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Fibroblast Growth Factor signaling regulates the expansion of A6-expressing hepatocytes in association with AKT-dependent β -catenin activation

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Abstract

Background & Aims—Fibroblast Growth Factors (FGFs) promote the proliferation and survival of hepatic progenitor cells (HPCs) via AKT-dependent β -catenin activation. Moreover, the emergence of hepatocytes expressing the HPC marker A6 during 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury is mediated partly by FGF and β -catenin signaling. Herein, we investigate the role of FGF signaling and AKT-mediated β -catenin activation in acute DDC liver injury.

Methods—Transgenic mice were fed DDC chow for 14 days concurrent with either *Fgf10* over-expression or inhibition of FGF signaling via expression of soluble dominant-negative FGF Receptor (R)-2IIIb.

Results—After 14 days of DDC treatment, there was an increase in periportal cells expressing FGFR1, FGFR2, and AKT-activated phospho-Serine 552 (pSer552) β -CATENIN in association with up-regulation of genes encoding FGFR2IIIb ligands, *Fgf7*, *Fgf10*, and *Fgf22*. In response to *Fgf10* over-expression, there was an increase in the number of pSer552- β -CATENIN^{(positive)+ive} periportal cells as well as cells co-positive for A6 and hepatocyte marker, Hepatocyte Nuclear Factor-4 α (HNF4 α). A similar expansion of A6^{+ive} cells was observed after *Fgf10* over-expression with regular chow and after partial hepatectomy during ethanol toxicity. Inhibition of FGF signaling increased the periportal A6^{+ive}HNF4 α ^{+ive} cell population while reducing centrolobular A6^{+ive} HNF4 α ^{+ive} cells. AKT inhibition with Wortmannin attenuated FGF10-

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mediated A6⁺HNF4 α ⁺ cell expansion. *In vitro* analyses using FGF10 treated HepG2 cells demonstrated AKT-mediated β -CATENIN activation but not enhanced cell migration.

Conclusion—During acute DDC treatment, FGF signaling promotes the expansion of A6-expressing liver cells partly via AKT-dependent activation of β -CATENIN expansion of A6⁺ periportal cells and possibly by reprogramming of centrolobular hepatocytes.

Keywords

Fibroblast Growth Factor 10; hepatic progenitor cells; DDC; beta-catenin

Introduction

Human diseases such as congenital biliary atresia and primary biliary cirrhosis are marked pathologically with periportal fibrosis surrounding expanding biliary ductular reactions, the cells of which exhibit characteristics consistent with those of epithelial progenitor and stem cells [1, 2]. Treatment of rodents with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induces an analogous fibrosis with ductular reactions populated with hepatic progenitor cells (HPCs) expressing SOX9 [3], IC3 [3], PROMININ-1 (CD133) [4], and A6 [5]. Recent lineage tracing studies suggest that the HPC transdifferentiation toward a hepatocyte cell fate during DDC injury is negligible [6-8]. Instead, hepatocytes undergo some degree of reprogramming or transdifferentiation with *de novo* expression of A6, SOX9, CK19, and OPN, which are conventionally considered HPC or biliary epithelial cell (BEC) markers [9].

Fibroblast Growth Factor (FGF) signaling regulates hepatogenesis [10-12], progenitor cell expansion, and liver regeneration [13, 14]. The FGF family comprises 22 polypeptide ligands that bind to 4 promiscuous tyrosine kinase FGF receptors (FGFR), each expressed as two isoforms [15]. FGFRs are principally located in the cell membrane although nuclear localization has been described [16, 17]. We previously demonstrated that during early hepatogenesis, FGF10, expressed by embryonic mesenchymal hepatic stellate cells, promotes HPC proliferation via β -catenin activation [10]. Postnatally, hepatocyte proliferation following liver injury is regulated in part by activation of FGF signaling via FGFR2IIIb [14]. During DDC-induced liver injury, mesenchymal cell expression of FGF7 is known to regulate HPC expansion, and over-expression of *Fgf7* reduces hepatocyte damage and cholestatic liver injury [13].

Wnt/ β -catenin signaling has been implicated in HPC-mediated liver regeneration [18, 19]. Binding of the Wnt ligand to Frizzled receptor leads to dephosphorylation, activation, and nuclear translocation of the transcriptional regulator β -CATENIN. Using *ex vivo* embryonic liver cultures, Sekhon *et al* showed that FGF signaling promotes β -catenin-mediated proliferation of hepatoblasts [20]. Activation of β -CATENIN can also occur non-canonically via receptor tyrosine kinase (RTK) activation through AKT-dependent [21, 22] and Protein Kinase A (PKA) mediated [23] phosphorylation of β -CATENIN at Serine-552 (pSer552- β -CATENIN). FGF signaling promotes HPC proliferation *in vitro* via AKT-dependent β -CATENIN activation [24]. Postnatal HPC proliferation induced by DDC treatment is mediated in part via β -catenin activation through increased expression of Wnt ligands [19]. Hyper-activation of liver-specific β -catenin during chronic DDC-induced liver injury leads

to increased expansion of A6-expressing hepatocytes in association with improved hepatic repair and resolution of cholestasis [25].

In this study, we further investigate the role of FGF signaling in the emergence and expansion of A6^{ive} cells during DDC-induced liver injury. We also demonstrate a link between FGF signaling and β -catenin activation during acute DDC liver injury, during which the initial expansion of A6^{ive} cells is induced, analogous to what is observed in embryonic liver development.

Materials and Methods

Experimental Animals and Procedures

Six-week old, C57BL/6J (wild-type, WT) male mice (Jackson Laboratories) were fed either a standard diet or 0.1% DDC diet (Test Diet, Richmond) up to 14 days. Inducible transgenic and littermate control mice were given water with 1% doxycycline (Clontech) 2 days prior to and throughout DDC treatment. *CMV^{cre};Rosa26^{rtTA}^{-/-};tet(O)-sFGFR2-IIIb^{+/-}* mice (dnFGFR) exhibited induced ubiquitous expression of dominant-negative soluble FGFR2-IIIb as confirmed by qPCR (Supp. Fig. 1B) [26]. *CMV^{cre};Rosa26^{rtTA}^{-/-};tet(O)-Fgf10^{+/-}* mice (*Fgf10*-induced) exhibited induced *Fgf10* over-expression in uninjured, and DDC treated mice [27]. In a separate experiment, *Fgf10*-induced mice were given daily intra-peritoneal injections of the AKT inhibitor, Wortmannin (*Fgf10*+Wort, 0.7 mg/kg) or vehicle solution (*Fgf10*+Vehicle) in DMSO with 1× phosphate buffered saline (PBS) (Sigma-Aldrich), two days prior to and throughout DDC treatment. *Fgf10* was also induced two days prior to and throughout 70% partial hepatectomy (PHx) combined with ethanol (EtOH) gavage (1g/kg, every 12 hrs pre- and post-PHx). All procedures were done in compliance with the IACUC of Children's Hospital Los Angeles/Saban Research Institute guidelines for use of laboratory animals.

Tissue Collection

After carbon dioxide euthanasia, 1× PBS was flushed through the portal vein. Portions of the right lobe were collected for histology and total RNA. Single cell suspension of whole liver and non-hepatocyte fractions were obtained by mechanical and enzymatic digestion followed by serial centrifugation [4] to enrich the non-hepatocyte fractions (NHF). qPCR for *Albumin* and *Hnf4* was performed to confirm depletion of hepatocytes from the NHF (Supp Figure. 1A).

Immunohistochemistry and Immunofluorescence Analysis

Collected tissues were fixed in 4% paraformaldehyde (PFA) or 30% sucrose for 4 hours. 5 μ m sections of paraffin-embedded and OCT-embedded tissues were utilized for immunostaining (Supp. Table 1). Hematoxylin & Eosin (H&E) was utilized for morphologic analyses. Quantification of A6^{ive}HNF4 α ^{ive} cells per lobular region was performed by separating each lobule into periportal (0-96 μ m), mid (97-194 μ m), and central regions (195-290 μ m) (Figure 2E). Immunohistochemistry was performed with the Dako EnVision+ Dual Link System-HRP (DAB+) kit (Dako). Immunofluorescence (IF) images were taken

using a Leica DM5500B microscope (Leica). All IF quantifications were performed on at least three images per animal with three animals per treatment (n=3).

Quantitative PCR Analysis of Gene Expression

Total RNA was isolated with the Qiagen RNeasy Mini kit (Qiagen). Complementary DNA (cDNA) was prepared with iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qPCR) was performed using the Light-Cycler Taqman Master (Roche Applied Science) and probes from the Universal Probe Library (Roche Applied Science) against intron spanning, gene specific primers (Supp. Table 2). *18s* was selected as the optimal internal control for all analyses.

Cell Migration Assays

HepG2 cells were cultured in DMEM with 1% L-Glutamine, 1% Pen/Strep, and 10% Fetal Bovine Serum (Gibco). Scratch assays in serum free conditions were used to determine the dose response of HepG2 cell migration to recombinant FGF10 (rFGF10) (n=8). Images were taken at 0, 24, and 48 hours post scratch and the width determined by three measurements per image. An optimal dose of rFGF10 (10ng/mL) was determined from a generated standard curve for trans-well cell migration assays. 40,000 cells were cultured in 8 µm pore trans-well inserts (BD Falcon) for 24 hours and stained with crystal violet to quantify cell migration by cell counting and spectrophotometer analysis (Victor3, Perkin Elmer). Western blot analysis was performed as previously described to confirm signaling pathway activation in rFGF10 treated HepG2 cells (Supp. Table 1) [24].

Statistical Analysis

ANOVA-Post hoc Fisher's PLSD test or Mann-Whitney Rank Sum was performed using Statview (SAS Institute, Inc.) to calculate statistical significance (p<0.05).

Results

Expression of FGF ligands and receptors is up-regulated during acute DDC liver injury

FGF ligands expressed by mesenchymal cells regulate HPCs and liver regeneration during chemically induced liver injury [13, 16, 17]. Thus, qPCR was performed on RNA extracted from a density centrifugation-enriched, non-hepatocyte cell fraction (NHF) and from whole liver tissue in order to assess FGF ligand and receptor gene expression during acute DDC injury. Partial enrichment of small non-hepatocyte cells was validated by qPCR with a marked reduction in expression of hepatocyte genes *Albumin* and *Hnf4a* relative to the hepatocyte cell fraction (Supp. Figure 1A) [4]. Within the first 14 days of DDC treatment, we observed 11.6-fold and 12.4-fold increases in *Fgfr1IIIb* and *Fgfr2IIIb* expression, respectively, in the NHF (Figure 1A, p<0.05), indicating FGFR activation as previously described [24]. FGF1, FGF2, FGF3, and FGF10 possess high binding affinity for FGFR1IIIb while FGF1, FGF3, FGF7, FGF10, and FGF22 exhibit high binding affinity for FGFR2IIIb [28]. qPCR was performed in lieu of western blot analyses given the lack of reliable antibodies against FGF ligands. *Fgf3* expression was not detected by qPCR. *Fgf10* expression was significantly up-regulated 14 days after DDC injury while *Fgf7* and *Fgf22* were upregulated throughout the entire observation period (Figure 1B, p<0.05). *Fgf1*

expression was transiently down-regulated at 3 days and significantly upregulated by 14 days of DDC treatment (Figure 1B, $p < 0.05$). In contrast, gene expression profiles from whole liver samples for *Fgfr2IIIb* and all *Fgf*'s, with the exception of *Fgf10*, demonstrated no significant upregulation, which indicates that these genes are predominantly expressed by cells within the NHF (Supp. Figure 2A, B, $p < 0.05$).

Using non-isoform specific antibodies, we observed increased nuclear, cytoplasmic, and membrane-bound FGFR1 and FGFR2 in periportal cells and hepatocytes at 7d and 14d DDC injury (Fig. 1C,D). There was an increase in the fraction of FGFR1⁺PCNA⁺ cells in the periportal region throughout acute DDC treatment (Fig. 1D-F, $p < 0.05$). FGFR1 was expressed by CK19⁺ biliary epithelial cells, and small periportal HNF4 α ⁺ hepatocytes but not by α SMA⁺ mesenchymal cells (Supp. Figure 2C-E). Thus, the majority of proliferating FGFR⁺ periportal cells are either biliary epithelial cells or hepatocytes in acute DDC injury.

Fgf10 promotes expression of A6 in HNF4 α ⁺ hepatocytes

In order to assess the effects of FGF signaling on DDC liver injury, transgenic mouse models were utilized to inhibit FGF signaling with dominant-negative FGFR2b (dnFGFR) mice and to hyper-activate FGF signaling with *Fgf10* over-expressing mice (Figure 2A). After 14 days, there were no observable differences in liver-to-body weight ratios, serum AST, ALT levels (Table 1), or fibrosis amongst WT, dnFGFR and *Fgf10*-induced animals (Supp. Figure 3A). There was, however, a significant reduction in total serum bilirubin levels in *Fgf10*-induced mice similar to that observed by Takase *et al* with *Fgf7* over-expression [13].

Using A6, a putative HPC marker, and HNF4 α as a marker of hepatocyte differentiation, we observed an increase in the number of nonbiliary A6⁺HNF4 α ⁺ cells near the portal vein after *Fgf10* over-expression for 14 days in uninjured mice (Supp. Figure 4A). We also observed a marked increase in nonbiliary A6⁺CK19⁻ cells with *Fgf10* over-expression in 14 day DDC-treated livers (Supp. Figure 3C). Specifically there were increases in both A6⁺HNF4 α ⁻ cells and A6⁺HNF4 α ⁺ hepatocytes in *Fgf10*-induced livers (Figure 2B-D, $p < 0.0001$ and $p < 0.05$ respectively) without a significant change in the relative distribution of cells across the periportal, mid, and central regions of the hepatic lobule (Figure 2E-F, Supp. Figure 3B). A similar increase in periportal A6⁺HNF4 α ⁺ cells was observed with *Fgf10* over-expression in the setting of HPC expansion associated with acute ethanol-induced impairment of hepatocyte proliferation [29-31] following partial hepatectomy (Supp. Figure 4B). Conversely, inhibition of FGF signaling during DDC treatment did not affect the number of A6⁺HNF4 α ⁻ or A6⁺ HNF4 α ⁺ cells compared to WT (Figure 2C-D). However, dnFGFR mice exhibited altered intralobular distribution of A6⁺HNF4 α ⁺ cells with an increase in the number of A6⁺HNF4 α ⁺ cells in the periportal region and a reduction in the cells in the central region relative to WT and *Fgf10*-induced mice (Figure 2F, $p < 0.05$).

We next sought to determine if FGF signaling might enhance migration of hepatocytes, throughout the liver lobule. Given the lack of options for *in vivo* lineage tracing of A6⁺ cells, we evaluated the effect of FGF signaling on cell migration *in vitro*. Scratch assay on

HepG2 cells, a transformed human hepatocyte cell line which expresses HPC markers and FGFRs, determined the dose responsiveness of these cells to recombinant (r) FGF10 (Supp. Figure 5A-B). Although HepG2 cells are known to exhibit evidence of elevated levels of basal activation of β -catenin at baseline [32], we detected an increase in protein levels of pAKT and pSer552- β -catenin within 30 minutes after media supplementation with 10 ng/mL of rFGF10 (Supp. Figure 5C,D). Thus, despite high basal activation of β -catenin, HepG2 cells are still responsive to FGF10. We then performed Boyden chamber trans-well migration assays \pm rFGF10, and observed no significant change in HepG2 cell migration compared to controls (Supp. Figure 5E,F). Thus, either FGF signaling does not regulate HPC-like hepatocyte migration or it does so via a non-AKT-mediated β -catenin pathway.

FGF signaling mediates periportal cell proliferation and AKT-dependent β -catenin activation

Centrolobular cell proliferation, as assessed by phospho-Histone-H3 (pHH3) staining, was not significantly different amongst treatment groups (data not shown). However, proliferation of the periportal cells (Figure 3A) exclusive of large hepatocytes but inclusive of A6⁺ cells, BECs, mesenchymal cells, and small HNF4 α ⁺ hepatocytes, was reduced in dnFGFR livers and increased with *Fgf10* over-expression compared to WT (Figure 3A-B, $p < 0.01$). Given that β -catenin activation regulates the expansion of the A6⁺ cell population with DDC injury and that FGF promotes AKT-dependent β -catenin activation of embryonic and tumor initiating HPC *in vitro*, we sought to determine if FGF signaling induces AKT-mediated β -catenin activation in DDC liver injury. We observed a ~10% increase in the fraction of pSer552- β -catenin⁺ periportal cells in *Fgf10*-induced livers compared to WT (Figure 3C-D, $p < 0.0001$). In contrast, there was a marked reduction in the fraction of pSer552- β -catenin⁺ periportal cells in dnFGFR livers compared to WT and *Fgf10*-induced livers (Figure 3C-D, $p < 0.0001$, and $p < 0.01$ respectively). Collectively, these observations indicate that FGF signaling promotes AKT-dependent β -catenin activation in periportal cells during the initial two weeks of DDC-induced liver injury.

Inhibition of PI3K signaling disrupts A6⁺HNF4 α ⁺ cell expansion

To further validate the role of FGF signaling in β -catenin-dependent expansion of A6⁺ cells during acute DDC liver injury, *Fgf10*-induced animals were injected daily with the AKT inhibitor Wortmannin (*Fgf10*+Wort). No significant difference was observed with serum AST, ALT, total bilirubin, and fibrosis between *Fgf10*+Wort and *Fgf10*+vehicle controls (Table 2, Supp. Figure 6A). *Fgf10*+Wort treatment did not affect the expansion of the A6⁺HNF4 α ⁻ cell population (Figure 4A-B) but significantly reduced the number of A6⁺HNF4 α ⁺ cells compared to controls (Figure 4C, $p < 0.05$). The distribution of A6⁺HNF4 α ⁺ cells within the hepatic lobule was also altered, in a pattern similar to that seen in dnFGFR livers in which the percent of cells near the portal vein is greater than that near the central vein (Figure 4D, Supp. Figure 6B, $p < 0.05$). Collectively these data indicate that inhibition of FGF/AKT signaling disrupts hepatocyte expression of A6⁺ cells, an indication of hepatocyte transdifferentiation towards biliary cells as seen with DDC injury.

IF analysis of AKT-dependent β -catenin activation revealed a ~30% reduction in the percentage of pSer552- β -catenin⁺ periportal cells in the *Fgf10*+Wort livers compared to

Fgf10+Vehicle (Fig. 4F, $p<0.05$). We also observed a 50% reduction in periportal cell proliferation in *Fgf10*+Wort compared to *Fgf10*+Vehicle (Supp. Figure 6C,D, $p<0.01$). These data indicate that AKT-dependent β -catenin activation plays a key role in regulating the $A6^{+ive}HNF4\alpha^{+ive}$ cell population during cholestatic liver injury.

Discussion

FGF signaling is a key regulator of endoderm specification toward a liver-specific fate [11] as well as embryonic HPC survival and proliferation [10, 24]. Postnatally, FGF signaling regulates liver regeneration following injury [13, 14, 33]. In this study, we show that FGF signaling promotes the initial expansion of a DDC-induced periportal $A6^{+ive}HNF4\alpha^{-ive}$ cell and $A6^{+ive}HNF4\alpha^{+ive}$ hepatocyte populations in association with AKT-mediated activation of β -catenin.

Takase et al. observed that FGF7, expressed by the periportal $Thy1^{+ive}$ mesenchymal cells, induces the expansion of periportal HPCs during adult cholestatic liver injury [13]. We observed a similar increase in $A6^{+ive}HNF4\alpha^{-ive}$ cell expansion with over-expression of *Fgf10*, a member of the FGF7 ligand sub-family [34]. Knockout of *Fgf7* in the liver leads to impairment of $CK19^{+ive}$ HPC proliferation during DDC injury and is lethal in 80% of mice by 8 weeks compared to nearly zero mortality in WT mice [13]. We utilized a sublethal inhibition of FGF signaling during acute DDC treatment and observed a reduction in the number of centrolobular $A6^{+ive}HNF4\alpha^{+ive}$ cells. Inhibition of AKT by Wortmannin in *Fgf10*-induced mice similarly disrupted hepatocyte expression of A6 consistent with previous observations of reduced $A6^{+ive}$ cell expansion with decreased phosphoinositide 3-kinase (PI3K)/AKT activation, knockout of *c-met*, the HGF Receptor gene, [35] or liver-specific conditional knockout of β -catenin [18]. Our *in vitro* data indicated a lack of enhanced hepatocyte migration with rFGF10 treatment. Although we did not observe migration of HepG2 cells in response to FGF10 treatment, it does not fully preclude the possibility of FGF-mediated cell migration. Our observations are consistent with the emergence of $A6^{+ive}$ hepatocytes as a result of hepatocyte reprogramming and not downstream migration of $A6^{+ive}$ HPCs.

Using hepatocyte-specific, adeno-associated virus vector-mediated Cre expression, Malato *et al* demonstrated minimal progenitor cell contribution to the population of newly formed hepatocytes with DDC injury [8]. Instead, Yanger *et al* showed evidence of hepatocyte reprogramming/transdifferentiation to BECs during DDC injury under the regulation of Notch signaling [9]. The authors also demonstrated that hepatocytes, which express BEC markers such as A6, OPN, and SOX9, acquire apical-basal polarity after 3 weeks of DDC injury [9]. In this study, we observed hepatocyte expression of A6 during acute DDC injury is partly regulated by *Fgf10* and β -catenin signaling. This increase in A6 expressing hepatocytes was also associated with a significant increase in cell proliferation, consistent with the findings of Yanger *et al* that hepatocyte reprogramming is associated with high cellular turnover [36]. It is also reasonable to speculate that FGF signaling may also be a regulator of hepatocyte reprogramming to BECs, although further studies would be required to prove this. We also observed A6 expression by hepatocytes after *Fgf10* over-expression in the absence of injury and during alcohol-impaired liver regeneration after partial

hepatectomy, thus, implying a more generalizable role for FGF signaling in hepatocyte reprogramming.

β -catenin activation promotes the proliferation of a variety of cells in the liver, including HPCs. Increased Wnt ligand expression with subsequent downstream β -catenin activation promotes the proliferation of DDC-induced HPCs [19]. Additionally, liver-specific expression and activation of β -catenin increases the population of A6-expressing hepatocytes throughout the hepatic lobule and improved liver function with chronic DDC treatment [25]. FGF signaling induces β -catenin activation in embryonic HPC *in vitro* [10, 20] in part via downstream activation of AKT [24]. In this study, we observed increased AKT-mediated β -catenin activation and cell proliferation in the periportal region with two weeks of *in vivo Fgf10* over-expression concurrent with DDC treatment. We extrapolate that FGF signaling regulates the initial expansion of the A6⁺HNF4 α ⁻ HPCs. Despite the increase in pSer552- β -catenin⁺ periportal cells and A6-expressing hepatocytes seen with *Fgf10* over-expression, there was no improvement in hepatocyte injury as assessed by serum AST and ALT consistent with observations by Thompson *et al*, in which excessive β -catenin expression and activation did not improve serum biochemistry markers for liver injury during the initial two weeks of DDC treatment [25]. While the role of hepatocyte reprogramming in liver regeneration remains unclear, the authors observed improved liver function tests with hyperactivation of β -catenin at 150 days indicating that these cells may improve liver regeneration long term. Given this finding, we speculate that prolonged *Fgf10* over-expression, were it feasible, may similarly ameliorate liver injury with prolonged DDC treatment. Unfortunately, the utility of our transgenic model of ubiquitous over-expression of *Fgf10* is limited to less than 3 weeks due to gastrointestinal complications which limit mouse viability [36]. Liver-specific *Fgf10* over-expression has not been performed. In our study, inhibition of FGF signaling through soluble FGFR2b expression and Wortmannin inhibition resulted in a decrease in pSer552- β -catenin⁺ periportal cells without reducing the A6⁺HNF4 α ⁻ cell population. Taken together, these data suggest that AKT-mediated β -catenin activation is sufficient but not necessary to expand this cell population. Retention of pSer552- β -catenin⁺ periportal cells may be due to incomplete inhibition of FGF signaling with dominant-negative FGFR2IIIb expression or it may be due to PKA-mediated phosphorylation of β -catenin at Serine-552, which was not analyzed in this study [23]. Hence, our data are consistent with reprogramming of hepatocytes with A6 expression towards a biliary epithelial phenotype during DDC-induced liver injury.

In conclusion, we demonstrate that FGF signaling, partly through AKT-mediated β -catenin activation, regulates the expansion of the A6⁺ HPCs and A6⁺HNF4 α ⁺ hepatocytes. Further investigation into the transdifferentiation of hepatocytes into BECs and their function in response to injury will provide a better understanding of liver regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HPCs	hepatic progenitor cells
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
CD133	Prominin-1
FGF	Fibroblast Growth Factors
FGFR	Fibroblast Growth Factor Receptor
RTK	receptor tyrosine kinase
WT	wild type
dnFGFR	dominant negative Fibroblast Growth Factor Receptor
PCNA	Proliferating Cell Nuclear Antigen
NHF	non-hepatocyte fraction
PFA	paraformaldehyde
H&E	hematoxylin and eosin
cDNA	complementary DNA
qPCR	quantitative PCR
HGF	Hepatocyte Growth Factor
HNF4α	Hepatocyte Nuclear Factor-4 α
GSK3β	glycogen synthase kinase-3 beta
PI3K	Phosphoinositide 3-kinase

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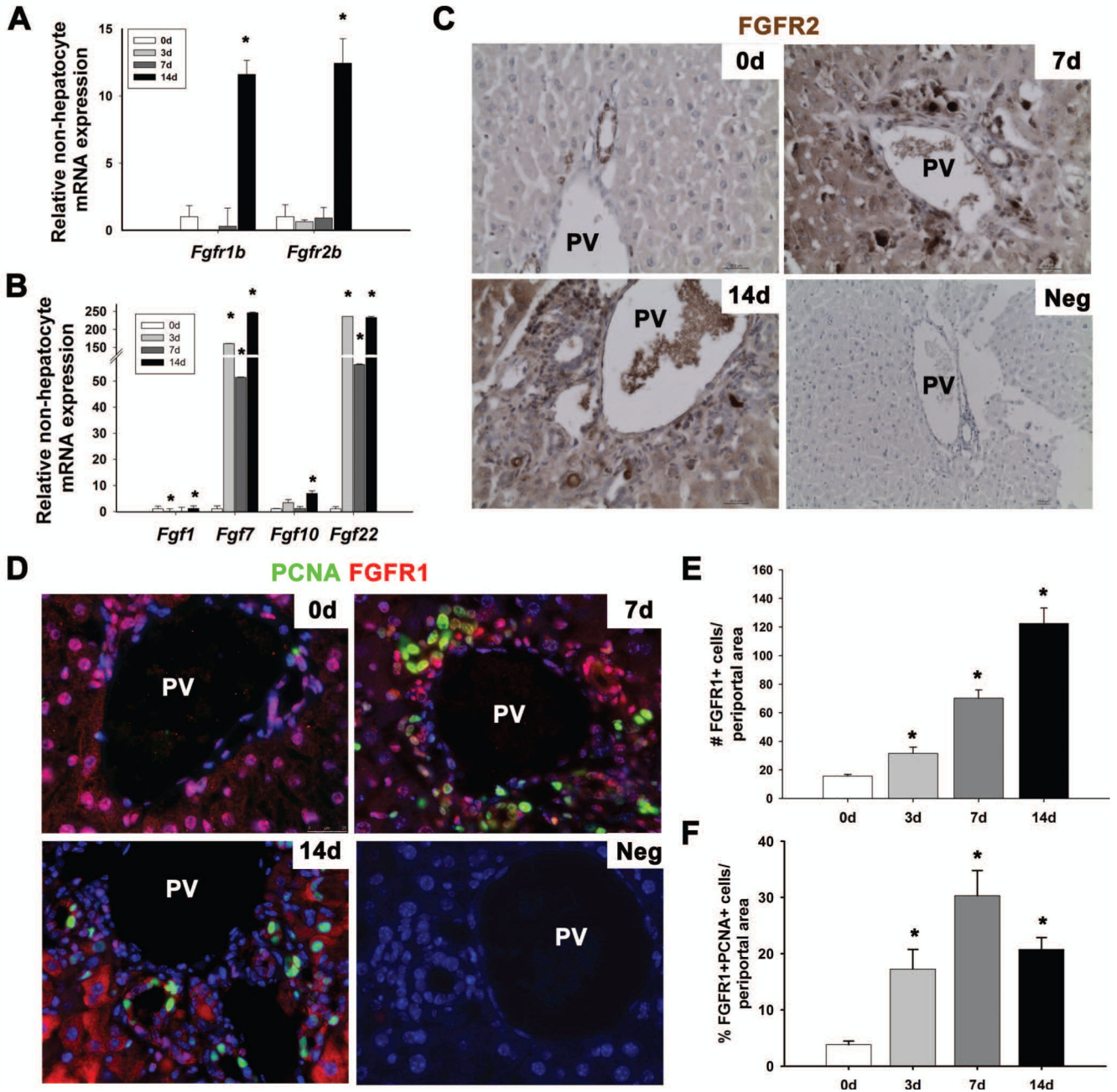


Fig. 1. FGF signaling is up-regulated during DDC injury

Relative expression levels of *Fgfr1IIIb* and *Fgfr2IIIb* (A) and *Fgf1*, *Fgf7*, *Fgf10*, and *Fgf22* (B) was performed on the NHF. (C) Immunohistochemistry for FGFR2 (brown) in the periportal region at 0d, 7d, and 14d DDC injury (20×) and representative negative control (10×). (D) Immunofluorescence staining for PCNA (green) and FGFR1 (red) at 0d, 7d, and 14d DDC injury and representative negative control (40×). (E) Quantification of the number of FGFR1⁺ periportal cells at 0d, 3d, 7d, and 14d DDC injury. (F) Quantification of the % of FGFR1⁺PCNA⁺ periportal cells. Statistical significance was determined by Mann-Whitney Rank Sum test (n=3, *p<0.05).

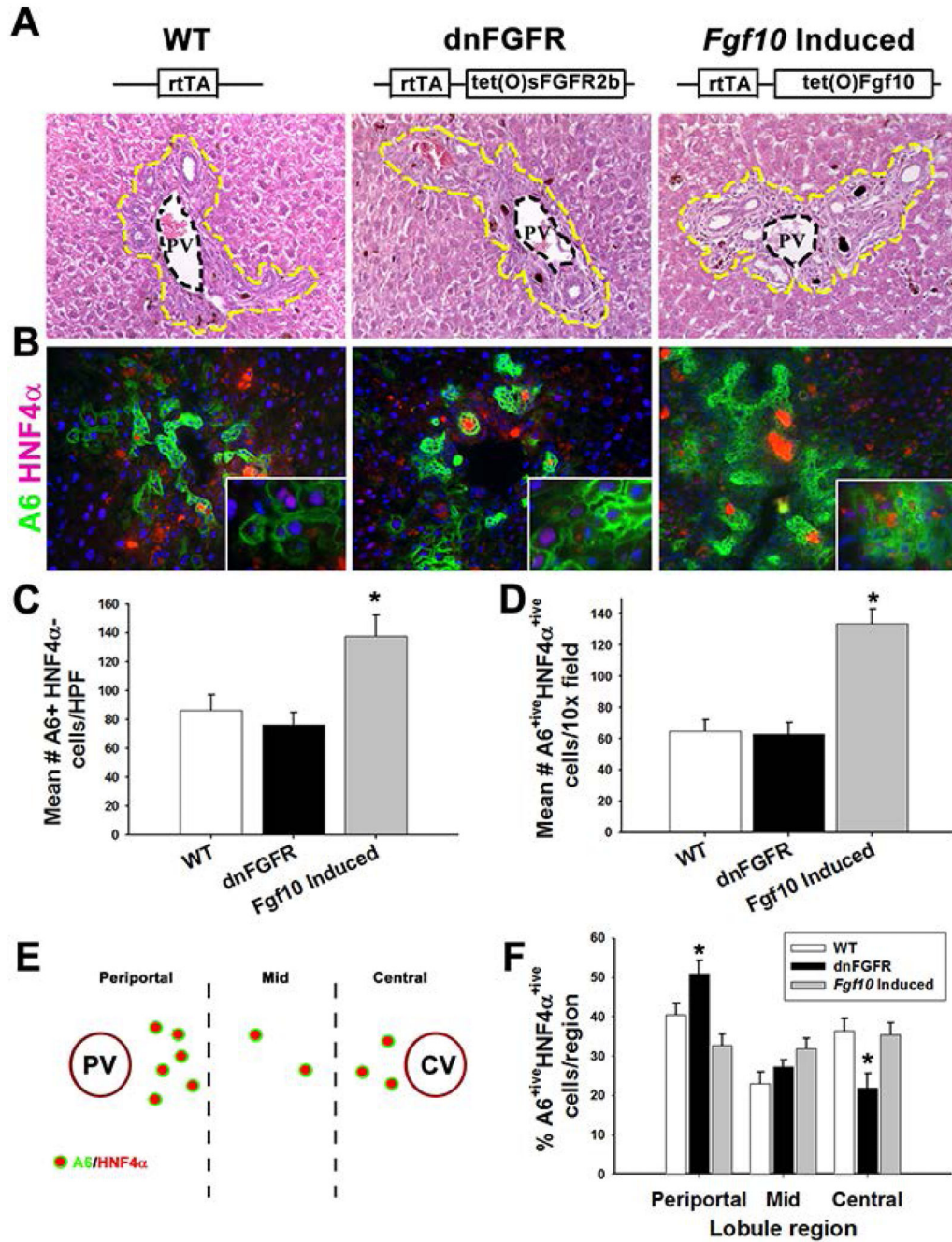


Fig. 2. FGF signaling regulates A6 expression in HNF4α^{+ive} hepatocytes

(A) WT, dnFGR, and *Fgf10*-induced H&E staining of periportal regions (20×, yellow dashed line indicates periportal cells). (B) IF staining for A6 (green), and HNF4α (red) (20× with 40× inset). (C) Quantification of the mean number of A6^{+ive}HNF4α^{-ive} cells per high powered field (HPF). (D) Quantification of mean number of A6^{+ive}HNF4α^{+ive} cells per HPF. (E) Schematic for quantification of A6^{+ive}HNF4α^{+ive} cells lobular region. (F) Quantification of percent of A6^{+ive}HNF4α^{+ive} cells per lobular region. Statistical significance was determined by ANOVA with Fisher’s post-hoc test (n=3, * *p*<0.05).

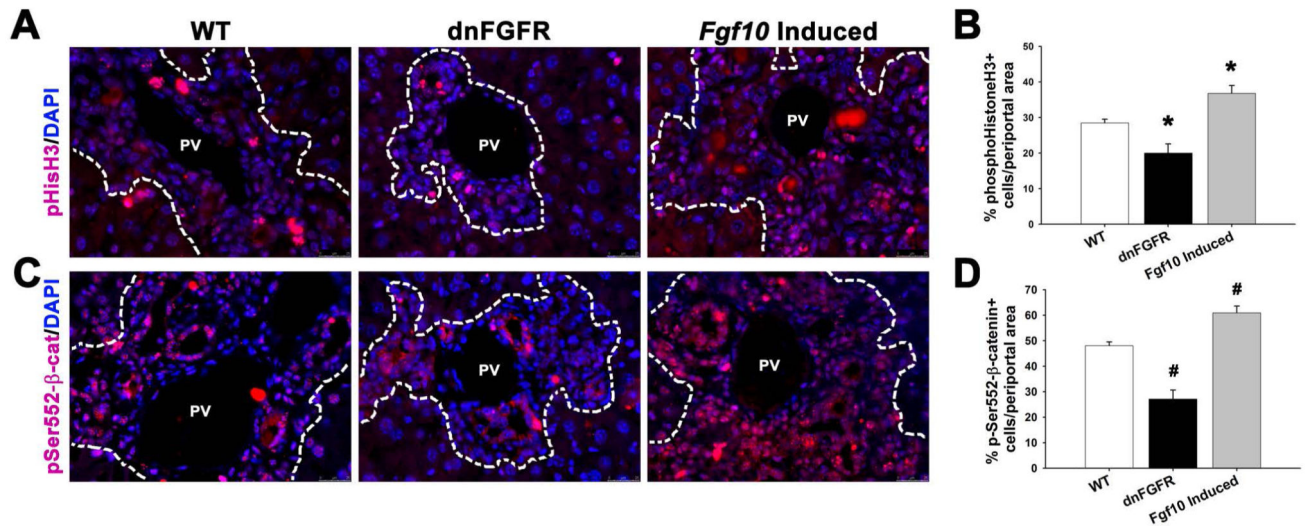


Fig. 3. FGF signaling regulates periportal cell proliferation and AKT-dependent β -catenin phosphorylation

(A) IF staining for cell proliferation by phospho-Histone H3 (red) (pHH3) and DAPI (blue) (40 \times). (B) Quantification of the percent of pH3H3⁺ periportal cells. (C) IF staining for pSer552- β -catenin⁺ (red) cells with DAPI (blue) per periportal area (40 \times). (D) Quantification of the percent of pSer552- β -catenin⁺ periportal cells. White dashed lines delineate periportal cell population. Statistical significance was determined by ANOVA with Fisher's post-hoc test (n=3, * $p < 0.05$, # $p < 0.0001$).

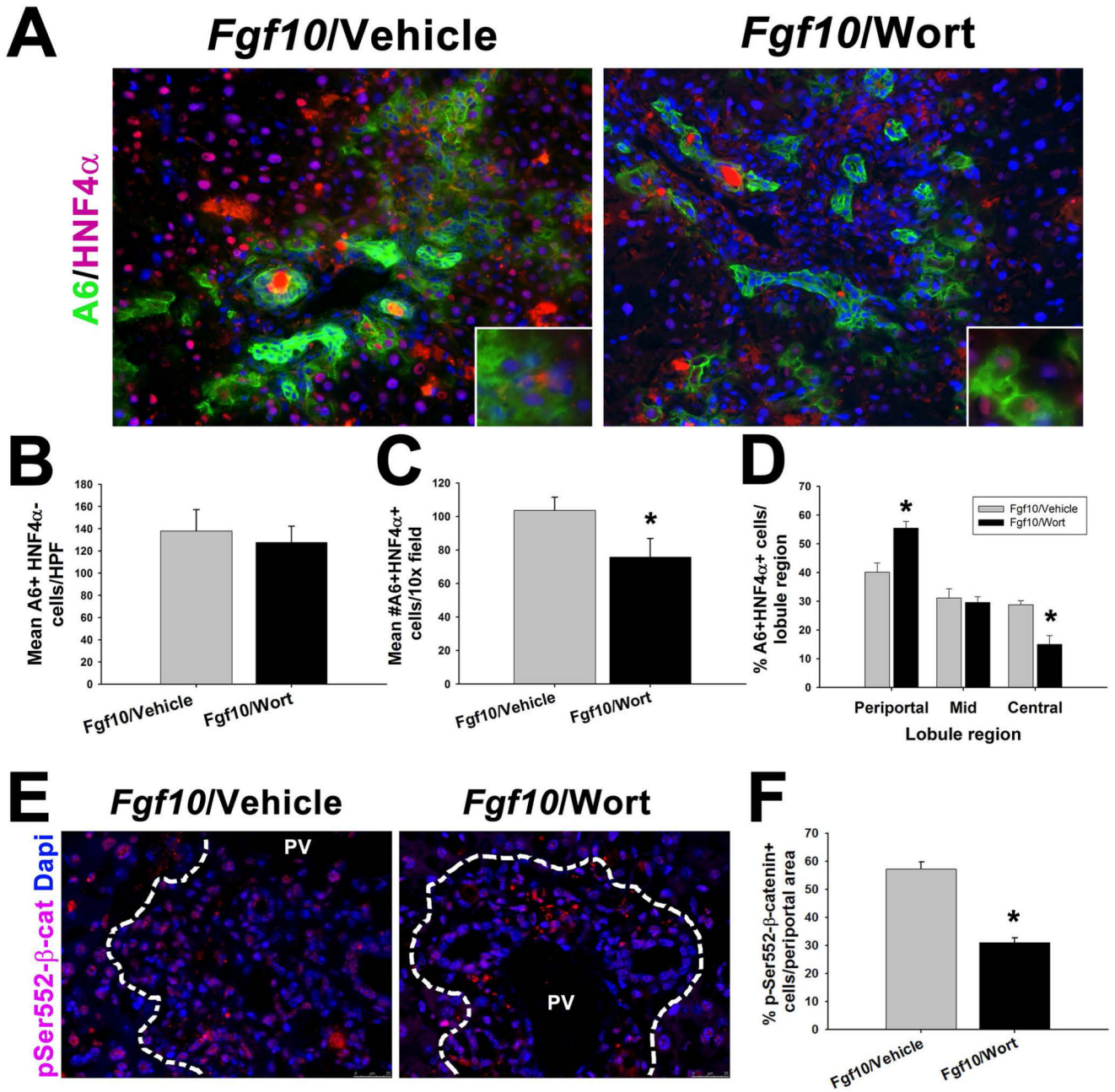


Fig. 4. Wortmannin inhibition of *Fgf10* induced livers disrupts the A6+HNF4α⁺ cell differentiation and migration
 (A) IF staining for A6 (green), and HNF4α (red) (20× with 40× inset). (B) Quantification of mean number of A6⁺HNF4α⁻ cells per HPF. (C) Quantification of average number of A6⁺HNF4α⁺ cells per HPF. (D) Quantification of % of A6⁺HNF4α⁺ cells per lobular regions. (E) IF staining for pSer552-β-catenin⁺ (red) cells with DAPI (blue) (40×). (F) Quantification of the % of pSer552-β-catenin⁺ periportal cells. Statistical significance was determined by Mann-Whitney Rank Sum test (n=3, * p<0.05).

Table 1
Effects of dnFGFR and *Fgf10* over-expression on DDC liver injury Treatment

Treatment	Liver/Body Weight Ratio	AST (U/L)	ALT (U/L)	Total Bilirubin (mg/dL)
WT	0.08±0.003	2171.7± 230.3	2336.8± 342.5	17.4± 3.6
dnFGFR	0.07±0.005	2245.2 ± 112.2	2404.3± 458.0	15.5± 2.8
<i>Fgf10</i> Induced	0.08±0.002	1446.5± 383.8	2588.8± 639.0	5.2± 1.2*

Values are given as mean ± standard error of mean (SEM). Statistical analysis was determined using ANOVA with Fisher's post-hoc test.

Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransferase.

* denotes P<0.05 compared to WT and dnFGFR.

Table 2
Effects of pAKT inhibition with *Fgf10* over-expression on DDC liver injury

Treatment	Liver/Body Weight Ratio	AST (U/L)	ALT (U/L)	Total Bilirubin (mg/dL)
<i>Fgf10</i> /Vehicle	0.07± 0.004	1173.0± 315.8	2238.3± 15.5	4.6± 1.6
<i>Fgf10</i> /Wort	0.11± 0.028	1604.7± 282.8	1987.7± 439.1	6.7± 2.1

Values are given as mean ± standard error of mean (SEM). Statistical analysis was determined using the Mann-Whitney Rank Sum test. Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransferase.