammary gland tumor formation in the absence of FAK. Altogether, we show that FAK is involved in $p53^{lox/lox}$-induced mammary gland tumor development.

Introduction

The tumor suppressor p53 has been implicated in diverse biological processes such as apoptosis, cell-cycle arrest, DNA repair, cellular differentiation and senescence; and has been shown to be altered in over 50% of spontaneous tumors in humans (reviewed in (1))(2, 3). In addition to spontaneous tumors, germ-line mutations in p53 are observed in Li-Fraumeni patients which predisposes them to a wide spectrum of early-onset cancers, including breast cancers (4, 5, 5, 6). Normally, p53 is expressed in a latent form and is maintained at low levels through targeted degradation. However, in response to DNA damage or stress, p53 is activated to function as a transcription factor, resulting in a cascade of events that eventually prevents tumor development. P53 is the most frequently mutated gene in spontaneous breast tumors. Approximately 30% of sporadic breast tumors acquire p53 mutations, which are related to breast cancer progression and resistance to doxorubicin therapy in breast cancer patients (1, 3, 6).

Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, specifically associates with cell-matrix adhesions where it integrates signals from integrins and growth factor receptors to coordinate and control processes such as actin cytoskeleton dynamics and migration. FAK expression is elevated in numerous cancers including colon-, cervix- and breast cancer (2, 7-10). In addition, a correlation of FAK and p53 mutations in human breast cancer is present (10). Recently several groups have used the MMTV-PyMT model for breast cancer to determine the role of FAK in mammary gland tumor development and progression. FAK deletion in these models reduced the incidence of MMTV-PyMT mammary tumors, suggesting an important role for FAK in mammary gland tumor development. The PyMT is a strong proto-oncogene inducing the onset of mammary gland tumors within 10 weeks. This precludes the analysis of the role of FAK in spontaneous mammary gland tumor development, which is more relevant to the human situation. Thus these studies were unable to address the role of FAK in the natural course of mammary tumorigenesis (9, 11-13). P53 has been shown to affect FAK levels by binding to the N-terminal region of FAK as well as to the promoter region of FAK to inhibit the transcription of FAK (14). Vice versa, FAK facilitates p53 turnover via enhanced mdm2-dependent p53 ubiquitination, and subsequent proteosomal degradation leading to inactivation of p53 (15). This mutual interaction suggests interplay in cancer development, but it has never been investigated.

Here, we used a spontaneous mammary tumorigenesis model based on conditional deletion of the p53 tumor suppressor protein, which induces mammary tumors starting around 45 weeks. This allowed us to determine the course of mammary gland tumor development using a clinically more relevant model. We generated $p53^{lox/lox}/FAK^{wt}$, $p53^{lox/lox}/FAK^{wt/-}$, $p53^{lox/lox}/FAK^{lox/wt}$, and $p53^{lox/lox}/FAK^{lox/-}$, in a WapCre background, and followed the development of

mammary gland tumors in time. Upon pregnancy and weaning the WapCre promoter is activated leading to the deletion of the coding sequences of p53, avoiding production of biologically active p53, and to the deletion of the FAK allele that is flanked by loxP sites. We show that FAK is essential for the progression of mammary epithelial hyperplasia to mammary gland tumors. Interestingly, in FAK-null tumors E-cadherin levels were decreased indicating that FAK-null tumors require an additional molecular switch for tumors to develop.

Results

Successful deletion of Fak and p53 in $p53^{lox/lox}$/WapCre mice

To investigate the role of FAK in spontaneous mammary gland tumor development we generated female $p53^{lox/lox}/FAK^{wt/wt}$/WapCre ($FAK^{wt/wt}$), $p53^{lox/lox}$/

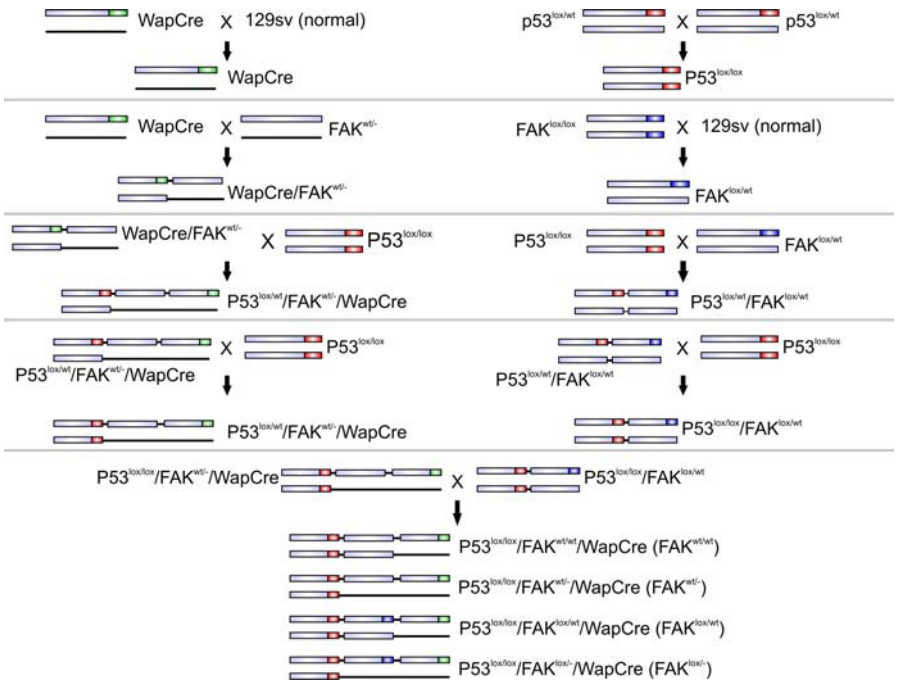

Figure 1. Breeding overview
Schematic representation of all the breeding steps required to generate $p53^{lox/lox}/FAK^{wt/wt}$/WapCre (referred to as $FAK^{wt/wt}$) and $p53^{lox/lox}/FAK^{Wt/-}$/WapCre (referred to as $FAK^{wt/-}$), and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre (referred to as $FAK^{lox/wt}$) and $p53^{lox/lox}/FAK^{lox/-}$ /WapCre (referred to as $FAK^{lox/-}$).

$FAK^{wt/-}$/WapCre ($FAK^{wt/-}$), $p53^{lox/lox}$/ $FAK^{lox/wt}$/WapCre ($FAK^{lox/wt}$) and $p53^{lox/lox}$/$FAK^{lox/-}$/WapCre ($FAK^{lox/-}$) (Fig. 1). $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice were generated to compare heterozygous FAK expression and complete deficiency of FAK. In both genotypes one allele was conditionally deleted upon WapCre activation. The mice were subjected to one pregnancy to induce WapCre-mediated deletion of *p53* and *Fak* genes flanked by two loxP sites, and subsequently followed the development of mammary tumors for a total time period of 90 weeks. This enabled us to determine whether FAK expression level was correlated to the number of mammary tumors formed. Mammary tumors were isolated and deletion of the *p53* gene and *Fak* gene was analyzed by PCR. Deletion of the *p53* gene was successful in the mammary tumors from all animal groups (Fig. 2A, 2D and Fig. S1). FAK recombination was confirmed in both $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice (Fig. 2B). PCR analysis confirmed the FAK heterozygous status of $FAK^{lox/-}$ and $FAK^{wt/-}$ mice (Fig. 2B and 2E). FAK staining on cryo-sections clearly shows reduction of FAK levels in $FAK^{lox/-}$ mammary tumors compared to $FAK^{lox/wt}$ (Fig. 2C). This was also observed in $FAK^{wt/-}$ tumors: FAK levels were lower compared to $FAK^{wt/wt}$ tumors (Fig. 2F). Despite FAK recombination in $FAK^{lox/wt}$ mice reduction of FAK levels was not clearly evident by immunofluorescence when compared to $FAK^{wt/wt}$ mice (Fig. 2C and 2F, respectively).

Mammary gland-specific Fak deletion inhibits spontaneous mammary gland tumor formation in p53 null mice

The first onset of mammary tumors in $FAK^{lox/-}$ mice was comparable to that in $FAK^{lox/wt}$ mice. However, mammary tumor formation reached a plateau in $FAK^{lox/-}$ mice after 60 weeks, but continued normally in $FAK^{lox/wt}$ animals (Fig 3A). This reflected in the effect on the total number of mammary tumors that developed: fifty-eight percent of $FAK^{lox/wt}$ mice developed mammary tumors (14 of a total of 24 mice) while only thirty-five percent of $FAK^{lox/-}$mice developed mammary tumors (9 of a total of 26 mice) (Fig. 3B). The mean latency time for spontaneous mammary tumor development was 75 ± 3 weeks for $FAK^{lox/wt}$ mice and 62 ± 5 weeks for $FAK^{lox/-}$ mice. The lower mean latency time in $FAK^{lox/-}$ mice can be explained by the lack of tumor formation in later stage. Possibly the tumors that arise in early stage still expressed low amounts of FAK or have acquired additional alterations enabling them to develop. These alterations may not occur later in mammary tumorigenesis. The percentage of $FAK^{lox/wt}$ mammary tumor bearing mice was comparable to $FAK^{wt/-}$ indicating that the deletion of *Fak* was successful. Thus the incidence of mammary tumors directly relates to FAK expression levels.

Decreased FAK expression in FAK heterozygous mice has been shown to suppress papilloma formation during experimental mouse skin carcinogenesis (16). To determine the correlation between FAK levels and incidence of mammary

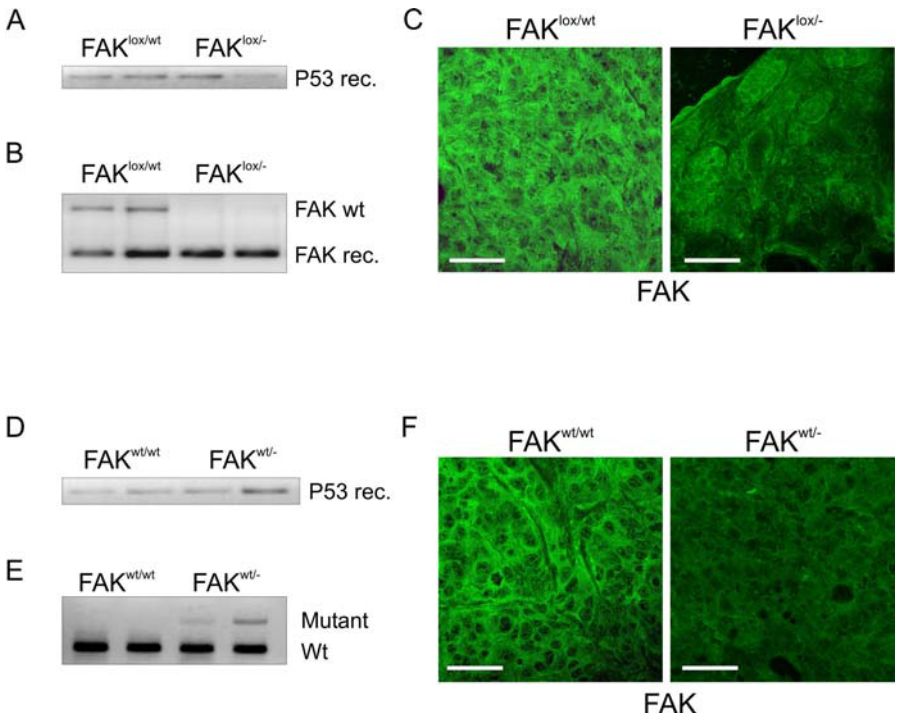

Figure 2. P53 and Fak gene recombination was successful in p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$/WapCre mice
(A) P53 recombination PCR on mammary tumors obtained from FAK$^{lox/wt}$ and FAK$^{lox/wt}$ mice. P53 recombined band runs at 612 bp. (B) FAK recombination PCR was used to determine recombination of the FAK allele flanked by loxP sites. FAK wt is 1.4 kb, FAK lox is 1.6 kb and FAK recombined is 327 bp. (C) FAK staining on cryo-sections of mammary tumors obtained from FAK$^{lox/-}$ and FAK$^{lox/wt}$ mice. Scale bar is 50 μm. (D) P53 recombination PCR on mammary tumors obtained from FAK$^{wt/wt}$ and FAK$^{wt/-}$ mice. P53 recombined band is 612 bp. (E) FAK PCR to determine FAK wt or FAK mutant (wt/-). FAK wt is 88 bp, FAK mutant is 170 bp. (F) FAK staining on cryo-sections of mammary tumors obtained from FAK$^{wt/wt}$ and FAK$^{wt/wt}$ mice. Scale bar is 50 μm.

tumors we evaluated the p53-dependent tumor formation in FAK homozygous and heterozygous mice. FAK$^{wt/wt}$ mice were more susceptible for mammary tumor development compared to FAK$^{wt/-}$ (Fig. 3C). Sixty-two percent of FAK$^{wt/wt}$ mice were mammary tumor-bearing (16 of a total of 26 mice), while only forty-one percent of FAK$^{wt/-}$ mice were mammary tumor-bearing (12 of a total of 29 mice) (Fig. 3D). The mean latency time for spontaneous mammary tumor development was 60 $\pm$ 5 weeks for FAK$^{wt/wt}$ mice and 73 $\pm$ 4 weeks for FAK$^{wt/-}$ mice. Altogether, our data indicate that FAK is involved in p53 null-induced mammary tumorigenesis.

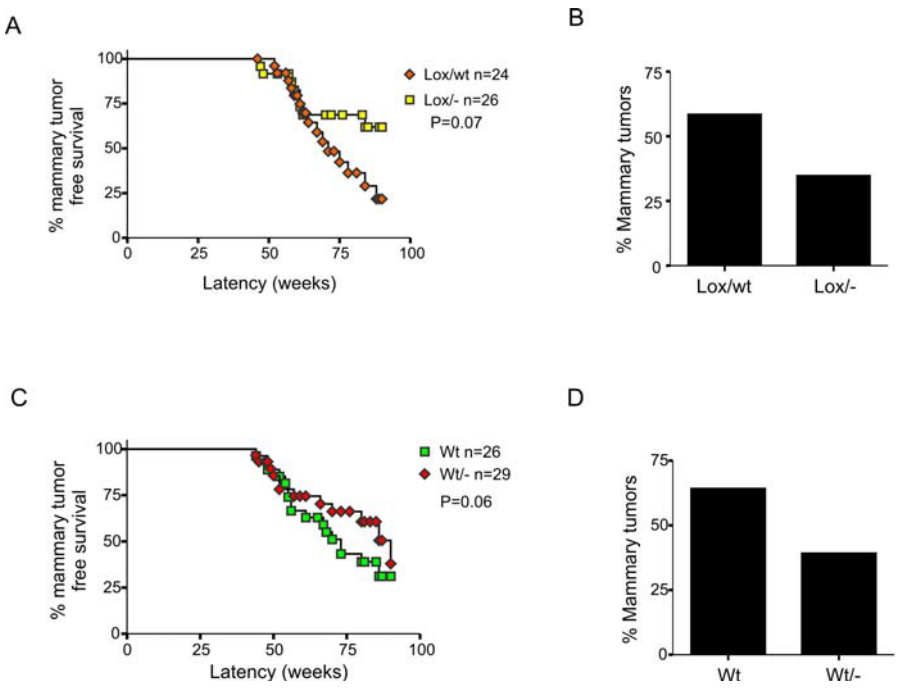

Figure 3. FAK is involved in $p53^{\Delta 2\text{-}10/\Delta 2}$-induced mammary tumorigenesis
Female $FAK^{lox/wt}$ (n=24) and $FAK^{lox/-}$ mice (n=26) were subjected to one pregnancy to induce WapCre-mediated deletion of *p53* and *Fak*, and subsequently followed for the development of mammary tumors for a total time period of 90 weeks. (A) Mammary tumor free survival (%). (P=0.07) (B) Percentage of mammary tumors that arose in $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. Female $FAK^{wt/wt}$ (n=26) and $FAK^{wt/-}$ mice (n=29) were subjected to one pregnancy to induce WapCre-mediated deletion of *p53*, and subsequently followed for the development of mammary tumors for a total time period of 90 weeks. (C) Mammary tumor free survival (%). (P=0.06) (D) Percentage of mammary tumors that arose in $FAK^{wt/wt}$ and $FAK^{wt/-}$ mice.

FAK does not affect formation of hyperplasias

Mammary tumors and contra lateral non-tumor bearing mammary glands were isolated, stained for hematoxylin/eosin and analyzed for tumor type or mammary gland hyperplasia, respectively (Fig. 4). Histological analysis of mammary glands isolated from the contra-lateral side of the mammary tumor showed signs of neo- or hyperplasia in all groups. Morphological differences were observed between the FAK expressing and FAK null group. $FAK^{lox/-}$ mammary glands did not have the dense structure of tumor cells but showed a more dispersed phenotype (Fig. 4A). Mammary glands contain two types of epithelial cells; luminal epithelial cells are the cells that are located surrounding the lumen of a duct. Luminal epithelial cells are surrounded by myoepithelial cells that are in their turn bound to the

extracellular matrix. WapCre is activated predominantly in the luminal compartment. To determine if the tumors developed from the luminal-epithelial compartment, we stained mammary glands and tumors for cytokeratin 8 (CK8), a luminal marker, and cytokeratin 5 (CK5), a myoepithelial marker. In contra lateral non-tumor bearing mammary glands, expansion of the luminal compartment was evident and with exception of one $FAK^{wt/wt}$ mice, all tumors were CK8 positive (Fig. 4A and 4B bottom panel), indicating that the tumors developed from the luminal compartment. Mammary gland tumors that developed were solid carcinomas, originating from breast epithelial cells; adenocarcinomas, originating in the milk ducts and/or lobules (glandular tissue) of the breast; and carcinosarcomas, tumors composed of intimately admixed malignant epithelial

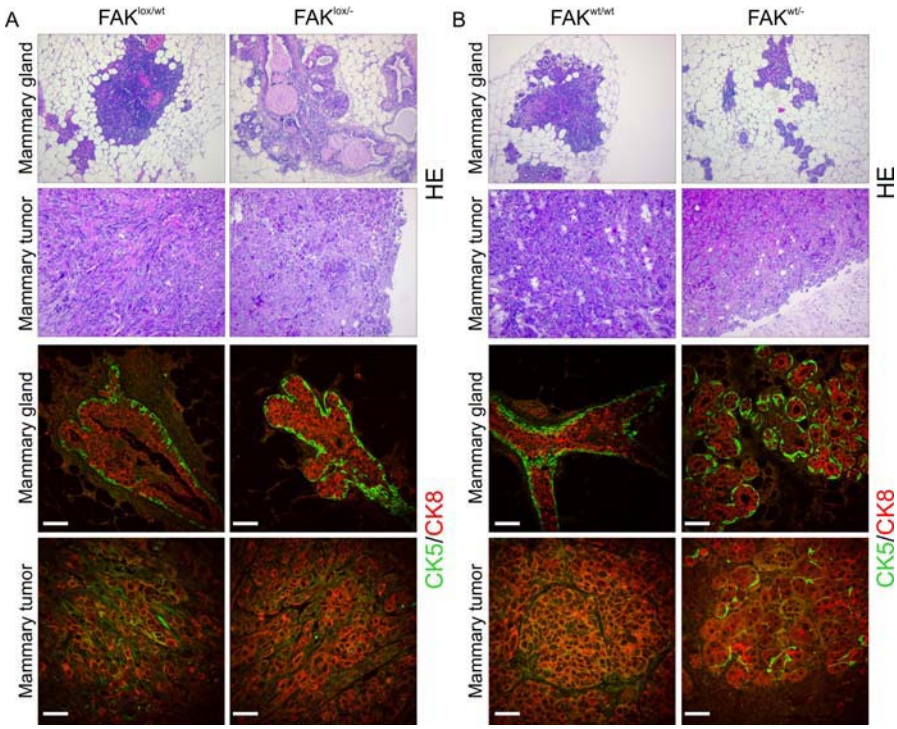

Figure 4. $p53^{lox/lox}$/WapCre mice develop luminal-like mammary gland tumors
Sections of formalin-fixed, paraffin embedded mammary tumors and non-tumor bearing mammary glands that were isolated from $FAK^{lox/-}$, $FAK^{lox/wt}$ (A), $FAK^{wt/wt}$, and $FAK^{wt/-}$,(B) mice, were stained for HE (upper panel) and luminal marker cytokeratin 8 (CK8) and myoepithelial marker cytokeratin 5 (CK5) (bottom panel). Fluorescent staining was analyzed by confocal laser scanning microscopy. Scale bar is 50 µm.

and stromal components. Since tumor types were not restricted to one experimental group, tumor type/grade could not be linked to differences in FAK expression between the groups.

Reduced E-cadherin expression in P53$^{lox/lox}$/WapCre FAK deficient tumors

FAK protein levels in tumors obtained from FAK$^{wt/wt}$, FAK$^{wt/-}$, FAK$^{lox/wt}$ and FAK$^{lox/-}$ mice determined on western blot indicated that FAK levels were reduced in FAK$^{wt/-}$, FAK$^{lox/wt}$ and FAK$^{lox/-}$ tumors compared to FAK$^{wt/wt}$ tumors (Fig. 5A and Fig. S2).

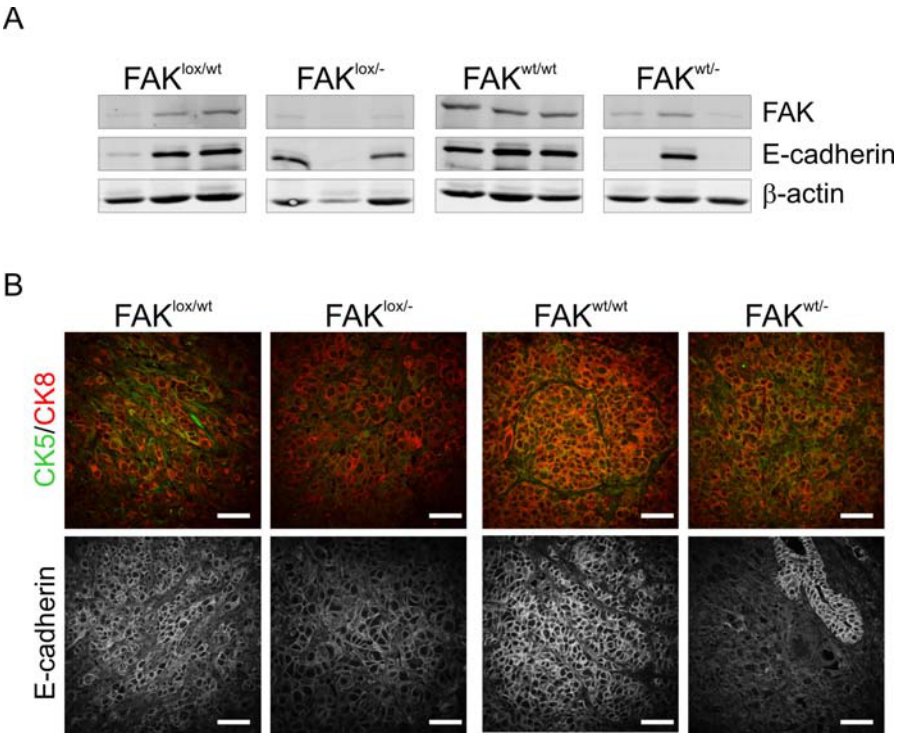

Figure 5. E-cadherin expression correlates with FAK protein levels

(A) Sections of formalin-fixed, paraffin embedded mammary tumors and non-tumor bearing mammary glands that were isolated from FAK$^{wt/wt}$, FAK$^{wt/-}$, FAK$^{lox/wt}$ and FAK$^{lox/-}$ mice, were stained for CK5 and CK and E-cadherin. Slides were analyzed using confocal laser scanning microscopy. Scale bar is 50 μm. (B) Western blot analysis of total lysates of tumor samples derived from FAK$^{wt/wt}$, FAK$^{wt/-}$, FAK$^{lox/wt}$ and FAK$^{lox/-}$ mice. Blots were incubated with antibodies for FAK, E-cadherin, cytokeratin 5, and β-actin was used as a loading control.

FAK is required for transcriptional up-regulation of mesenchymal and invasiveness markers and delocalization of membrane-bound E-cadherin (17). To determine the status of EMT-like markers in our spontaneous mammary gland tumor model, mammary tumors that developed in $FAK^{wt/wt}$, $FAK^{wt/-}$, $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice, were stained for E-cadherin and analyzed by confocal laser scanning microscopy. Interestingly, while the majority of FAK expressing tumors expressed E-cadherin (Fig. 5B), mammary tumors that developed in mammary glands with decreased FAK expression showed reduced or completely lacked E-cadherin expression. Western blot analysis confirmed that E-cadherin protein levels correlated to FAK protein levels (Fig. 5A and Fig. S2). These data suggest that in mammary glands with reduced FAK expression either an additional genetic modification and/or defected FAK signaling contributes to enhanced progression from hyperplasia to full mammary tumors, possibly in relation to an EMT-like related loss/down regulation of E-cadherin expression.

Discussion

Genetic alterations in the tumor suppressor protein p53 are common and p53 is mutated in over 50% of all human tumors, including breast cancer. In this study we determined the role of FAK in p53 null-induced spontaneous mammary gland tumor formation. We show that the incidence of mammary tumors directly correlates to the protein expression levels of FAK. Reduced number of mammary tumors was observed in FAK heterozygous mice and in mice with conditional *Fak* deletion, indicating that FAK is involved in p53 null-induced mammary tumorigenesis. In addition, we found a clear correlation between FAK expression levels and expression of E-cadherin. Tumors with reduced FAK expression showed significantly reduced E-cadherin levels, indicating that these tumors acquired an additional hit to develop.

In previous studies using a MMTV-PyMT mouse model for breast cancer, FAK was implicated to play an important role in the development of these tumors. These studies showed the importance of FAK expression in the induction of these tumors. The development of human breast cancer is a multistep process in which several hits are needed for a tumor to form. The MMTV-PyMT is a strong proto-oncogene inducing tumors within 10 weeks, without the need for additional alterations and is therefore not a clinically relevant model. In our spontaneous mammary gland tumor model based on conditional deletion of p53, mammary tumors arose after approximately 50 weeks. This suggests that loss of p53 alone is not sufficient to induce mammary tumors but that additional hits are required for mammary tumors to develop, indicating that our model better mimics the natural process of breast cancer development.

An interesting finding was that E-cadherin expression correlated to FAK protein levels. Though several studies have suggested that Src-induced FAK

signaling leads to suppression of cell-cell adhesion (reviewed in Avizienyte et al (18)) others have suggested that integrin/FAK signaling may stabilize the cell periphery and actin cytoskeleton, thereby promoting the formation of cadherin-mediated cell-cell adhesions (19). In addition, induction of E-cadherin expression by TGFβ was shown to be mediated through the activation of FAK (20). Though the role of FAK in mammary tumorigenesis was studied extensively in the last couple of years, no statements were made on the relationship between FAK and E-cadherin levels. In previous studies simultaneous loss of p53 and E-cadherin was associated with invasive lobular carcinoma and metastasis (21, 22). Though loss of E-cadherin was found in p53-/-/FAK-/- tumors, the tumors that developed in our model do not resemble those described in Derksen et al. In our model mostly adenocarcinomas, solid carcinomas and sarcinosarcomas developed, while in the model of Derksen a large portion of tumors were invasive lobular carcinomas. Most likely the time frame in which the loss of E-cadherin occurs affects the morphology and metastatic potential of these tumors. Since loss of E-cadherin expression was exclusively found in tumors with low or no expression of FAK, we believe that due to the loss of FAK expression tumor development is inhibited but once an additional factor such as E-cadherin is lost mammary tumors can be formed. What the effect is of loss of E-cadherin in these tumors is on metastases formation has yet to be determined. However, one of the key steps in metastases formation is loss of E-cadherin suggesting that in the tumors that do develop regardless of reduced FAK expression tumor cells might be more capable in escaping the primary tumor and form distant metastases. In addition to the effects observed on E-cadherin expression we wonder whether FAK deletion affects stem cell maintenance in our model as is described by others (23). In our spontaneous mammary gland tumor model the incidence of mammary tumors starts around the same time but reaches a plateau in mice with a complete FAK deletion in the mammary gland, while incidence of mammary tumors continues in FAK expressing mice. The sudden stop in mammary tumor incidence can be explained by the reduced survival and defects in maintenance of FAK deficient cancer stem cell population. This would in the end affect mammary tumor development in FAK deficient mammary glands. In the future we will determine the effect of FAK on the maintenance of stem cells in our spontaneous mammary gland tumor model.

Altogether, our data shows that FAK is involved in $p53^{lox/lox}$/WapCre-induced mammary gland tumor formation. In future studies we will unravel the mechanism by which FAK regulates $p53^{lox/lox}$/WapCre-induced mammary gland tumor formation and determine its role in the regulation of E-cadherin expression. For this purpose we are currently performing micro-RNA (miR) analysis to determine if the MiR200 family, a family of miRNAs that regulate the expression of E-cadherin suppressors, is down regulated. In addition, gene expression

profiling studies will provide data on signaling pathways that are differentially regulated.

Materials and Methods

Breeding of mice
All breeding steps necessary to generate $p53^{lox/lox}/FAK^{wt/wt}$/WapCre (referred to as $FAK^{wt/wt}$), $p53^{lox/lox}/FAK^{Wt/-}$/WapCre (referred to as $FAK^{wt/-}$), $p53^{lox/lox}/FAK^{lox/wt}$/WapCre (referred to as $FAK^{lox/wt}$) and $p53^{lox/lox}/FAK^{lox/-}$/WapCre (referred to as $FAK^{lox/-}$) are summarized in Fig. 1.

Genotyping of mice
Genotyping of $p53^{lox/lox}$ mice was performed using the following primers. 5'-CAC AAA AAC AGG TTA AAC CCA G-3' [p53-int1-fwd], 5'-AGC ACA TAG GAG GCA GAG AC-3' [p53-int1-rev], 5'-AAG GGG TAT GAG GGA CAA GG-3' [p53-int10-fwd], 5'GAA GAC AGA AAA GGG GAG GG-3' [p53-int10-rev]. Deletion of p53 was confirmed with primers: 5'-CAC AAA AAC AGG TTA AAC CCA G-3' [p53-int1-fwd] and 5'GAA GAC AGA AAA GGG GAG GG-3' [p53-int10-rev]. Genotyping of *fak* was performed using specific primers for FAK: 5'-GAGAATCCAGCTTTGGCTGTT-3'; 5'-GGCTTCTTGAAGGAACTTCTC-3'; 5'-TGATATTGCTGAAGAGCTTGGCG G-3'. PCR products derived were FAK wt 88 and/or FAK mutant 170bp; for $FAK^{lox/lox}$ mice the primers are fwd: 5'-GAGAATCCAGCTTTGGCTGTTG-3', rev: 5'-GAATGCTACAGGAACC AAATAAC-3', PCR products were Wt 290bp and lox 400bp. For Cre genotyping primers used were fwd: 5'-GTTCAGGGATCGCCAGGC G-3' and rev: 5'-GCTGGCTGGTGGCAGATGG -3'. FAK recombination was determined using the following primers fwd: 5'-GACCTTCAACTTCTCATTTCTCC-3' and rev: 5'-GAATGCTACAGGAACCAAATA-AC-3'; PCR products were Wt 1.4 kB, lox 1.6kB, and FAK recombined allele 327bp.

Analysis of spontaneous tumour development
Spontaneous tumor development was determined in $FAK^{wt/wt}$ (n=26), $FAK^{wt/-}$ (n=29), $FAK^{lox/-}$ (n=26) and $FAK^{lox/wt}$ (n=24). At the age of 8 weeks mice were placed in breeding; pregnancy and lactation stimulated the WapCre promoter. After three weeks of weaning the pups were removed from the mother. Weight was determined and mammary tumor formation was monitored by palpation once a week. Mice were sacrificed before tumors reached the size of $1cm^3$ or when mice lost more than 10% of their total body weight. Mammary tumor formation was followed up to 90 weeks, after which all remaining animals in the experiment were sacrificed. Tumor and mammary gland tissue were isolated and divided in

two; one part was snap-frozen in liquid nitrogen, the other part was fixed in 4% formaldehyde and subsequently processed and embedded in paraffin.

Tissue processing and histology
Tumors and/or mammary glands isolated from the mice were fixed in 4% formaldehyde and subsequently processed and embedded in paraffin or snap-frozen in liquid nitrogen. Paraffin sections of 3 μm were deparaffinized and antigen retrieval was performed when needed. Cryo-sections were fixed in ice-cold 4% formaldehyde. Sections were stained with primary antibody overnight at 4^{o}C and secondary antibody 1 hr at room temperature. Slides were mounted with aqua polymount.

Immunofluorescence microscopy and image analysis
Immunofluorescence staining of coverslips was performed as described previously. Primary antibody incubation was performed overnight at 4 °C and cells were subsequently incubated with Alexa-488 or Cy-3 conjugated secondary antibody (Molecular Probes). Microscopic analysis was performed using a Bio-Rad Radiance 2100 confocal system with a 60 X or 40 X Plan Apo (NA 1.4; Nikon) objective lens. Image acquisition was controlled using the Laser Sharp software (Bio-Rad). Images were processed and quantitative image analysis was performed using Image-Pro® Plus (Version 5.1; Media Cybernetics) in combination with in house developed macros to analyze focal adhesion size.

Western blot
A small biopsy from the tumors was lysed in lysis buffer (Tris-HCL (10mM), sucrose (250 mM), EDTA (1 mM). Primary antibody incubation was performed overnight at 4^{o}C. Thereafter blots were incubated with horseradish peroxidase conjugated secondary antibody (GE Healthcare) or Cy5-conjugated secondary antibody in TBS-T for 1h at room temperature. Protein signals were detected with ECL-plus method (GE Healthcare) followed by scanning of the blots or in case of Cy5-incubation, the blots were directly scanned with a Typhoon 9400 (GE Healthcare).

Antibodies
Anti-FAK clone 4.47 Upstate Biotechnology (#05-537), anti-E-cadherin BD transduction (#610263), anti-cytokeratin 5 Covance (#PRB-160P), anti-cytokeratin 8 Fitzgerald (rdi-pro61038), β-actin Santa Cruz (sc-47778).

Statistics
Survival curves were generated using Kaplan-Mayer statistics. Significance was estimated using unpaired two-tailed Student t-test. Significance is marked in the graphs.

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Supplemental figures

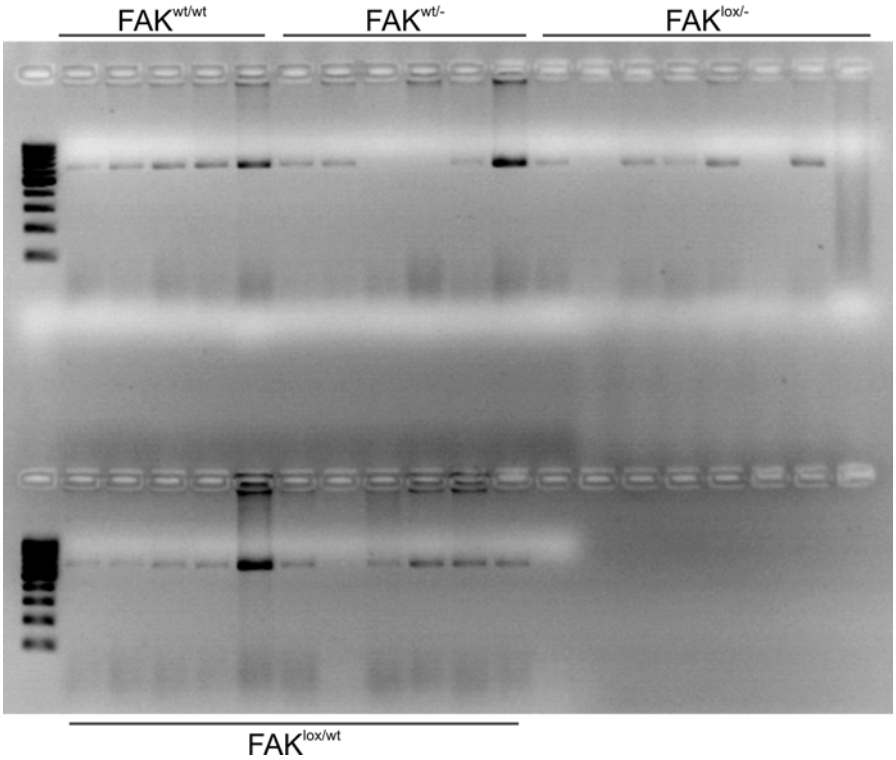

Figure S1. P53 gene recombination
PCR analysis of mammary tumor samples obtained from $FAK^{wt/wt}$, $FAK^{wt/-}$, $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice.

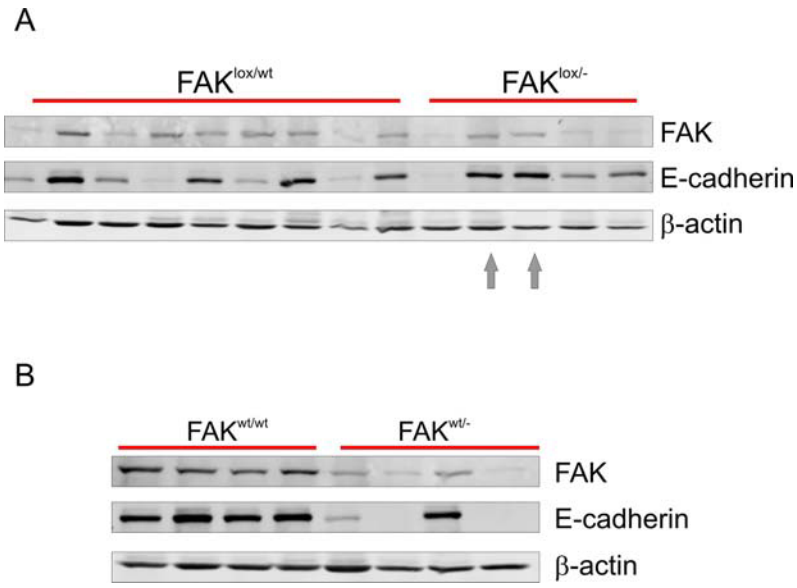

Figure S2. E-cadherin expression correlates with FAK protein levels
Western blot analysis of total lysates of tumor samples derived from $FAK^{wt/wt}$ and $FAK^{wt/-}$ (A), and $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice (B). Blots were incubated with antibodies for FAK, E-cadherin, cytokeratin 5, and β-actin was used as a loading control. Arrows indicate samples that were excluded from the group based on FAK recombination PCR.