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Liver epithelial focal adhesion kinase modulates fibrogenesis and hedgehog signaling

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Abstract

Focal adhesion kinase (FAK) is an important mediator of extracellular matrix-integrin mechano-signal transduction that regulates cell motility, survival, and proliferation. As such, FAK is being investigated as a potential therapeutic target for malignant and fibrotic diseases, and numerous clinical trials of FAK inhibitors are underway. The function of FAK in non-malignant non-motile epithelial cells is not well understood. We previously showed that hepatocytes demonstrated activated FAK near stiff collagen tracts in fibrotic liver. In this study, we examined the role of liver epithelial FAK by inducing fibrotic liver disease in mice with liver epithelial FAK deficiency. We found that mice that lack FAK in liver epithelial cells develop more severe liver injury and worse fibrosis as compared to controls. Increased fibrosis in liver epithelial FAK-deficient mice is linked to the activation of several pro-fibrotic pathways, including the hedgehog-smoothened pathway. FAK-deficient hepatocytes produce increased Indian hedgehog in a manner dependent on matrix stiffness. Furthermore, expression of the hedgehog receptor, smoothened, is increased in macrophages and biliary cells of hepatocyte-specific FAK-deficient fibrotic liver. These results indicate that liver epithelial FAK has important regulatory roles in the response to liver injury and progression of fibrosis.
**Introduction**

Mechano-signal transduction is the process through which physical stimuli are converted to biochemical signals within cells that orchestrate cell morphology and behavior. Focal adhesion kinase (FAK) is a key molecular mechano-sensor that connects the extracellular matrix with a cell’s cytoskeleton (1). Through its dual function as a tyrosine kinase and scaffolding protein, FAK mediates signals triggered by integrin binding to extracellular matrix ligands and links integrin activation, via adaptor proteins, to actin cytoskeletal responses (2). The downstream effects of FAK-mediated signals are multiple, and include stimulation of cell motility, proliferation, and survival (3, 4). FAK also broadly interacts with and cross-regulates other mechano-sensors and mediators of integrin signals, including Src (5, 6), Rho family GTPases (7, 8), integrin-linked kinase (ILK) (9, 10), and Yes-associated protein (YAP) (11, 12).

FAK is over-expressed in many types of cancers. Elevated FAK expression or activation correlates with increased invasiveness, metastases, and poorer prognosis (13). Stiffened tumor stroma activates FAK and downstream cell contractility, which cooperate with growth-factor-mediated signals to potentiate proliferation and invasive behavior of malignant cells (7, 8). Hepatocellular carcinoma is one cancer type that frequently demonstrates FAK over-expression and/or hyper-phosphorylation (14). Culture of hepatocellular carcinoma cell lines on stiff matrices induced FAK activation and promoted proliferation and chemotherapy resistance (15). Moreover, deletion of FAK in hepatocytes repressed the development of a mouse model of hepatocellular carcinoma (16). Accordingly, FAK is thought to be a promising anti-cancer therapeutic target (17). Numerous FAK-specific small molecule inhibitors have been developed
(18), and several ongoing clinical trials are testing the safety and efficacy of FAK inhibition in the treatment of aggressive solid tumors in humans (19, 20).

In addition to proliferative and pro-survival effects, tumor-intrinsic FAK activation has been shown to amplify fibrotic stiffening of the peri-tumor stroma, resulting in bidirectional tumor-stroma positive feedback of mechanics-induced signaling (21, 22). A stiffened microenvironment (23-25) and FAK activation (26, 27) are required for the differentiation of myofibroblasts, which are key fibrogenic effector cells that deposit pathological collagen matrix. In chronic fibrotic disorders, FAK activity is elevated in intra-lesional skin fibroblasts from patients with systemic sclerosis (28) and in lung myofibroblasts from patients with idiopathic pulmonary fibrosis (29). FAK signaling is required for fibroblast and hepatic stellate cell durotaxis, in which motile cells migrate directionally toward more rigid matrix (12, 30, 31). FAK activation is required to induce lung myofibroblast profibrotic phenotype and administration of FAK inhibitors in vivo attenuates the development of an experimental model of lung fibrosis (32). Likewise, FAK activation is required for hepatic stellate cell expression of α-smooth muscle actin and profibrotic collagens. Treatment with FAK inhibitors reduces the severity of carbon tetrachloride-induced liver fibrosis in mice (33). These preclinical studies have motivated considerable interest in developing FAK inhibitors as a novel therapeutic for fibrotic diseases (34). However, while activation of FAK signaling in myofibroblasts may be profibrotic, less is known regarding the function of FAK in parenchymal epithelial cells. It is possible that FAK serves important homeostatic functions in epithelial cells related to cell adhesion and mechanosensing. Global inhibition of FAK with systemic inhibitor administration may demonstrate a predominant effect on suppressing
myofibroblast activation, but mask concurrent detrimental effects on epithelial homeostasis. In this study, in order to delineate potentially differential roles of FAK signaling in different cell types during fibrogenesis, we investigated the effect of liver epithelial-specific deletion of FAK on liver fibrosis.

There is evidence to suggest that tonic integrin- and FAK-mediated signals are important for maintaining liver homeostasis. Prolonged knock-down of β1-integrin in hepatocytes results in distorted bile canaliculi morphology and liver injury (35, 36). Inhibition or deletion of hepatocyte β1-integrin severely impairs liver regeneration (36, 37). Hepatocytes exhibit FAK activation adjacent to stiff collagen tracts in fibrotic liver in vivo and when cultured on top of stiff matrix in vitro (38), indicating that hepatocytes are mechanically-responsive through FAK signaling. Moreover, data suggest that epithelial FAK signaling is protective in fibrotic disease, since deletion of FAK in alveolar epithelial cells promoted apoptosis in response to TGFβ (39, 40) and resulted in greater lung injury and mortality in experimental models of lung fibrosis (39).

Given the potential benefits of FAK inhibition in treating malignant and/or fibrotic conditions, it is important to determine whether there may be adverse off-target effects on normal and protective epithelial functions. We hypothesized that liver epithelial FAK played important roles in maintaining hepatic homeostasis and in regulating response to fibrotic injury. We designed a series of experiments with liver epithelial-specific FAK-deficient mice to examine the hypothesis.
**Results**

RNA-seq analysis suggests that FAK-deficient hepatocytes have decreased synthetic and proliferative functions, and increased cell stress and lipid metabolism. We bred FVB FAK^{fl/fl} mice (41) with albumin (Alb)-Cre mice (42) to generate liver epithelial-specific FAK-deficient mice (FAK^{fl/fl};Alb-Cre^{+} mice) and littermate controls (FAK^{fl/fl};Alb-Cre^{-}). Because albumin is expressed in bipotential progenitor cells in the fetal liver, Alb-Cre-mediated gene deletion occurs within the fetal liver and manifests both in hepatocytes and cholangiocytes (43-47). We found that FAK^{fl/fl};Alb-Cre^{+} mice were born in the expected Mendelian ratios and had normal lifespans (>1 year.) FAK^{fl/fl};Alb-Cre^{+} mice aged 6-8 weeks old had liver FAK protein expression less than 15% of wild-type levels in littermate controls (Supplemental Figure 1) and exhibited normal liver architecture histologically and normal serum liver function tests (Supplemental Figure 2).

To determine whether liver epithelial FAK deletion induced cellular changes not readily detectable by gross phenotypic characterization, we performed RNA-seq analysis on whole liver tissue of six-to-eight-week-old FAK^{fl/fl};Alb-Cre^{+} mice and control FAK^{fl/fl};Alb-Cre^{-} littermates. We also performed RNA-seq on isolated hepatocytes to distinguish hepatocyte-specific versus non-parenchymal cell-specific gene expression effects. Differential gene expression analysis of whole-liver tissue or isolated hepatocytes from FAK^{fl/fl};Alb-Cre^{+} compared to FAK^{fl/fl};Alb-Cre^{-} mice revealed 782 genes, which were hierarchically classified into 4 clusters based on expression pattern (Figure 1). Clusters 1 and 2 represented genes that were downregulated in FAK^{fl/fl};Alb-
Cre+ liver and hepatocytes, whereas clusters 3 and 4 contained genes that were upregulated in FAKfl/fl;Alb-Cre+ liver and hepatocytes.

Gene ontology (GO) analysis showed that coagulation factors (Figure 2A), which represented a major class of serum proteins produced by hepatocytes, and cell proliferation genes (Figure 2B) were downregulated in FAKfl/fl;Alb-Cre+ samples. Conversely, genes associated with cell stress (Figure 2C) and lipid metabolism (Figure 2D) were upregulated in FAKfl/fl;Alb-Cre+ samples. Many genes within these functional categories were up- or downregulated more markedly in isolated hepatocytes than in whole-liver tissue, suggesting that these gene expression changes were hepatocyte-specific. Several differentially regulated genes were nearly undetectable in isolated hepatocytes and showed differences only in whole-liver tissue, suggesting a non-parenchymal cell source. These included upregulation of extracellular matrix (Figure 2E) and biliary cell-specific genes (Figure 2F) in FAKfl/fl;Alb-Cre+ whole-liver tissues as compared to controls. These results suggest that liver epithelial FAK deficiency reduces hepatocyte-specific synthetic and proliferative functions, while increasing hepatocyte cell stress and lipid metabolism, and inducing activation of extracellular matrix and biliary cells within the liver.

Liver epithelial FAK-deficient mice show greater signs of liver injury and steatosis with increased age as compared to age-matched controls. To determine whether gene expression indicating increased cell stress and lipid metabolism in FAK-deficient hepatocytes manifested phenotypically with time, we analyzed the serum liver function tests and liver histology of FAKfl/fl;Alb-Cre+ mice and FAKfl/fl;Alb-Cre− littermate controls.
<40g at 6 months of age. We found that liver epithelial FAK deletion induced liver injury and steatosis and that the effect differed between male and female mice. Two-way ANOVA indicated significant interaction (p=0.025) between the effects of genotype (WT vs. FAK-/-) and sex (male vs. female) on serum alanine transaminase (ALT) levels (Figure 3A). Simple main effect analysis showed that FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice had significantly higher ALT than wild-type littermates (p=0.028), indicating greater hepatocellular injury. However, higher ALT was observed only in male FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> compared to male FAK<sup>fl/fl</sup>;Alb-Cre<sup>-</sup> mice; female FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> and FAK<sup>fl/fl</sup>;Alb-Cre<sup>-</sup> mice showed no difference. For alkaline phosphatase, sex was the main source of variation, with female mice showing higher levels than male mice (p=0.002). These results suggest that absence of liver epithelial FAK signaling induces liver injury with time and that manifestations are sexually dimorphic.

Histologic analysis demonstrated increased steatosis, without evidence of steatohepatitis or fibrosis, in the livers of 6-month-old FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice compared to age-matched controls (Figure 3B). A liver pathologist scored the severity of steatosis, and 2-way ANOVA indicated that FAK genotype was a significant source of variation (p=0.007). Post-hoc analysis demonstrated that male FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice had significantly more steatosis than male controls, and female FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice showed a similar trend compared to female controls (Figure 3C). These results show that increased expression of cell stress and lipid metabolism genes in FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice eventually correlate with development of liver injury and steatosis as mice age.
Liver epithelial FAK-deficient mice had significantly higher early mortality after bile duct ligation as compared to controls. Since liver epithelial FAK deficiency led to gradual hepatocellular damage with aging, we hypothesized that absence of epithelial FAK signaling would also impair adaptive responses to acute liver injury. Bile duct ligation is a surgical model of obstructive cholestasis that leads to severe liver fibrosis in 4 weeks (48). We performed bile duct ligation on male and female FAK^{fl/fl};Alb-Cre^{+} or FAK^{fl/fl};Alb-Cre^{-} mice between the ages of six and eight weeks. Whereas the majority of FAK^{fl/fl};Alb-Cre^{-} control mice survived the immediate post-operative period, the vast majority of FAK^{fl/fl};Alb-Cre^{+} mice died within the first few days after the procedure (Figure 4A). Eleven out of 20 FAK^{fl/fl};Alb-Cre^{-} mice (55%) survived up to the end of the 5-week experimental period and developed fibrotic liver disease consistent with the model. In contrast, the median survival of FAK^{fl/fl};Alb-Cre^{+} mice after bile duct ligation was only 2 days, and only 2 out of 14 mice (14%) remained alive at 5 weeks. Survival rates did not differ between male and female mice. Liver histology of FAK^{fl/fl};Alb-Cre^{+} mice that died one day after bile duct ligation did not show obvious signs of liver parenchymal disruption or damage (Figure 4B). FAK^{fl/fl};Alb-Cre^{+} mice that survived until week 5 showed similar patterns of liver inflammation, parenchymal necrosis, and fibrosis as compared to FAK^{fl/fl};Alb-Cre^{-} controls. Nevertheless, these results suggest that liver epithelial cell FAK expression is critical for the immediate adaptive response to acute obstructive cholestatic injury.

Liver epithelial FAK-deficient mice develop more severe liver injury and fibrosis in response to 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) as compared to
controls. Bile duct ligation caused rapid early mortality in FAK^{fl/fl};Alb-Cre^+ mice and very few mice survived long enough to develop fibrosis. Therefore, to determine the effect of absent liver epithelial FAK signaling on fibrogenesis, we turned to the 0.1% DDC diet model that induced more gradual obstructive cholestasis and biliary fibrosis (49). All FAK^{fl/fl};Alb-Cre^- and FAK^{fl/fl};Alb-Cre^+ mice on the DDC diet survived until the 4-week endpoint. Male FAK^{fl/fl};Alb-Cre^+ mice lost significantly more weight than male FAK^{fl/fl};Alb-Cre^- controls, whereas weight loss did not differ between female FAK^{fl/fl};Alb-Cre^+ and FAK^{fl/fl};Alb-Cre^- mice (Figure 5A). Compared to their littermate controls, both male and female FAK^{fl/fl};Alb-Cre^+ mice demonstrated increased cholestasis, as indicated by serum total bilirubin and alkaline phosphatase, and hepatocellular injury, as indicated by serum aspartate transaminase (AST) (Figure 5B). Histological analysis showed similar peri-portal inflammation, ductular reaction, and fibrosis between the 2 groups (Figure 5C). Quantitation of hydroxyproline to determine collagen content indicated that the livers of male FAK^{fl/fl};Alb-Cre^+ mice had significantly more collagen, and thus more fibrosis, than those of male littermate controls (Figure 5D). Male FAK^{fl/fl};Alb-Cre^+ mice also showed significantly more hydroxyproline than female FAK^{fl/fl};Alb-Cre^+ mice. Using quantitative real-time reverse transcription PCR (qRT-PCR), we further analyzed the mRNA expression of fibrillar (Col1a1, Col1a2, Col3a1), microfibrillar (Col6a1), and fibril-associated (Col12a1) collagen species that were elevated in fibrotic liver diseases (50). We found that female wild-type FAK^{fl/fl};Alb-Cre^- mice showed higher expression of these collagen genes than male wild-type FAK^{fl/fl};Alb-Cre^- mice, suggesting that female mice developed more severe fibrosis compared to male mice at baseline. Importantly, the livers of male FAK^{fl/fl};Alb-Cre^+ mice showed
greater mRNA expression of all of these collagen species than did livers of male wild-type littermates (Figure 5E). These results indicate that liver epithelial FAK deficiency leads to increased DDC-induced liver fibrosis in male mice.

**Hepatocyte-specific FAK deletion results in greater expression of fibrotic collagen genes in response to DDC-induced fibrosis as compared to controls.**

Studies suggest that FAK^{fl/fl};Alb-Cre+ mice delete FAK both from hepatocytes and cholangiocytes at the fetal liver stage (43-47). To determine the effect of absent hepatocyte FAK signaling immediately before pro-fibrotic liver injury, we injected FAK^{fl/fl} mice with adeno-associated virus serotype 8 (AAV8)-thyroxine binding globulin (TBG)-Cre vector to induce FAK deletion only in hepatocytes (51). Control FAK^{fl/fl} mice were given AAV8-TBG-Null that did not express the Cre recombinase. DDC diet was initiated 2 weeks after viral vector administration when liver FAK expression in AAV8-TBG-Cre treated mice was reduced to <15% of wild-type control levels (Supplemental Figure 3).

In contrast to FAK^{fl/fl};Alb-Cre+ mice, male FAK^{fl/fl} mice injected with AAV8-TBG-Cre did not have greater weight loss than male mice treated with AAV8-TBG-Null (Figure 6A). Female FAK^{fl/fl} mice treated with AAV8-TBG-Cre initially lost weight more rapidly than female controls, though weights became comparable later. Total bilirubin levels were slightly lower in AAV8-TBG-Cre treated mice compared to controls; alkaline phosphatase, AST, and ALT did not show significant differences (Figure 6B). Weight and serum liver function tests of Null- and Cre-virus treated mice did not significantly change when DDC diet was extended to 8 weeks as compared to 4 weeks (data not shown). Null- and Cre-virus treated FAK^{fl/fl} mice demonstrated similar levels of peri-
portal inflammation, ductular reaction, and fibrosis (Figure 6C). Although no differences in liver collagen were detected by hydroxyproline assay (Figure 6D), AAV8-TBG-Cre treated mice showed greater mRNA expression of fibrotic collagen genes as compared to controls (Figure 6E). Interestingly, in contrast to FAK\(^{+/+}\);Alb-Cre\(^{+}\) mice in which fibrotic collagen mRNA expression was increased only in males, AAV8-TBG-Cre mediated FAK deletion led to increased collagen mRNA expression in both sexes. These results indicate that AAV-mediated hepatocyte FAK deletion produced less severe DDC-induced liver injury and fibrosis than Alb-Cre-mediated liver epithelial FAK deletion, but both methods of FAK deletion led to increased expression of fibrotic collagens.

RNA-seq analysis shows upregulation of several fibrosis regulatory pathways in FAK\(^{+/+}\);Alb-Cre\(^{+}\) mice and FAK\(^{+/+}\) mice treated with AAV8-TBG-Cre, compared with their respective controls. We hypothesized that the absence of FAK signaling in hepatocytes activated profibrotic pathways, leading to increased transcription of fibrotic collagens. To identify these pathways, we performed RNA-seq analysis on DDC-induced fibrotic whole-liver tissue from FAK\(^{+/+}\);Alb-Cre\(^{+}\) and FAK\(^{+/+}\);Alb-Cre\(^{-}\) mice (“Bred” method of FAK deletion) as well as FAK\(^{+/+}\) mice treated with AAV8-TBG-Cre or AAV8-TBG-Null (“Virus” method of FAK deletion). We defined FAK genotype (WT vs. KO) and method of FAK deletion (Bred vs. Virus) as independent variables in 2-way ANOVA, which revealed 896 significantly differentially regulated genes. We hierarchically classified these genes into 8 clusters based on their expression patterns across the 4 groups: Virus WT, Bred WT, Virus KO, and Bred KO (Figure 7A and 7B). We were
most interested in genes that were highly upregulated in KO mice induced by both Bred and Virus methods of deletion as compared to their respective WT controls (i.e. cluster 7). We hypothesized that genes with this expression profile represented pathways that were most robustly upregulated as a result of FAK deficiency in hepatocytes. Indeed, GO analysis of genes highly upregulated in both Bred and Virus methods of FAK deletion revealed several important fibrosis regulatory pathways (Figure 7C and Table 1). Confirming qRT-PCR results reported in Figures 5 and 6, RNA-seq analysis identified collagen fibril-forming genes as a significant functional category upregulated in both Bred and Virus KO mice. In addition, smoothened (Smo), Wnt, TGFβ, epithelial-mesenchymal transition, and polarity pathways were significantly upregulated both in Bred and Virus KO mice, as compared to their respective controls. Smo, Wnt, and TGFβ are master regulatory pathways of fibrogenesis (52-54). Epithelial-mesenchymal transition and changes in planar cell polarity are cellular processes implicated in the progression of fibrosis (55, 56). Consistent with FAK^{fl/fl};Alb-Cre^{+} mice developing more severe disease than FAK^{fl/fl} mice treated with AAV8-TBG-Cre, upregulation of many of these pathways were significantly greater in the Bred model than in the Virus model. We examined the expression of key gene members of the Smo, Wnt, and TGFβ pathways (Figure 7D, 7E, and 7F, respectively), and they were significantly more highly expressed in KO than in WT in both Bred and Virus models. These results indicate that multiple cross-regulatory pro-fibrotic pathways are upregulated in the liver as a result of hepatocyte FAK deficiency in response to chronic fibrogenic injury.
FAK-deficient hepatocytes show greater stiffness-dependent expression of Indian hedgehog (Ihh); macrophages and biliary cells express elevated Smo in fibrotic hepatocyte-specific FAK-deficient liver. The hedgehog (Hh)-Smo pathway is a major pro-fibrotic signaling pathway (53), and hepatocyte expression of Ihh has been shown to promote liver fibrosis (57). Our RNA-seq data indicated that, compared to controls, hepatocyte-specific FAK-deficient fibrotic liver expressed significantly higher levels of both the ligand Ihh and the receptor Smo (Figure 7D). We hypothesized that pro-fibrotic injury stimulated greater Ihh expression from FAK-deficient hepatocytes, leading to greater activation of Smo-expressing target cells and more severe fibrosis. To test this, we isolated hepatocytes from the livers of six-to-eight-week-old FAKfl/fl;Alb-Cre− and FAKfl/fl;Alb-Cre+ mice and cultured isolated hepatocytes on top of collagen-coated polyacrylamide gels of 140Pa, 1kPa, or 6kPa stiffness. Previously, we had shown that 140Pa was the matrix rigidity of normal liver, and 1-6kPa was the stiffness of fibrotic liver matrix (38). Wild-type hepatocytes isolated from FAKfl/fl;Alb-Cre− mice showed uniformly low expression of Ihh on all 3 matrix stiffnesses. FAK-deficient hepatocytes demonstrated significantly higher levels of Ihh compared to wild-type when cultured on top of 1kPa and 6kPa gels (Figure 8A). Whole-liver tissue and freshly isolated hepatocytes from FAKfl/fl;Alb-Cre− and FAKfl/fl;Alb-Cre+ mice did not show significant differences in Ihh expression (data not shown). These results indicate that absent FAK signaling in hepatocytes leads to increased Ihh expression in response to rigid fibrotic matrix.

Next, we wanted to identify the target cell(s) in fibrotic liver responsive to increased Ihh signals derived from FAK-deficient hepatocytes. Stellate cells are a major
target of Hh signals and the producers of fibrotic matrix (58-63). We isolated stellate cells from FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{-} and FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{+} mice with DDC-induced liver fibrosis and determined fibrotic and Hh target gene expression (Figure 8B). Stellate cells from FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{+} fibrotic livers showed higher activation (Acta2) and collagen expression (Col1a1 and Col1a2) than in controls, consistent with the more clinically severe fibrosis that developed. However, expression of Smo and other Hh-Smo pathway genes (Gli2, Gli3, and Ptch1) was similar in stellate cells isolated from fibrotic FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{-} and FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{+} liver. These findings suggest that increased Ihh production by FAK-deficient hepatocytes promoted stellate cell activation and fibrillar collagen deposition through an indirect mechanism.

In order to identify the Smo\textsuperscript{+} Ihh-responsive cells, we performed multi-plexed RNAscope analysis on DDC-induced fibrotic liver tissue from FAK\textsuperscript{fl/fl} mice treated with AAV8-TBG-Null or AAV8-TBG-Cre. We found that Smo\textsuperscript{+} cells were localized to peri-portal areas of inflammation and fibrosis (Figure 8C). Consistent with greater Smo expression as demonstrated by RNA-seq (Figure 7), RNAscope showed that there were higher numbers of Smo\textsuperscript{+} dots in the peri-portal regions of AAV8-TBG-Cre-treated mice as compared to controls. Smo\textsuperscript{+} dots co-localized with cells that expressed Adgre1 (also known as F4/80), which marked activated macrophages. In addition, Smo\textsuperscript{+} dots co-localized with cells that expressed Krt19 (cytokeratin 19), which marked biliary cells, proliferating ductular cells, and progenitor cells within ductular reactions. We quantified the numbers of Smo\textsuperscript{+} dots in Adgre1\textsuperscript{+} cells (Figure 8D) and Krt19\textsuperscript{+} cells (Figure 8E), and found that these cells in AAV8-TBG-Cre-treated mice expressed significantly more Smo than did controls. These results suggest that absence of
hepatocyte FAK signaling induces greater hepatocyte *Ihh* expression, which activates Smo-dependent signaling in macrophages and biliary cells, leading to more severe fibrosis.
Discussion

Our study shows that liver epithelial-specific deletion of FAK led to hepatocyte dysfunction and cell stress, which eventually manifested as liver injury as mice aged. Mice with liver epithelial FAK deficiency died rapidly when challenged with acute biliary obstruction and developed more severe fibrosis in response to chronic cholestatic injury. Absence of hepatocyte FAK induced upregulation of several pro-fibrotic signaling pathways, including the Hh-Smo pathway. Moreover, FAK-deficient hepatocytes showed increased expression of Ihh that likely stimulated Smo+ macrophages and biliary cells to promote fibrogenesis. These findings indicate that FAK signaling has critical roles in the homeostatic function of hepatocytes and in regulating liver fibrosis progression.

Our results are consistent with previously published observations that FAKfl/fl;Alb-Cre+ mice did not develop obvious liver abnormalities at the typical adult age investigated in laboratory studies (i.e. 6-8 weeks-old) (16). We showed that liver damage due to absent liver epithelial FAK signaling accumulated over time and was triggered by acute or chronic insults. Accordingly, our findings are also consistent with data indicating that integrin-mediated signals in hepatocytes have important homeostatic and protective functions. Prolonged hepatocyte β1-integrin knock-down distorted bile canaliculi morphology, induced liver dysfunction, and inhibited liver regeneration (35-37). Moreover, our results add to the body of evidence that epithelial FAK signaling has a regulatory role in response to organ injury and fibrosis. Alveolar epithelial FAK deletion induced greater epithelial apoptosis, lung injury, and mortality in experimental lung fibrosis (39, 40). Similarly, we showed that liver epithelial FAK
deletion led to greater liver injury, fibrosis, and activation of pro-fibrotic pathways in experimental liver fibrosis.

The two methods, “Bred” and “Virus,” that we used to induce liver epithelial FAK deletion yielded different results. Overall, in response to DDC, FAK deletion through the Bred method demonstrated greater liver injury, fibrosis, and activation of pro-fibrotic pathways than the Virus method. There could be at least 2 non-mutually exclusive reasons for this finding. Our results indicated that the absence of hepatocyte FAK signaling caused increased cell stress that manifested as hepatocellular injury over time. Since the Bred method induced FAK deletion in the fetal liver, FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice might have shown more marked liver injury and fibrosis because their livers were already under significant stress due a life-long absence of FAK. In contrast, in the Virus method, FAK<sup>fl/fl</sup> mice treated with AAV8-TBG-Cre developed FAK deficiency just before initiation of DDC-induced injury; the livers of these mice might have been relatively more robust and more able to withstand the pro-fibrotic insult. Another consideration is that the Bred method deleted FAK both in hepatocytes and cholangiocytes (43-47), whereas the Virus method deleted FAK only in hepatocytes (51). It is possible that FAK plays an important role in cholangiocytes as well as in hepatocytes (64), and thus, the absence of FAK in both cell types induces more severe liver injury and fibrosis in FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> mice. Importantly, our results show that AAV8-TBG-Cre-mediated FAK deletion was sufficient to induce greater fibrotic collagen expression and activation of pro-fibrotic pathways, indicating that the absence of FAK in hepatocytes alone was enough to promote more severe liver fibrosis.
Our results showed that differences between male FAK$^{fl/fl};$Alb-Cre$^+$ mice and male controls were more significant than between female FAK$^{fl/fl};$Alb-Cre$^+$ mice and female controls. Six-month-old male FAK$^{fl/fl};$Alb-Cre$^+$ mice demonstrated significantly increased serum ALT and steatosis, whereas female mice did not, indicating that sex modified the manifestations of liver epithelial FAK deficiency. Sexual dimorphism in liver function and diseases is well-documented (65, 66). Sex-specific differences we observed in DDC-induced liver fibrosis may be at least partially attributable to differential cytochrome-mediated metabolism of DDC in male and female mice (67). Importantly, regulation of FAK signaling may be sexually dimorphic in ways both dependent and independent of sex hormones. Estrogen receptor activation in human ovarian cancer cells activates FAK (68). In mice, there is a female-specific vitronectin-FAK-IL-6 regulatory circuit that is independent of sex hormones (69). Vascular smooth muscle cells from female rats are more resistant to anoikis and exhibit increased FAK activation and survival by autophagy compared to cells from male rats (70). These observations, along with our results, suggest that sexual dimorphic effects of FAK signaling may be due to a variety of different mechanisms. Greater understanding of the sex-specific effects of FAK activation is of interest for future research, and will provide crucial insight for the clinical translation of FAK-based therapies to female and male patients.

Finally, our results indicate that hepatocyte FAK deficiency activates Hh-Smo, a pleiotropic signaling pathway with key regulatory roles in embryonic development, cancer, and fibrosis (53, 71, 72). Hh-Smo signaling is activated in the course of fibrogenesis in multiple organs, including the liver, and inhibition of Hh-Smo reduces
fibrosis progression (53). Whereas normal adult liver produces very little Hh ligand, injured hepatocytes are an important source of Ihh (57, 73, 74). Many different cell types respond to Hh signals in the inflammatory fibrotic liver microenvironment, including stellate cells (58-63), macrophages (73, 75), cholangiocytes (76-78), and progenitor cells (59, 73, 74, 76). Our results suggest that FAK-deficient hepatocytes upregulate Ihh expression and stimulate Smo⁺ macrophages and bile ductular cells in peri-portal areas of inflammation. Other studies have shown that Hh-responsive macrophages and cholangiocytes secondarily activate stellate cells through paracrine cytokines/chemokines, and thereby increase fibrotic collagen production (73, 75-78). Our data are consistent with a model in which FAK-deficient hepatocytes produce Ihh that stimulates Smo⁺ macrophages and biliary cells, which in turn stimulate stellate cells to increase production of fibrotic collagens. Although we showed that stellate cells in FAK-deficient fibrotic liver did not upregulate Smo and other Hh-Smo pathway genes, hepatocyte-derived Ihh could still activate stellate cells through non-canonical Smo-independent Gli-independent signaling (73). Future studies are needed to elucidate the precise mechanisms of how hepatocyte FAK signaling regulates Hh-Smo inter-cellular communication and subsequent fibrosis progression. Moreover, upregulated Ihh in FAK-deficient hepatocytes might represent a generalized response of injured hepatocytes (74), or alternatively, might be due to dysregulated mechanosensing in the absence of FAK signaling, as suggested by our results that FAK-deficient hepatocytes expressed greater Ihh when cultured on matrix of higher rigidity. Compensatory and/or parallel mechanosensing mechanisms, such as ILK, Src, Rho, and/or YAP/tafazzin (TAZ), might be stimulated in the absence of FAK and induce aberrant Ihh expression.
Interestingly, TAZ has been shown to induce hepatocyte expression of Ihh in non-alcoholic steatohepatitis (57), and ILK has been shown to interact with Smo to mediate Hh signaling (79).

In conclusion, our findings show that liver epithelial FAK has important roles in maintaining liver homeostasis and in regulating liver fibrosis. Although these findings raise caution for the use of FAK inhibitors in treating malignant and fibrotic conditions, it is likely that oncogenic cells and myofibroblasts have different setpoints of FAK activity than normal epithelial cells. FAK inhibition may remain a promising strategy if specific cell types of interest can be targeted, while sparing normal epithelial cells. Additional studies to determine the safety, efficacy, and potential off-target effects of FAK inhibitor-based therapies are needed.
Methods

Mice

Liver epithelial-specific FAK-deficient mice (FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{+}) and littermate controls (FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{-}) were generated by breeding FVB FAK\textsuperscript{fl/fl} mice (gift from Hilary E. Beggs) (41), in which the FAK gene was flanked by loxP sites, with Alb-Cre mice (gift from Derek LeRoith) that carry the Cre recombinase under the albumin promoter (42). Hepatocyte-specific FAK deletion was induced by tail-vein injection of FAK\textsuperscript{fl/fl} mice with 2.5x10\textsuperscript{11} AAV8-TBG-Cre (gift from James M. Wilson; Addgene viral prep # 107787-AAV8; http://n2t.net/addgene:107787; RRID:Addgene_107787; Addgene, Watertown, MA). Littermates controls were injected with 2.5x10\textsuperscript{11} viral particles/mouse of control viral vector that did not express Cre recombinase (AAV8-TBG-Null). All experimental mice were cared for in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.”

Liver fibrosis models

Acute obstructive cholestasis was induced by bile duct ligation using standard described surgical techniques (80). Gradual cholestatic injury was induced by providing mice with 0.1\% w/w DDC diet (TestDiet, St. Louis, MO) ad libitum for 4-8 weeks (49).

Serum and tissue analyses

Blood and liver were collected from mice under inhaled isoflurane anesthesia. Fresh unfrozen sera were analyzed by ADVIA Chemistry XPT System (Siemens, Malvern, PA) for AST, ALT, total bilirubin, and alkaline phosphatase. The entire liver was recovered
and apportioned for histologic, RNA, protein, and total collagen content analyses. For histology, liver tissues were fixed in 10% buffered formalin and the UCSF Liver Center Imaging and Pathology Core (San Francisco, CA) performed H&E and Sirius Red staining using standard techniques. A liver pathologist qualitatively evaluated all histology samples for steatosis, inflammation, and fibrosis.

**RNA-seq**

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD), and RNA quantity and integrity measured by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library construction, high-throughput sequencing, quality control, and sequence alignment were performed by Novogene Inc. (Davis, CA) per the company’s standard procedures. Briefly, 1μg of total RNA was used for the sample preparations. rRNA was removed by the Ribo-zero rRNA removal kit (Epicentre, Madison, WI) and the residual RNA cleaned by ethanol precipitation. Sequencing libraries were generated by NEBNext Ultra directional RNA library prep kit (Illumina, San Diego, CA) following the manufacturer’s protocols. Library fragments were purified with AMPure XP system (Beckman Coulter, Brea, CA) to attain 250-300bp cDNA fragments. Library quality was assessed by Agilent Bioanalyzer 2100, and high-throughput sequencing was performed on the Illumina Hiseq 4000 platform using HiSeq 3000/4000 SBS Kit (300 cycles) with 20 million reads per sample. Raw reads in FASTQ format were processed by removing the adaptor reads or poly-N and low-quality reads. Error rate distribution, Q20, Q30 and GC content of the cleaned data were calculated. Quality controlled reads were mapped to the mouse reference genome (mm10) with
STAR alignment software (81). Gene expression levels were analyzed according to FPKM (Fragments Per Kilobase of transcript per Millions base pairs sequenced). MINSEQE-compliant dataset is available through the Gene Expression Omnibus public repository (accession #: GSE157156).

**Total liver collagen quantification**

Total collagen content in liver tissues was determined by using a hydroxyproline assay kit (MilliporeSigma, St. Louis, MO). Briefly, 100-150mg tissue per liver sample was hydrolyzed at 110°C for 20 hours in 1ml 6N HCl per 100mg tissue. Hydroxyproline standards and 10ul of hydrolyzed samples were added to 96-well flat-bottom plates and evaporated to dryness on a 60°C heat block. The sediment was dissolved in 100ul of chloramine-T/oxidation buffer mixture for 10mins, then 100ul of DMAB/perchloric acid/isopropanol mixture was added to each well, and incubated at 60°C for 90min. Samples were cooled to room temperature and absorbance measured at 562nm.

**Quantitative real-time reverse transcription PCR (qRT-PCR)**

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration was measured by the NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and RNA purity verified by 260/280 absorbance, which consistently ranged between 1.8-2.0. Reverse transcription was carried out with 100ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) following the manufacturer’s instructions. One µl of the resulting cDNA was added to a final 10µl mixture containing
5µl of 2x SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) and 3pmol oligonucleotide primers. Quantitative PCRs were carried out in a 7300 Real-Time PCR System (Applied Biosystems) using the thermal profile 50°C for 2min, 95°C for 10min, followed by 40 amplification cycles consisting of 95°C for 15s, 60°C for 30s, and 72°C for 30s. Samples were normalized to rRNA 18S internal standard. Relative quantification of gene expression was calculated by using the $2^{\Delta\Delta Ct}$ equation. Sequences of primers used in qRT-PCRs are listed in Supplemental Table 1.

**Primary mouse hepatocyte and stellate cell isolation**

Hepatocyte isolation was achieved through two-step perfusion of the liver in situ using Liver Perfusion and Liver Digest Media (Thermo Fisher Scientific) and then density separation by 50% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) gradient. Stellate cell isolation was performed following previously published protocols (82). Briefly, fibrotic livers were perfused in situ with 1.2mg/ml pronase (Roche, Indianapolis, IN) and 0.25U/ml collagenase (Crescent Chemical, Islandia, NY) and then digested in vitro with 0.5mg/ml pronase, 0.1U/ml collagenase, and 0.1mg/ml DNase I (Roche) for 10min in a 250rpm shaker at 37°C. Stellate cells were separated by density gradient centrifugation using 12% Accudenz (Accurate Chemical, Tempe, AZ) at 1380g for 17min at 4°C.

**Collagen-conjugated polyacrylamide gels**

Circular 22mm glass coverslips (VWR, Brisbane, CA) were activated by 0.5% 3-aminopropyl trimethoxysilane and glutaraldehyde (MilliporeSigma). Different ratios of
40% acrylamide and 2% bis acrylamide (Bio-Rad, Hercules, CA) were combined to generate 140Pa, 1kPa and 6kPa stiffness gels as calibrated previously (83). Polymerization was initiated by TEMED (Bio-Rad) and potassium persulfate (MilliporeSigma). Then 0.01% bis-acrylamide, 0.002% Di(trimethylolpropane) tetraacrylate (MilliporeSigma), 0.025% Irgacure (BASF Resins, Laramie, WY), 0.006% acrylic acid N-hydroxysuccinimide ester (MilliporeSigma), and 25% ethanol in 50 mM HEPES (pH 6.0) were applied, and gels were exposed to 245nm ultraviolet light for 300s for photoactivation. Gels were thoroughly washed in phosphate buffered saline to remove excess reagent and incubated with 150mg/ml rat-tail collagen I (VWR) solution overnight at 4°C for conjugation. Before cells were plated, the gels were equilibrated in DMEM (Mediatech, Manassas, VA) at 37°C overnight. Primary hepatocytes were cultured at 50,000 cells/gel/well in 12-well plates (Eppendorf, Hauppauge, NY) in DMEM supplemented with 5% FBS (Hyclone, Logan, UT), L-glutamine, antibiotics, insulin-transferrin-selenium, and HEPES (Mediatech).

**RNAscope in situ hybridization**

Multiple-plex RNAscope assay of mouse liver tissue was performed by using probes and reagents from Advanced Cell Diagnostics (Newark, CA) and following the manufacturer’s protocol. Briefly, 5μm thickness tissue sections were baked at 60°C for 1h, deparaffinized, and treated with hydrogen peroxide at room temperature for 10min. Target retrieval was performed by putting slides into 100°C target retrieval reagent for 30min, followed by protease treatment at 40°C for 30min. Mouse Smo (318411-C1), Adgre1(460651-C2), and Krt19 (402941-C3) probes were then hybridized at 40°C for
2 hrs. Hybridization with pre-amplifier, amplifier, and fluorescent labeling probes was performed by using RNAscope Multiplex Fluorescent Detection Kit v2 (Advanced Cell Diagnostics) and opal520, opal570, and opal650 (Akoya Biosciences, Menlo Park, CA) at 40°C. Images were acquired using a Leica DM6B microscope with a 4.2MP CMOS camera (Leica Microsystems, Buffalo Grove, IL). For quantification, 10 high-power-field images were randomly taken per sample and fluorescent Smo spots were counted from 20 randomly selected Adgre1+ or Krt19+ cells per image, i.e. Smo spots were counted in 200 randomly selected Adgre1+ and Krt19+ cell per sample.

Statistics

RNA-seq differential gene expression and clustering analyses using FPKM were performed with GeneSpring GX v14.9 (Agilent). We used moderated t-test with corrected p-value <0.05 to determine differential gene expression between baseline WT and FAK-/- samples (n=8 per group; pooled whole-liver tissue n=4 and isolated hepatocyte n=4 for each genotype). For fibrotic liver samples, genotype (WT vs. KO) and method of FAK deletion (Bred vs. Virus) were assigned as independent variables in 2-way ANOVA with Benjamini-Hochberg multiple testing correction (correct p-value <0.05 and n=3 per group). Heatmap hierarchical clustering on normalized intensity values was performed on genes and conditions using Pearson centered or Euclidean similarity measure and Ward’s linkage. Violin plots show median, interquartile range, and range. GO analysis was performed with the GeneSpring software or GOrilla web-based tool (84) using p-value threshold <0.001.
Additional statistical analyses, including survival analysis, log-rank test, regression analysis, 2-way ANOVA, and 2-tailed Student’s t-tests, were performed with Prism v8.4.2 (GraphPad, La Jolla, CA). Where appropriate, 2-way ANOVA was performed with genotype (WT vs. KO) and sex (male vs. female) as independent variables. Otherwise, 2-tailed Student’s t-test was used to test significance. Box plots show individual data points, median, interquartile range, and range. Bar graphs show individual data points and mean, with error bars representing SEM.

Study approval

Animal studies were approved by UCSF IACUC (Approval #: AN176137).
Author contributions

YW designed and conducted experiments, analyzed data, and wrote the manuscript; TL, VXZ, MLI, MAP, TB, and MCY conducted experiments; WTC evaluated histology; TTC conceived the research, designed and conducted experiments, analyzed data, and wrote the manuscript.

Acknowledgements

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References


**Figures and figure legends**

**Figure 1.** Whole-liver tissue (Tissue) or isolated hepatocytes (Hep) from adult six-to-eight-week-old FAK^{fl/fl};Alb-Cre{+} mice (FAK-/-) or FAK^{fl/fl};Alb-Cre{-} littermate controls (WT) were analyzed by RNA-seq. Heatmap shows hierarchical clustering of 782 significantly differentially expressed genes. Each column represents the averaged normalized expression of 4 samples (n=4). Four major clusters emerged based on expression comparison to WT.
Figure 2. Gene Ontology (GO) analysis of RNA-seq revealed numerous gene categories that are differentially regulated in whole-liver tissue (Tissue) and/or isolated hepatocytes (Hep) of adult six-to-eight-week-old FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{+} mice (FAK\textsuperscript{-/-}) compared to FAK\textsuperscript{fl/fl};Alb-Cre\textsuperscript{-} littermate controls (WT). Expression of representative genes are shown for the GO terms: (A) coagulation, (B) epithelial cell proliferation, (C) response to stress, and (D) lipid metabolism. Some genes are predominantly expressed in whole-liver tissue and not hepatocytes, indicating a non-parenchymal cell source, and include (E) extracellular matrix and (F) biliary-specific genes. Sample size n=4 per group; *p<0.05, **p<0.01, and ***p<0.001 by Student’s t-test. Data represent individual data points and mean ± SEM.
Figure 3. Male and female FAK^{fl/fl};Alb-Cre^{+} mice (FAK-/-) and FAK^{fl/fl};Alb-Cre^{-} littermate controls (WT) were aged to 6 months. (A) Serum liver function tests: aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (Alk Phos). (B) Representative liver H&E and trichrome histology of 6-month-old male WT and FAK-/- mice. Scale bar=200µm. (C) The degree of steatosis as scored by a liver pathologist. For (A) and (C), sample sizes are WT male n=6, WT female n=7, FAK-/- male n=7, and FAK-/- female n=7. Two-way ANOVA was performed with genotype (WT vs. FAK-/-) and sex (male vs. female) as independent variables; *p<0.05 by post-hoc Tukey’s HSD test. Data represent individual data points and mean ± SEM.
Figure 4. Male and female FAK^{fl/fl};Alb-Cre^{+} mice (FAK/-) and FAK^{fl/fl};Alb-Cre^{-} littermate controls (WT) were subject to bile duct ligation. (A) Kaplan-Meier survival analysis indicated significantly higher early mortality in FAK/- mice (male n=8, female n=6) compared to WT (male n=12, female n=8) after bile duct ligation, p<0.01 by log-rank test. (B) Representative liver histology of FAK/- mice that died one day after bile duct ligation or survived until day 35. WT controls were sacrificed at the same timepoints for comparison. Scale bar=200µm.
Figure 5. Male and female FAK<sup>0/0</sup>;Alb-Cre<sup>+</sup> mice (FAK<sup>-/-</sup>) and FAK<sup>0/0</sup>;Alb-Cre<sup>-</sup> littermate controls (WT) were given 0.1% DDC diet to induce liver fibrosis. (A) Male FAK<sup>-/-</sup> mice (n=7) lost significantly more weight than sex-matched controls (n=12). Linear regression analysis showed that slopes of the two lines for male mice were significantly different (p<0.05). Percentage weight loss in male mice was significantly different comparing WT and FAK<sup>-/-</sup> by Student’s t-test at days 21 and 28, **p<0.01. Weight loss
was not significantly different between female WT (n=4) and FAK-/- (n=8). Data represent mean ± SEM. (B) Serum liver function tests for WT (male n=8, female n=4) and FAK-/- mice (male n=7, female n=7). Data show individual data points and mean ± SEM. (C) Representative liver histology. Scale bar=100µm. (D) Liver hydroxyproline content of WT male (M; n=7) and female (F; n=4) compared to FAK-/- male (n=5) and female (n=7) mice. (E) mRNA expression of various collagen species as determined by qRT-PCR in WT (M, n=8; F, n=4) and FAK-/- (M, n=7; F, n=7) mice. Two-way ANOVA showed significant interaction between FAK genotype and sex (p<0.05) for (D) and every collagen species except Col12a1 in (E); genotype contributed significantly to the overall variation for Col5a1, Col6a1, and Col12a1 (p<0.05). Groups were also compared by Student’s t-test in (B), (D), and (E), *p<0.05, **p<0.01, and ***p<0.001. Box plots show individual data points, median, interquartile range, and range.
Figure 6. Male and female FAK^{floxed} mice were treated with AAV8-TBG-Cre (AAV-Cre) or AAV8-TBG-Null (AAV-Null) and then given 0.1% DDC diet to induce liver fibrosis. (A) Weight changes were not significantly different between male AAV-Null (n=7) and AAV-Cre (n=7) mice. Percentage weight-loss in female mice was significantly different between AAV-Null (n=5) and AAV-Cre (n=5) at days 7, 14, and 21 by Student’s t-test (*p<0.05, **p<0.01). Linear regression analysis showed that elevations of the AAV-Null
and AAV-Cre female weight-loss lines were significantly different (p<0.0001). Data represent mean ± SEM. (B) Serum liver function tests for AAV-Null (male n=4, female n=5) and AAV-Cre mice (male n=4, female n=5). Data show individual data points and mean ± SEM. (C) Representative liver histology. Scale bar=100µm. (D) Liver hydroxyproline content of AAV-Null male (M; n=4) and female (F; n=5) compared to AAV-Cre male (n=4) and female (n=5) mice. (E) mRNA expression of various collagen species as determined by qRT-PCR in AAV-Null (M, n=4; F, n=5) and AAV-Cre (M, n=4; F, n=5) mice. For all collagen genes, 2-way ANOVA showed no significant interaction between AAV genotype and sex, whereas genotype contributed significantly to the overall variation (p<0.05). For (B) and (E), *p<0.05 and **p<0.01 by Student’s t-test. Box plots show individual data points, median, interquartile range, and range.
Figure 7. FAK^{fl/fl} mice treated with AAV8-TBG-Null (Virus WT) or AAV8-TBG-Cre (Virus KO), as well as FAK^{fl/fl};Alb-Cre^- (Bred WT) and FAK^{fl/fl};Alb-Cre^+ (Bred KO) mice, were given 0.1% DDC diet to induce liver fibrosis. Whole-liver tissue from these mice was analyzed by RNA-seq. (A) Heatmap shows hierarchical clustering of 896 significantly differentially expressed genes. Each column represents the averaged normalized expression of 3 samples (n=3). (B) There were 8 gene clusters based upon expression patterns across the 4 groups. Cluster 1 contained 16 genes, cluster 2 – 2 genes, cluster 3 – 82 genes, cluster 4 – 110 genes, cluster 5 – 156 genes, cluster 6 –
327 genes, cluster 7 – 110 genes, and cluster – 93 genes. (C) GO analysis identified several processes and pathways that were upregulated in KO mice in both Virus and Bred methods of FAK deletion. Two-way ANOVA showed that there was significant interaction between the method of FAK deletion (Virus vs. Bred) and the genotype (WT vs. KO) for differentially regulated genes in the collagen fibril and epithelial-mesenchymal transition (EMT) categories (p<0.05). The method of FAK deletion was a significant source of variation for collagen fibril, Smo, TGFβ, and EMT pathways (p<0.01). For all 6 categories, genotype was a significant source of variation (p<0.0001). Post-hoc Tukey’s HSD test between the groups showed *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Expression levels of representative genes in the (D) Smo, (E) Wnt, and (F) TGFβ pathways were compared by Student’s t-test, *p<0.05 and **p<0.01. For (D-F), sample size was 3 per group (n=3). Data represent individual data points and mean ± SEM.
Figure 8. Hh-Smo pathway gene expression was determined in FAK-deficient hepatocytes and fibrotic hepatocyte-specific FAK-deficient liver. (A) Isolated hepatocytes from six-to-eight-week-old FAK<sup>fl/fl</sup>;Alb-Cre<sup>-</sup> (WT) and FAK<sup>fl/fl</sup>;Alb-Cre<sup>+</sup> (FAK-/-) mice on normal chow diet were cultured on collagen-coated polyacrylamide gels of 140Pa, 1kPa, or 6kPa stiffness. *Ihh* mRNA expression was determined by qRT-PCR 24 hours later. Sample size n=5-6 per group. (B) Stellate cells were isolated from WT and FAK-/- mice with DDC-induced fibrosis. Expression of Hh-Smo pathway genes and...
stellate cell activation markers were determined by qRT-PCR. Sample size n=4 per group. (C) Three-color multi-plexed RNAscope analysis of Smo, Adgre1, and Krt19 was performed on DDC-induced fibrotic liver tissues of FAK$^{fl/fl}$ mice treated with AAV8-TBG-Null (Null) or AAV8-TBG-Cre (Cre). Images are representative of n=4 per group. Scale bar=20 µm. (D) Number of Smo dots that were co-localized with Adgre1$^+$ macrophages. (E) Number of Smo dots that were co-localized with Krt19$^+$ biliary cells. For (D) and (E), 10 portal areas were analyzed per mouse and at least 20 Adgre1$^+$ or Krt19$^+$ cells were analyzed per portal area; sample size was n=4 per group. *p<0.05, **p<0.01, and ***p<0.001 by Student’s t-test. Data represent individual data points and mean ± SEM.
### Table 1. Differentially expressed genes within GO categories shown in Figure 7.

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<th>GO Term</th>
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<tr>
<td>Collagen Fibril</td>
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