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FAK Kinase Activity Is Required for the Progression of c-MET/β-Catenin-Driven Hepataocellular Carcinoma

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There is an urgent need to develop new and more effective therapeutic strategies and agents to treat hepatocellular carcinoma (HCC). We have recently found that deletion of *Fak* in hepatocytes before tumors form inhibits tumor development and prolongs survival of animals in a c-MET (MET)/β-catenin (CAT)-driven HCC mouse model. However, it has yet to be determined whether FAK expression in hepatocytes promotes MET/CAT-induced HCC progression after tumor initiation. In addition, it remains unclear whether FAK promotes HCC development through its kinase activity. We generated hepatocyte-specific inducible *Fak*-deficient mice (Alb-creERT2; *Fak*$^{flox/flox}$) to examine the role of FAK in HCC progression. We reexpressed wild-type and mutant FAK in *Fak*-deficient mice to determine FAK's kinase activity in HCC development. We also examined the efficacy of a FAK kinase inhibitor PF-562271 on HCC inhibition. We found that deletion of *Fak* after tumors form significantly repressed MET/CAT-induced tumor progression. Ectopic FAK expression restored HCC formation in hepatocyte-specific *Fak*-deficient mice. However, overexpression of a FAK kinase-dead mutant led to reduced tumor load compared to mice that express wild-type FAK. Furthermore, PF-562271 significantly suppressed progression of MET/CAT-induced HCC. *Fak* kinase activity is important for MET/CAT-induced HCC progression. Inhibiting FAK kinase activity provides a potential therapeutic strategy to treat HCC.

Key words: Hepatocellular carcinoma (HCC); Sleeping Beauty Transposon system; Proliferation; AKT; Extracellular receptor kinase (ERK)

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and is the third leading cause of cancer deaths worldwide[1]. The overall survival of patients with HCC is less than 12%, and most patients with HCC have limited treatment options[2]. There is an urgent need to develop new and more effective therapeutic strategies and agents to treat HCC.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that can be phosphorylated and activated by integrins and growth factors[3,4]. FAK targets multiple downstream signaling pathways to regulate different cellular functions[5]. Unlike another integrin-associated regulatory protein [integrin-linked kinase (ILK)], which has growth suppressor properties in the liver[6], FAK is a growth enhancer[7]. FAK is overexpressed in more than 50% of HCC specimens[8,9], offering a potential target for the treatment of HCC. We have recently reported that deletion of *Fak* in hepatocytes inhibits tumor proliferation and development and prolongs survival of animals in a c-MET/β-catenin-driven HCC mouse model[7]. The oncoproteins c-MET and β-catenin play critical roles in hepatocarcinogenesis. Overexpression of c-MET occurs in about 50% of HCC specimens, and about 40% of HCC tissues contain aberrant activation of β-catenin (mainly due to mutation of the *β-catenin* gene)[10–12]. Coactivation of c-MET and β-catenin often occurs in HCC[12]. It was reported that 60% of HCC specimens containing activated c-MET have activation of β-catenin, and over 60% of HCC samples carrying mutant *β-catenin* contain activated c-MET[12]. In addition, codelivery of both c-MET (MET) and constitutively active β-catenin (ΔN90-β-catenin; CAT), but not MET or CAT alone, into mouse livers using the Sleeping Beauty Transposon system efficiently induces HCC within several weeks[12–14]. Therefore, this model (referred to here as MET/CAT) is useful to study the functions of

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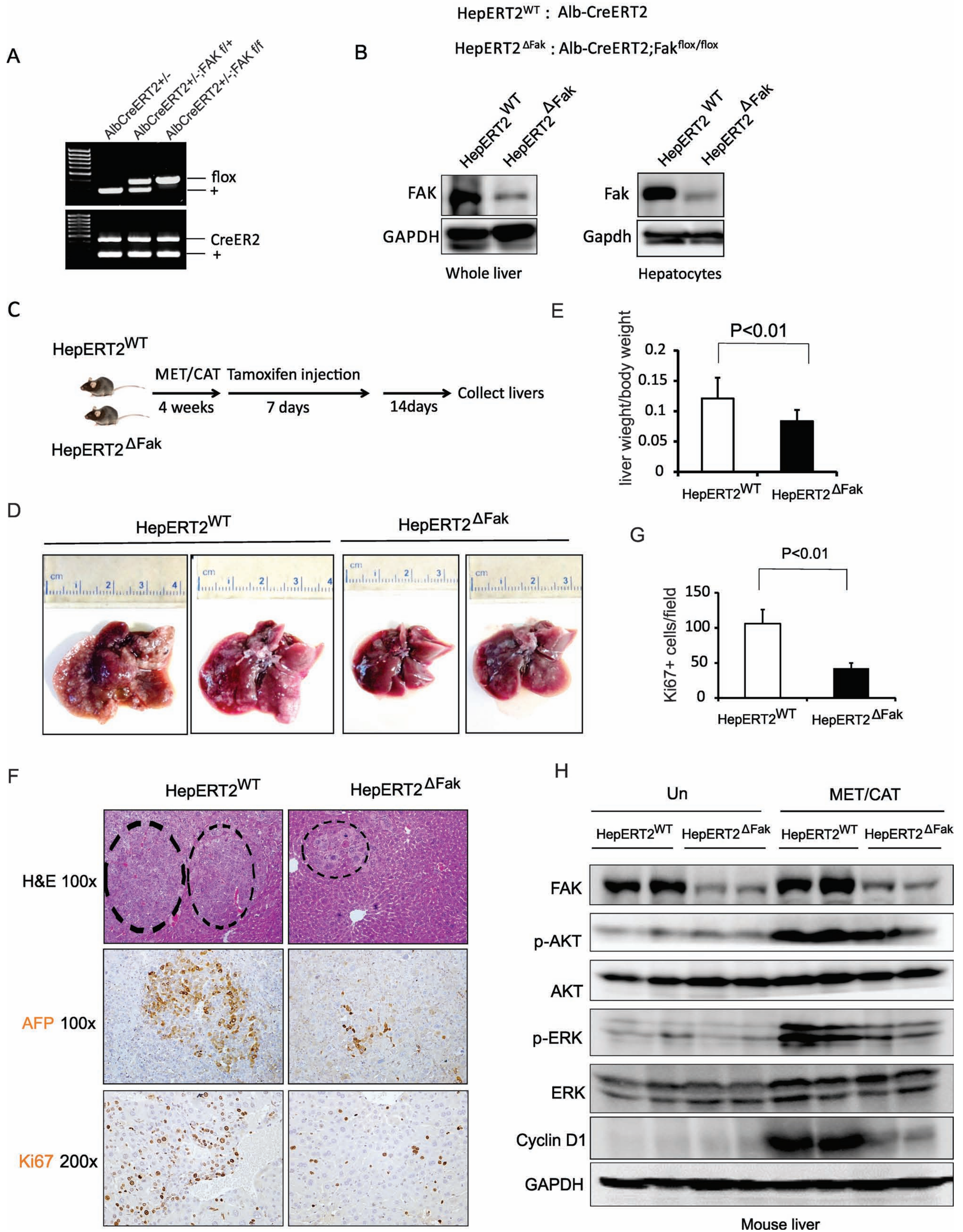

HepERT2WT : Alb-CreERT2
HepERT2ΔFak : Alb-CreERT2;Fakflox/flox
A
AlbCreERT2+/-
AlbCreERT2+/-;FAK f/+
AlbCreERT2+/-;FAK f/f
flox
+
CreER2
+
B
HepERT2 WT
HepERT2 ΔFak
FAK
GAPDH
Whole liver
Fak
Gapdh
Hepatocytes
C
HepERT2WT
HepERT2ΔFak
MET/CAT
4 weeks
Tamoxifen injection
7 days
14days
Collect livers
E
P<0.01
liver wieght/body weight
D
G
P<0.01
Ki67+ cells/field
F
H&E 100x
AFP 100x
Ki67 200x
H
Un
MET/CAT
FAK
p-AKT
AKT
p-ERK
ERK
Cyclin D1
GAPDH
Mouse liver

genes in hepatocarcinogenesis because of its clinical relevance and efficiency in inducing HCC.

Our data suggest that FAK is involved in the pathogenesis of HCC and that inhibition of FAK may be a promising strategy to prevent and/or treat HCC. However, further study is needed in order to understand how FAK promotes HCC development. For example, it remains to be determined whether inhibition of FAK is sufficient to suppress MET/CAT-induced tumor initiation, tumor progression, or both. If inhibition of FAK suppresses HCC initiation but not HCC progression, targeting FAK might provide a means to prevent HCC development in populations at risk. However, if inhibition of FAK suppresses HCC progression, FAK inhibitors may prolong survival in patients with advanced HCC and could potentially slow tumor progression in patients with HCC who are awaiting liver transplantation. In addition, FAK executes its functions through both kinase-dependent and kinase-independent mechanisms in a cell context-dependent manner[15,16]. The roles of FAK's kinase activity and scaffolding functions in HCC development remain unknown. Understanding these processes will provide a mechanistic basis to reduce protumor effects by inhibiting either FAK's kinase and/or kinase-independent activities.

In this study, we generated hepatocyte-specific inducible *Fak*-deficient mice. We found that deletion of *Fak* after tumor initiation significantly repressed MET/CAT-induced tumor maintenance and proliferation. In addition, ectopic FAK expression restored HCC formation in hepatocyte-specific *Fak*-deficient mice. Overexpression of a FAK kinase-dead mutant led to reduced tumor load compared to mice with expression of wild-type (WT) FAK. Furthermore, a FAK inhibitor, PF-562271, significantly suppressed MET/CAT-induced hepatocarcinogenesis. Overall, our data suggest that FAK kinase activity is required for MET/CAT-induced HCC development.

MATERIALS AND METHODS

Plasmids

The plasmids pENTR1A (#17398) and MIGR1 (#27490) were obtained from Addgene. pENTR1A-IRES-GFP was generated by cloning IRES-GFP (digested with *Eco*R1 and *Sal*1) into the pENTR1A vector. The pBabe FAK and pBabe FAK (K454R) plasmids were gifts from Dr. Fillippo Giancotti[17]. The pENTR1A-FAK-IRES-GFP and pENTR1A-FAK(K454R)-IRES-GFP plasmids were generated by cloning FAK, FAK(K454R), and FAK (FERM) coding region into the pENTR1A-IRES-GFP vector. The pT3EF1α plasmid was obtained from Dr. Xin Chen (UCSF)[14]. The pT3-IRES-GFP, pT3-FAK-IRES-GFP, and pT3-FAK(K454R)-IRES-GFP were then generated by gateway cloning using pENTR1A-IRES-GFP, pENTR1A-FAK-IRES-GFP, and pENTR1A-FAK(K454R)-IRES-GFP as entry vectors and pT3EF1α as destination vector, respectively. The plasmids were purified using *GeneJET* Plasmid Maxiprep Kit (Thermo Fisher Scientific) for hydrodynamic tail vein injection.

Mice and Treatments

All animals received humane care according to the "Guide for the Care and Use of Laboratory Animals" (http://oacu.od.nih.gov/ac_cbt/guide3.htm). The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago. To generate hepatocyte-specific inducible *Fak*-deficient mice, $Fak^{flox/flox}$ mice[7] were mated with Alb-CreERT2 mice[18]. The resulting offspring, Alb-CreERT2; $Fak^{flox/+}$ mice, were then mated to generate Alb-CreERT2 and Alb-CreERT2; $Fak^{flox/flox}$ littermates. Detailed information on genotyping of the mice can be found in the supporting information. The mice were housed in microisolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-h light/dark cycle) and were allowed access to water and chow ad libitum.

Mice were genotyped by polymerase chain reaction (PCR) using genomic DNA extracted from tail snips. The $Fak^{flox/flox}$ allele was identified using primers as previously described[7]. The ERT2Cre allele was detected using the sense primer 5′-GGAACCCAAACTGATGACCA-3′ and the antisense primers 5′-TTAAACAAGCAAAACCAAAT-3′ and 5′-ATCATTTCTTTGTTTTCAGG-3′, as previously described[18].

The MET/CAT-induced HCC model was utilized as previously described[7,19]. In brief, 50 μg of total plasmids, encoding the Sleeping Beauty transposase (HSB2) and transposons with oncogenes MET/CAT [22.5 μg of pT3-EF1α-c-MET (human) + 22.5 μg of pT3-EF1α-ΔN90-

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Figure 1. Inducible deletion of *Fak* following tumor formation suppresses tumor progression in a MET/CAT-induced HCC mouse model. (A) Genotyping of Alb-CreERT2, Alb-CreERT2; $Fak^{flox/+}$, and Alb-CreERT2; $Fak^{flox/flox}$ mice. (B) Western blotting showing expression of FAK and GAPDH proteins in whole livers and isolated hepatocytes of Alb-CreERT2 and Alb-CreERT2; $Fak^{flox/flox}$ mice after 7 days of tamoxifen treatment. (C) Diagram of the experimental protocol. (D) Photographs of livers of Alb-CreERT2 and Alb-CreERT2; $Fak^{flox/flox}$ mice 7 weeks after injection of MET/CAT followed by tamoxifen injection as indicated in (A). (E) Liver weight/body weight ratio was analyzed in mice from (D) ($n = 8$). (F) Histological analysis [hematoxylin and eosin (H&E) staining], AFP (an HCC marker), and Ki-67 staining of the livers of Alb-CreERT2 and Alb-CreERT2; $Fak^{flox/flox}$ mice 7 weeks after injection of MET/CAT followed by tamoxifen injection. (G) Quantification of Ki-67 staining for (F) ($n = 5$). (H) Expression of FAK, p-AKT, AKY, p-ERK, ERK, cyclin D1, and GAPDH proteins in the livers of Alb-CreERT2 and Alb-CreERT2; $Fak^{flox/flox}$ mice 7 weeks after injection with MET/CAT.

β-catenin (human) + 5 μg of HSB2][12,13], were injected hydrodynamically into 10 age- and gender-matched Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice. Four weeks after MET/CAT injection, 120 mg/kg tamoxifen was administrated to Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice by intraperitoneal (IP) injection daily for 7 days. Mice were sacrificed after 14 days. The liver weight and body weight of each mouse were measured.

Fifteen Alb-Cre; Fak$^{flox/flox}$ mice were injected hydrodynamically with FAK/MET/CAT [22.5 μg of pT3-EF1α-c-MET (human) + 22.5 μg of pT3-EF1α + 22.5 μg of pT3-EF1α-FAK + 7.5 μg of HSB2] or FAK(K454R)/MET/CAT [22.5 μg of pT3-EF1α-c-MET (human) + 22.5 μg of pT3-EF1α + 22.5 μg of pT3-EF1α-FAK(454R) + 7.5 μg of HSB2] to study the role of FAK kinase activity in HCC development.

Ten Alb-CreERT2 and 10 Alb-CreERT2; *Fak*$^{flox/flox}$ mice were injected hydrodynamically with MET/CAT and, 4 weeks later, with 33 mg/kg PF-562271 (dissolved in 12.5% DMSO and 12.5% PEG400)[20] twice daily for 3 weeks.

Western Blotting

Western blotting was performed as previously described[7]. Primary antibodies, including those against FAK (#3285), p-FAK (Y397) (#3283), p53 (2524), p-AKT (ser473) (#4060), AKT (#9272), p-ERK (#4670), ERK (#4695), and cyclin D1 (#2978), were purchased from Cell Signaling Technology (Danvers, MA, USA). The p21 antibody (#6246) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the GAPDH antibody (#G8795) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end labeling (TUNEL) staining was performed as previously described[7]. The apoptotic index was scored in at least five fields at 400× magnification/mouse and reported as mean ± standard deviation (SD). Three mice were used for each group.

Immunohistochemical (IHC) Staining

IHC was performed as previously described[7]. Cells with positive staining were scored in at least five fields at 400× or 200× magnification and reported as mean ± SD. Three mice were used per group.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism V software. Data are presented as mean ± SD. Statistical significance was calculated using the Student's *t*-test. A value of $p < 0.05$ was considered to be significant. The mean ± SD values are shown in the figures where applicable.

RESULTS

Deficiency of Fak *in Hepatocytes After Tumor Initiation Suppresses MET/CAT-Induced HCC Maintenance*

To determine the role of FAK in HCC maintenance, we generated hepatocyte-specific inducible *Fak*-deficient mice (Alb-CreERT2; *Fak*$^{flox/flox}$) (Fig. 1A). We confirmed that the administration of 120 mg/kg tamoxifen by IP injection daily for 7 days efficiently induces *Fak* deletion in hepatocytes (Fig. 1B). We then hydrodynamically injected age- and gender-matched Alb-CreERT2 mice and Alb-CreERT2; *Fak*$^{flox/flox}$ mice with MET/CAT. Four weeks after MET/CAT injection, we treated the mice with tamoxifen to induce *Fak* deletion (Fig. 1C). Because MET/CAT-induced tumors (microscopic tumor lesions) are beginning to form in the liver after 4 weeks[7,12,13], we are therefore able to determine whether FAK is required for HCC progression. We found that multiple HCC modules varying in size from 200 to 500 μM^3 formed in the Alb-CreERT2 mice (Fig. 1D and F). The tumors show a trabecular growth pattern with increased cellular density, cytological atypia, enlarged pleomorphic nuclei, and frequent mitoses. No significant inflammation was observed in these livers (Figs. 1F and 2). We found that the overall tumor load in Alb-CreERT2; *Fak*$^{flox/flox}$ mice was significantly decreased compared to Alb-CreERT2 mice (Fig. 1D–F). The relative liver weight versus body weight in Alb-CreERT2; *Fak*$^{flox/flox}$ mice was significantly decreased by 1.5-fold compared to that in Alb-CreERT2 mice (0.12 ± 0.02 vs. 0.08 ± 0.03) (Fig. 1E). The sizes of the tumors were also significantly decreased in the livers of Alb-CreERT2; *Fak*$^{flox/flox}$ mice compared to those of Alb-CreERT2 mice (Fig. 1D and F). Immunohistochemical staining signals for α-fetoprotein (AFP), a common HCC marker, were significantly lower in the livers of Alb-CreERT2; *Fak*$^{flox/flox}$ mice compared to those of Alb-CreERT2 mice (Fig. 1F). These data indicate that FAK deficiency in hepatocytes suppresses MET/CAT-induced HCC progression.

Suppression of HCC progression by deletion of *Fak* could be due to decreased proliferation or increased apoptosis of tumor cells[21]. To determine whether *Fak* deficiency affects proliferation in MET/CAT-induced HCC, we analyzed proliferation in MET/CAT-injected livers from Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice by Ki-67 staining. The number of Ki-67-positive cells was significantly decreased in FAK-deficient livers compared to WT livers (Fig. 1F and G). We then performed TUNEL staining on liver tissues from Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice that had been injected with MET/CAT. We did not find significant differences in apoptosis in livers when comparing Alb-CreERT2 to Alb-CreERT2; *Fak*$^{flox/flox}$ mice (Fig. 2B and C). These results suggest that *Fak* deficiency in hepatocytes suppresses MET/CAT-induced HCC maintenance by decreasing cell proliferation.

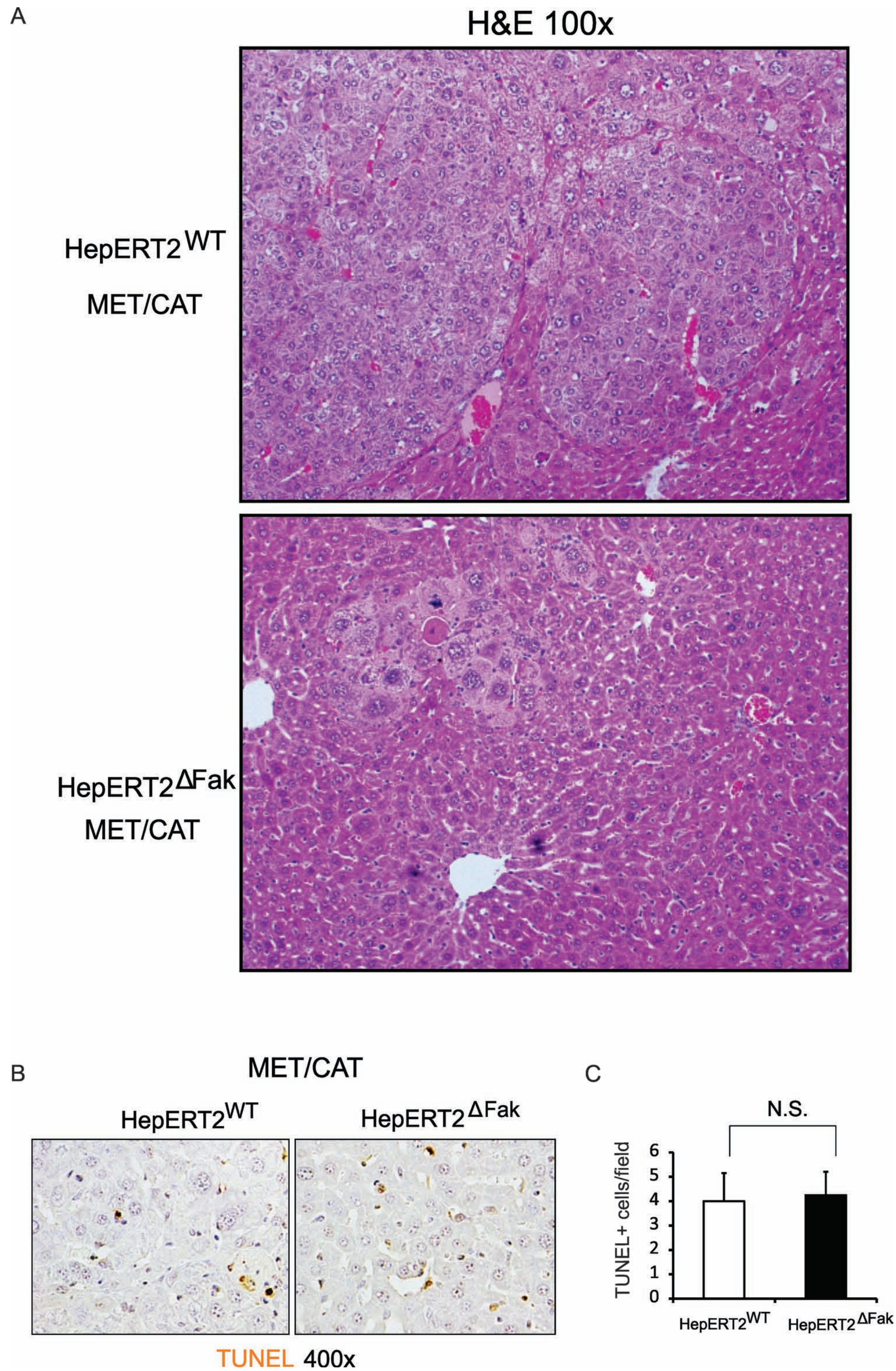

Figure 2. Inducible deletion of *Fak* following tumor formation does not affect liver inflammation or apoptosis in a MET/CAT-induced HCC mouse model. (A) H&E staining for the livers of Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice 7 weeks after injection of MET/CAT followed by tamoxifen injection. (B) TUNEL staining of the livers of Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice 7 weeks after injection of MET/CAT followed by tamoxifen injection. (C) Quantification of TUNEL staining for (B) ($n=5$).

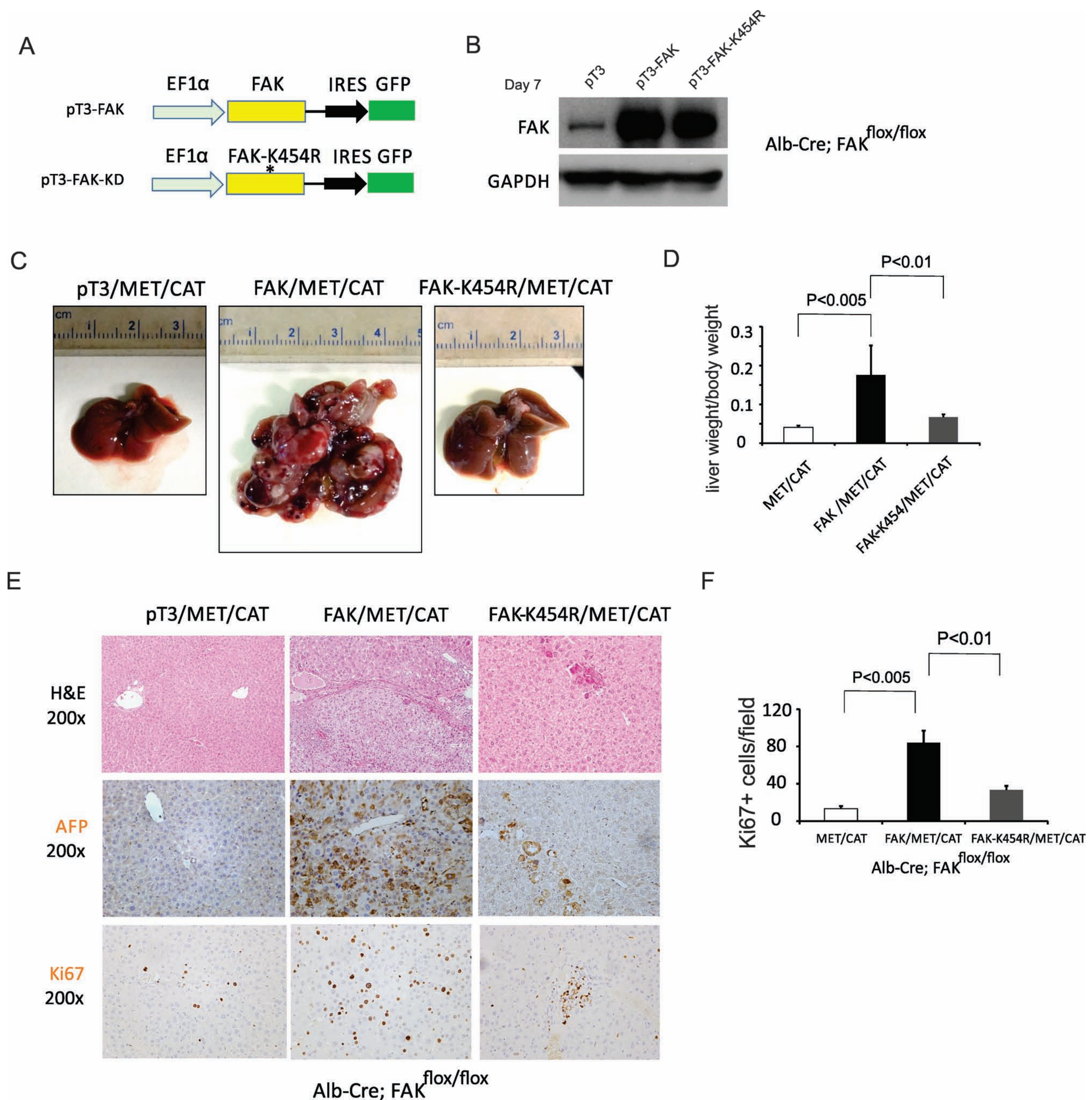

Figure 3. FAK kinase activity is critical for MET/CAT-induced hepatocarcinogenesis. (A) Diagrams of pT3-EF1α-FAK-IRES-GFP and pT3-EF1α-FAK (FERM)-IRES-GFP plasmids. (B) Expression of FAK and GAPDH proteins in the livers of Alb-CreERT2; *Fak*$^{flox/flox}$ mice that had been injected with pT3, pT3-FAK, or pT3-FERK-K454R for 7 days. (C) Photographs of the livers of Alb-Cre; *Fak*$^{flox/flox}$ mice 7 weeks after injection of pT3/MET/CAT, FAK/MET/CAT, and FAK-K454R/MET/CAT. (D) Liver weight/body weight ratio was analyzed in the mice from (D) (n=5). (E) Staining of the livers of Alb-CreERT2 and Alb-CreERT2; *Fak*$^{flox/flox}$ mice by H&E and also for AFP and Ki-67 at 7 weeks after injection of pT3/MET/CAT, FAK/MET/CAT, or FAK-K454R/MET/CAT. (F) Quantification of Ki-67 staining for (E) (n=5).

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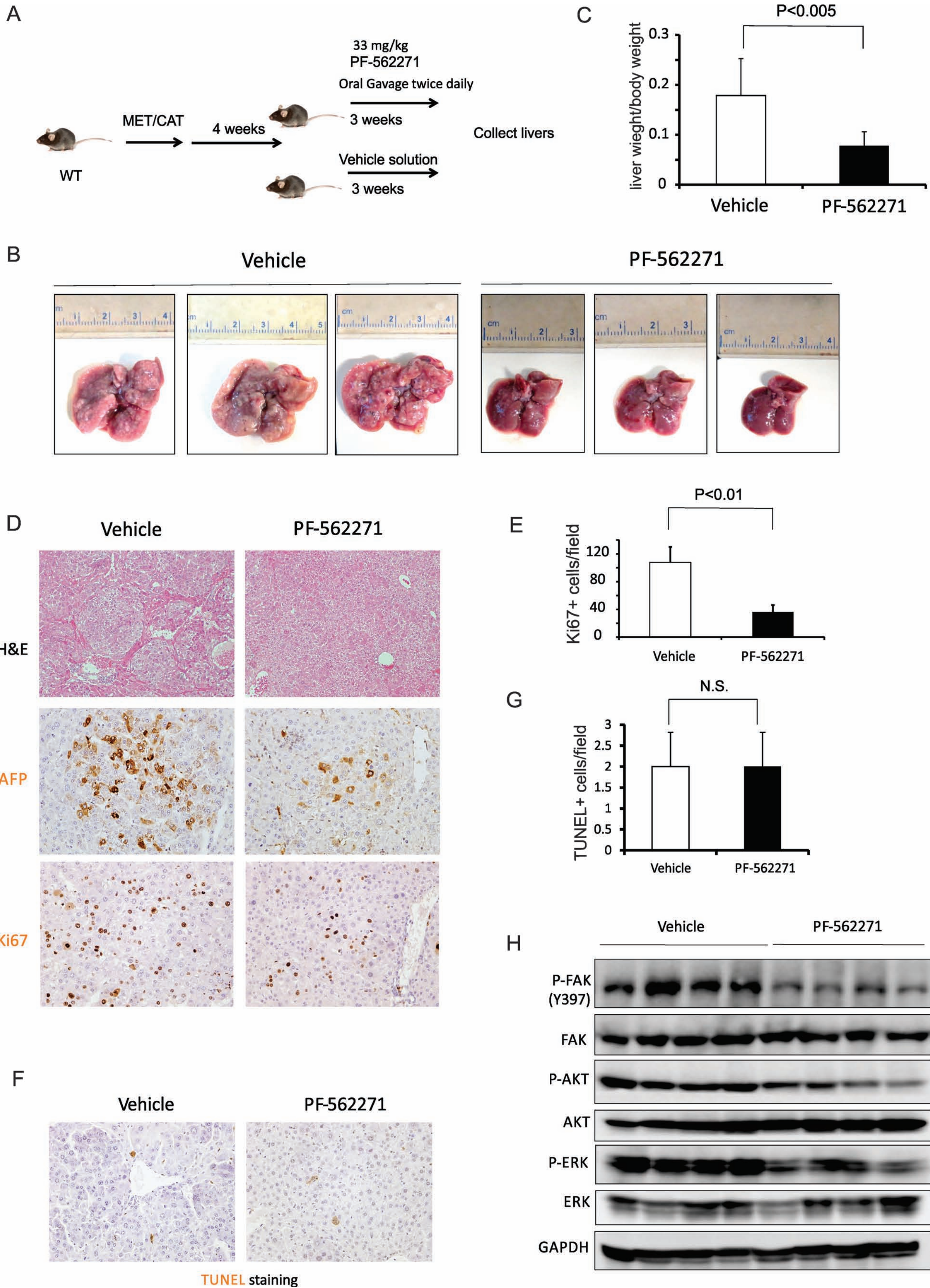
Figure 4. FAK inhibitor PF-562271 inhibits MET/CAT-induced hepatocarcinogenesis. (A) Diagram of the experimental protocol. (B) Photographs of the livers of WT C57/B6 mice after treatment with vehicle or PF-562271 as indicated in (A). (C) Liver weight/body weight ratio was analyzed in the mice from (B) (n=10). (D) H&E, AFP, and Ki-67 staining of the mouse livers from (C). (E) Quantification of Ki-67 staining for (D) (n=4). (F) Apoptosis in the livers of WT C57BL/6 mice after treatment with vehicle or PF-562271 was examined by TUNEL staining. (G) Quantification of TUNEL staining for (F) (n=4). (H) Expression of FAK, p-FAK (Y397), p-AKT, AKY, p-ERK, ERK, and GAPDH proteins in the livers of WT C57/B6 mice 7 weeks after treatment with vehicle or PF-562271.


A
WT
MET/CAT
4 weeks
33 mg/kg
PF-562271
Oral Gavage twice daily
3 weeks
Vehicle solution
3 weeks
Collect livers
C
P<0.005
liver wieght/body weight
0.3
0.2
0.1
0
Vehicle
PF-562271
B
Vehicle
PF-562271
D
Vehicle
PF-562271
H&E
AFP
Ki67
E
P<0.01
Ki67+ cells/field
120
80
40
0
Vehicle
PF-562271
G
N.S.
TUNEL+ cells/field
3
2.5
2
1.5
1
0.5
0
Vehicle
PF-562271
F
Vehicle
PF-562271
TUNEL staining
H
Vehicle
PF-562271
P-FAK (Y397)
FAK
P-AKT
AKT
P-ERK
ERK
GAPDH

We showed previously that *Fak* deficiency in hepatocytes before tumor formation decreases the activation of AKT and ERK[7]. We therefore examined whether *Fak* deficiency in hepatocytes after tumor formation also decreases the activation of AKT and ERK expression. We found that the levels of both phosphorylated-AKT and -ERK were significantly increased in Alb-CreERT2 livers after MET/CAT treatment. However, the phosphorylation levels of these two proteins were reduced in the livers of Alb-CreERT2; *Fak*$^{flox/flox}$ mice compared to those of Alb-CreERT2 mice (Fig. 1H), suggesting that deletion of *FAK* may suppress MET/CAT-induced HCC through the inhibition of AKT and ERK. Consistent with this, expression of cyclin D1, a downstream target of the PI3K/AKT and MAPK pathways, was also reduced in the livers of Alb-CreERT2; *Fak*$^{flox/flox}$ mice compared to those of Alb-CreERT2 mice (Fig. 1H).

Ectopic FAK Expression Restored HCC Formation in Hepatocyte-Specific Fak*-Deficient Mice*

We have shown that deletion of *Fak* in hepatocytes significantly suppresses MET/CAT-induced tumor development[7]. FAK is a nonreceptor tyrosine kinase, and its kinase activity plays a critical role in many of its functions[15,16]. In mouse livers and HCC cell lines, FAK was activated by MET, which induced the activation of AKT/ERK and upregulated cyclin D1[7]. CAT enhances MET-stimulated FAK activation[7]. Therefore, we hypothesized that the kinase activity of FAK is critical for MET/CAT-induced HCC development. However, FAK also has kinase-independent functions in different cellular processes[22,23]. To determine whether the kinase activity of FAK is critical for MET/CAT-induced HCC development, we examined whether the ectopic expression of *FAK* or a FAK kinase-dead mutant can restore MET/CAT-induced tumor formation to Alb-Cre; *Fak*$^{flox/flox}$ (Hep$^{\Delta Fak}$) mice.

We first generated a FAK transposon vector, pT3-EF1α-FAK-IRES-GFP (referred to here as pT3-FAK) (Fig. 3A). We then hydrodynamically injected pT3-FAK or the control pT3 plasmid with the transposase (HSB2) into experimental mice and collected the livers 1, 3, and 7 days after injection. Western blotting and IHC/IF staining indicated overexpression of FAK in the livers injected with pT3-FAK compared to those injected with the control pT3 (Fig. 3B). We next hydrodynamically injected pT3-FAK with MET and CAT transposons into Alb-Cre; *Fak*$^{flox/flox}$ mice, in which *Fak* is deleted in hepatocytes[7]. We found that the combination of FAK/MET/CAT restored HCC formation to Alb-Cre; *Fak*$^{flox/flox}$ mice (Fig. 3C–E). No tumors showed significant inflammation. These data indicate that ectopic FAK expression restores HCC formation in hepatocyte-specific *Fak*-deficient mice.

FAK Kinase Activity Is Important for MET/CAT-Induced Hepatocarcinogenesis

Mutation of *FAK* at residue 454 from lysine (K) to arginine (R) results in the inactivation of FAK kinase[24,25]. We generated a FAK kinase-dead mutant (K454R) transposon plasmid to test whether FAK kinase activity is required for HCC development (Fig. 3A). We hydrodynamically injected mice with FAK-K454R and confirmed overexpression of this plasmid in mouse livers (Fig. 3B). We hydrodynamically injected FAK or FAK-K454R along with MET and CAT into Alb-Cre; *Fak*$^{flox/flox}$ mice. Interestingly, there were considerably fewer tumors and less proliferation in Hep$^{\Delta Fak}$ mice injected with FAK-k454R/MET/CAT compared to FAK/MET/CAT (Fig. 3C–F), indicating that FAK kinase activity is required for MET/CAT-induced hepatocarcinogenesis.

FAK Inhibitor PF-562271 Inhibits MET/CAT-Induced Hepatocarcinogenesis

PF-562271, which inhibits FAK's tyrosine kinase activity, has been developed and used in clinical trials for other solid tumors[26]. It has been shown to effectively suppress tumor growth in prostate, pancreatic, colon, glioblastoma, and H460 lung xenotropic tumor models[27,28]. We therefore tested the efficacy of PF-562271 in suppressing HCC in our MET/CAT model. We hydrodynamically injected mice with MET/CAT to induce HCC and 4 weeks later treated these mice with 33 mg/kg PF-562271 daily for 3 weeks (Fig. 4A). Such a dose of PF-562271 has been shown to effectively inhibit FAK and tumor growth in a pancreatic cancer mouse model[27]. We found reduced HCC in mice treated with PF-562271 when compared to mice treated with vehicle solution (Fig. 4B–D). The sections of the livers treated with PF-562271 show multifocal dysplastic changes featuring increased cellular density and nuclear atypia. However, there were no trabecular or nested growth patterns, nor was there any significant increase in hepatocyte cord thickening, indicating that these remaining tumors contain dysplastic changes and are thus precursors of HCC. No significant inflammation was found in the livers from mice treated with either vehicle or PF-562271. In addition, less cell proliferation was found in livers of mice treated with PF-562271 compared to mice treated with vehicle solution (Fig. 4D and E). There was comparable apoptosis in the livers of mice treated with PF-562271 and mice treated with vehicle solution (Fig. 4F and G). Furthermore, Western blotting results confirmed that FAK phosphorylation was significantly decreased in the livers of mice treated with PF-562271 compared to mice treated with vehicle solution (Fig. 4H), which indicates efficiency of PF-562271 in inhibiting FAK activity. Consistently, phosphorylation of AKT and ERK was also decreased by treatment with

PF-562271 in the mouse livers (Fig. 4H). In general, these results demonstrate that PF-562271 effectively inhibits FAK activity and MET/CAT-induced hepatocarcinogenesis in mouse livers.

DISCUSSION

Treatment options for HCC are limited[2]. Currently, the most effective targeted therapeutic agent for advanced HCC, sorafenib, an inhibitor of several tyrosine protein kinases (VEGFR and PDGFR) and Raf kinases, only increases survival in patients with advanced HCC by 3 months[29]. Therefore, it is important to develop more effective therapeutic strategies and agents to treat HCC. In this study, we found that deletion of *Fak* after tumors have formed significantly repressed MET/CAT-induced tumor development, suggesting that FAK is critical for HCC progression. We previously reported that deletion of *Fak* before tumor formation inhibited MET/CAT-induced HCC formation[7]. Therefore, inhibition of FAK may be useful to prevent HCC in at-risk populations or to treat HCC in patients at advanced stages.

FAK can function through kinase-dependent and kinase-independent (scaffolding) mechanisms in a cell context-dependent manner. However, the role of FAK's kinase activity and scaffolding functions in HCC development remains largely unknown. Understanding these processes will provide a mechanistic basis to reduce protumor effects by inhibiting both/either FAK kinase and/or kinase-independent activities. In this study, our data indicate that the kinase activity of FAK is critical for MET/CAT-induced HCC development. Consistent with this, a FAK inhibitor, PF-562271, which inhibits FAK's tyrosine kinase activity[26], significantly suppressed MET/CAT-induced hepatocarcinogenesis. Our data provide a preclinical basis for the use of FAK inhibitors to treat patients with HCC. Several FAK inhibitors (including PF-562271) are currently in preclinical and clinical trials[5]. It will be exciting to test these inhibitors to treat HCC patients, particularly those with overexpression of c-MET and dysregulated β-catenin.

Although FAK kinase inactivation largely abolished FAK functioning in MET/CAT-induced hepatocarcinogenesis, there were more tumors in the Alb-Cre; *Fak*$^{flox/flox}$ mice injected with FAK-k454R/MET/CAT compared to pT3/MET/CAT (Fig. 3C–F). These data suggest that a FAK kinase-dead mutant can partially restore HCC formation to hepatocyte-specific *Fak*-deficient mice. Therefore, FAK kinase-independent functions might also play a role in MET/CAT-induced hepatocarcinogenesis. The FERM domain of FAK has been previously shown to promote cell proliferation and survival through a kinase-independent mechanism[22]. We intend to investigate the mechanisms by which FAK promotes the development of kinase-independent MET/CAT-induced HCC in future studies.

In conclusion, our study shows that FAK is critical for MET/CAT-induced tumor progression. FAK kinase activity is important for MET/CAT-induced HCC development. FAK inhibitors that inhibit FAK kinase activity (such as PF-562271) may have good efficacy against HCC in the clinical setting.

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