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Loss of integrin αvβ8 in murine hepatocytes accelerates liver regeneration

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ABSTRACT

Recent fate-mapping studies in mice have provided substantial evidence that mature adult hepatocytes are a major source of new hepatocytes following liver injury. In other systems, integrin αvβ8 has a major role in activating transforming growth factor beta (TGFβ), a potent inhibitor of hepatocyte proliferation. We hypothesized that depletion of hepatocyte integrin αvβ8 would increase hepatocyte proliferation and accelerate liver regeneration following injury. Using *Itgb8*$^{flox/flox}$*;Alb-Cre* mice to deplete hepatocyte αvβ8, following partial hepatectomy, hepatocyte proliferation and liver-to-body weight ratio were significantly increased in *Itgb8*$^{flox/flox}$*;Alb-Cre* mice compared to control. Antibody-mediated blockade of hepatocyte αvβ8 *in vitro*, with assessment of TGFβ signaling pathways by qPCR array, supported the hypothesis that integrin αvβ8 inhibition alters hepatocyte TGFβ signaling towards a pro-regenerative phenotype. A diethylnitrosamine-induced model of hepatocellular carcinoma, employed to examine the possibility that this pro-proliferative phenotype might be oncogenic, revealed no difference in either tumor number or size between *Itgb8*$^{flox/flox}$*;Alb-Cre* and control mice. Immunohistochemistry for integrin αvβ8 in healthy and injured human liver demonstrated that human hepatocytes express integrin αvβ8. Depletion of hepatocyte integrin αvβ8 results in increased hepatocyte proliferation and accelerated liver regeneration following partial hepatectomy in mice. These data demonstrate that targeting integrin αvβ8 may represent a promising therapeutic strategy to drive liver regeneration in patients with a broad range of liver diseases.

INTRODUCTION

Although the liver has a unique ability to regenerate, in many cases of liver disease this regenerative capacity is overwhelmed. A successful pro-regenerative therapy for the liver could have widespread application, reducing the need for transplantation in both acute and chronic liver failure, and potentially allowing more patients with primary or metastatic liver cancer to be treated successfully. Recent fate-mapping studies in mice have provided strong evidence that, in most murine models of liver injury and regeneration, restoration of liver mass occurs predominantly through self-duplication of hepatocytes.[1,2] Hence, identifying targets that promote proliferation and expansion of the pre-existent hepatocyte population represents an attractive therapeutic approach to drive liver regeneration.

Transforming growth factor beta (TGFβ) has pleiotropic roles in liver disease. In addition to its role as a major pro-inflammatory cytokine,[3] TGFβ is also a potent repressor of hepatocyte proliferation.[4-7] Therefore, in principle, TGFβ inhibition appears an attractive therapeutic strategy to promote hepatocyte proliferation and liver regeneration. An ideal therapy would target TGFβ with precision, allowing hepatocytes to escape the mito-inhibitory effects of TGFβ, while not abrogating the positive effects of TGFβ on extracellular matrix production and vascular remodeling during the regenerative process.[8,9] Furthermore, pan-TGFβ blockade may result in a number of unwanted, off-target effects, such as induction of autoimmunity and hepatocarcinogenesis.[10-12] Therefore, a more nuanced, selective approach targeting the TGFβ pathway to promote liver regeneration is required.

TGFβ is predominantly stored within the extracellular matrix in a latent state, and much of the regulation of TGFβ function results from precise, temporally and spatially restricted, extracellular activation of this latent complex.[13] The αv integrins, transmembrane heterodimeric proteins comprising an αv subunit and one of the five β subunits, bind to an arginine-glycine-aspartate (RGD) motif present on the tip of an exposed loop within the latency-associated peptide that maintains TGFβ in an inactive state.[14] All five αv integrins have been shown to interact with the RGD motif present in the latency-associated peptide.[15-19] This integrin-RGD interaction, in the presence of mechanical force supplied by the integrin-expressing cell, enables the release of the active TGFβ homodimer.[20]

Inhibition of myofibroblast αv integrins in mice reduces fibrosis in multiple organs via a reduction in TGFβ activation.[21] Furthermore, combined global knockout of integrins αvβ6 and αvβ8 phenocopies the developmental effects of loss of TGFβ-1 and -3.[22] In the liver, expression of integrin αvβ6 appears restricted to activated cholangiocytes, transitional hepatocytes, and oval cells during biliary and portal fibrosis.[23,24] Conversely, αvβ8 expression by hepatic cell types has not been well-characterized. Integrin αvβ8 has been shown to play an important role in TGFβ activation in other systems, including dendritic cells,[25-27] regulatory T cells,[28] neuroepithelium,[29] and in fibroinflammatory airway disease.[30] Further, integrin αvβ8 inhibits proliferation of lung epithelium via TGFβ activation.[31] Therefore, given the important role of αvβ8 in mediating TGFβ activation in other organ systems and pathologies, we investigated the role of hepatocyte integrin αvβ8 in the context of liver regeneration. We hypothesized that depletion of integrin αvβ8 from hepatocytes would reduce local activation of TGFβ and result in increased hepatocyte proliferation and accelerated liver regeneration following liver injury.

MATERIALS AND METHODS

Mice

Albumin-Cre (*Alb-Cre*) mice[32] were obtained from the Jackson Laboratory (Bar Harbor, ME), crossed with *Itgb8*$^{flox/flox}$ mice[33] obtained from L. Reichardt, and the resulting *Itgb8*$^{flox/flox}$*;Alb-Cre* mice were maintained on a C57BL/6 background. *Pdgfrb-Cre* mice (also on a C57BL/6 background) were obtained from R. Adams.[34] Mice used for all experiments were 8- to 16-week–old and were housed under specific pathogen–free conditions in the Animal Barrier Facility of the University of California, San Francisco, or the University of Edinburgh, UK. Genotyping of all mice was performed by PCR. Sample size was determined statistically prior to experimentation. Age- and sex-matched littermate controls were used for all experiments. Investigators were blinded to mouse genotype and experimental order was decided randomly. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, or performed in accordance with the UK Home Office regulations.

Two-thirds partial hepatectomy

Two-thirds of the liver was surgically removed under isoflurane anesthesia as previously described.[35] All surgeries were performed in the first half of the day. To label proliferating hepatocytes, 5-bromo-2-deoxyuridine (BrdU (Roche), Sigma-Aldrich, Gillingham, UK) was injected two hours prior to liver harvest (100 mg/kg intraperitoneally). Mice and livers were weighed at harvest to calculate liver-to-body weight ratio.

Hepatocarcinogenesis model

Male mice were injected with diethylnitrosamine (DEN, Sigma-Aldrich) at 12 to 14 days (25 mg/kg intraperitoneally). Mice were sacrificed at 40 weeks, and macroscopic tumors counted and measured.

Liver biochemistry

Whole blood was collected immediately post mortem, allowed to clot, and serum obtained by centrifugation (9391g for 5 minutes twice). Samples were frozen at -20 °C pending analysis. Serum albumin, total bilirubin, alanine transaminase (ALT), and alkaline phosphatase (ALP) measurements were determined using commercial kits (Alpha Laboratories, Eastleigh, UK [albumin, bilirubin, ALT]; Randox Laboratories, Crumlin, County Antrim, UK [ALP]) adapted for use on a Cobas Fara centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, UK).

Immunohistochemistry

Liver samples were fixed overnight at room temperature in either methacarn (60% methanol, 30% chloroform, 10% acetic acid), for BrdU immunohistochemistry (IHC), or 10% neutral buffered formalin. Samples were then paraffin-embedded prior to sectioning.

BrdU IHC: endogenous peroxidases were quenched with 0.3% H_2O_2 in methanol, followed by consecutive 10-minute incubation steps with 0.1% trypsin (Sigma-Aldrich, T7168), warm 1.8M HCl, and 0.1M sodium tetraborate decahydrate (Sigma-Aldrich, S9640). Blocking and subsequent incubation steps utilized the Mouse on Mouse Elite Peroxidase Kit (Vector Laboratories, Peterborough, UK, PK2200). Primary antibody was mouse anti-BrdU (Dako, Agilent Technologies, Cheadle, UK, M0744 – 1:40). Detection was performed using the Elite

Vectastain ABC kit (Vector, PK7100) and DAB (Dako, K3468), before counterstaining, dehydration, and mounting. For each liver sample, approximately 3,000 hepatocytes were counted to calculate the percentage of proliferating hepatocytes.

GR1 (neutrophil)/F4/80 (Kupffer cell)/PDGFRβ (hepatic stellate cell (HSC)) IHC: antigen retrieval was performed with Tris-EDTA (PDGFRβ only), endogenous peroxidases were quenched with 3% H_2O_2, Avidin/Biotin block was applied (Vector, SP-2001) before blocking with 20% goat serum (GR1/PDGFRβ) or rabbit serum (F4/80). Primary antibodies were applied for two hours at room temperature (PDGFRβ, Abcam, Cambridge, UK, ab32570 – 1:500) or overnight at 4 °C (GR1, R&D, Abingdon, UK, Mab 1037 – 1:750; F4/80, Abcam, ab6640 – 1:200). Secondary antibody (PDGFRβ – biotinylated goat anti-rabbit (Vector, BA-1000 – 1:1000); GR1 – biotinylated goat anti-rat (Vector, BA-9401 – 1:1000); F4/80 – biotinylated rabbit anti-rat (Vector, BA-4001 – 1:200)) was applied for 30 minutes at room temperature. Detection was performed using the Elite Vectastain ABC kit and DAB, before counterstaining, dehydration, and mounting. For each sample, 10 sequential fields were acquired at x20 magnification and percentage positive staining calculated using FIJI.[36]

β8 integrin subunit/Cleaved Caspase-3 IHC: antigen retrieval was performed with Tris-EDTA (β8 integrin subunit) or sodium citrate (cleaved caspase-3), endogenous peroxidases were quenched with 3% H_2O_2, before blocking with 20% horse serum. Primary antibody (β8 integrin subunit, Abcam, ab80673 – 1:500; Cleaved Caspase-3, Cell Signaling Technology, Leiden, The Netherlands, 9664 – 1:1000) was applied overnight at 4 °C. Detection was performed using the ImmPRESS Polymerized Reporter Enzyme Staining System (Vector, MP7401) and DAB, before counterstaining, dehydration, and mounting.

Hematoxylin and eosin staining: sections were baked at 55 ^{o}C overnight, before de-waxing and rehydration. Slides were then placed in Harris Hematoxylin (Thermo Fisher Scientific, Paisley, UK) for five minutes. After washing, slides were placed in 1% acid alcohol for five seconds, followed by Scott's tap water for two minutes. Slides were then transferred to Eosin Y solution (Thermo Fisher Scientific) for two minutes, followed by washing, dehydration, and mounting. For quantification of mitotic figures, a minimum of 1,000 hepatocytes were counted per sample.

No image processing was performed prior to quantitative analysis. Images presented in figures were contrast-enhanced by adjusting intensity minima and maxima. Images to be compared were processed identically and in a manner that preserved the visibility of dim and bright structures in the original image.

Primary mouse hepatocyte isolation

Primary mouse hepatocytes were isolated by retrograde perfusion of the liver with Liver Perfusion Medium (Thermo Fisher Scientific), followed by Liver Digest Medium (Thermo Fisher Scientific) at 37 °C. When hepatocytes were visually dispersed within the liver capsule, the liver was removed to a sterile dish and minced with scissors to release the crude cell isolate. The cells were then suspended in DMEM/F-12 (Thermo Fisher Scientific) and pelleted twice. Hepatocytes were purified from the washed pellets by resuspension in culture medium and centrifugation through 50% equilibrated Percoll (GE Healthcare Life Sciences, Little Chalfont, UK).

Standard primary hepatocyte culture

Primary hepatocytes were isolated as described above, resuspended in low-serum medium (DMEM (Thermo Fisher Scientific), 2.5% Fetal Bovine Serum (Thermo Fisher Scientific), 2% L-Glutamine (Thermo Fisher Scientific), 1% Penicillin Streptomycin (Thermo Fisher Scientific)), and plated onto collagen-coated wells (Collagen Type I, Millipore, Watford, UK) in a 6-well plate at a density of 500,000 cells per well. Either β8 integrin subunit blocking antibody[26] or non-binding control antibody were added at 20µg/mL and samples were incubated for 24 hours at 37 ºC in 5% CO_2. Wells were then washed with PBS and cells lyzed as described below.

RT-qPCR

RNA was isolated from whole mouse liver, primary hepatocytes, or liver sinusoidal endothelial cells using an RNeasy Mini Kit (whole liver, hepatocytes) or RNeasy Plus Micro Kit (liver sinusoidal endothelial cells) (Qiagen, Manchester, UK). cDNA transcription and qPCR were performed using a SYBR-GreenER Two-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific) or QuantiTect Reverse Transcription and SYBR Green PCR Kits (Qiagen). Samples were amplified on an ABI 7900HT thermocycler (Applied Biosystems, Thermo Fisher Scientific) and normalized to *Actb* and/or *Gapdh* expression. Primers used were as follows: *Actb* forward: 5'-TGTTACCAACTGGGACGACA-3', reverse: 5'-GGGGTGTTGAAGGTCTCAAA-3'; *Itgb8* forward: 5'-CTGAAGAAATACCCCGTGGA-3', reverse: 5'-ATGGGGAGGCATACAGTCT-3'. Quantitect Primer Assays (Qiagen, 249990) were used for the following genes: *Ccna2* (QT00102151); *Ccnb1* (QT00152040); *Ccnd1* (QT00154595); *Ccne1* (QT00103495); *Cdkn1a* (QT00137053); *Cdkn1b* (QT01058708); *Gapdh* (QT01658692); and *Plat* (QT00133630).

To assess TGFβ signaling, a custom RT^2 Profiler PCR array (Qiagen, 330171) was designed containing primer sequences for the genes shown in Supplementary Table S1. RNA was isolated following primary hepatocyte culture as described above and reversed transcribed using the RT^2 First Strand Kit (Qiagen, 330401). qPCR was performed using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, 330522) on an ABI 7900HT thermocycler (Applied Biosystems), normalized to *Actb* and *Gapdh* expression.

Hepatocyte adhesion assay

Adhesion was assessed using a colorimetric ECM Cell Adhesion Array Kit (Millipore, ECM540) according to the manufacturer's instructions. Primary mouse hepatocytes were isolated as described above, plated in triplicate at 50,000 cells per well, and incubated for two hours at 37 °C in 5% CO_2. Absorbance was measured at 570nm using a Synergy HT microplate reader (BioTek, Swindon, UK). Relative absorbance was calculated by standardizing to absorbance in the Collagen I well, prior to calculation of mean relative absorbance for each extracellular matrix protein for each sample.

Hepatocyte proliferation assay

Primary mouse hepatocytes were isolated as above and plated at 10,000 cells per well in 24-well plates (Primaria, Corning, St David's Park, UK, 353847) in DMEM/F-12 supplemented with 15mM HEPES (Sigma-Aldrich, H3375), 10% Fetal Bovine Serum, 1% Insulin-Transferrin-Selenium (Thermo Fisher Scientific, 41400-045), and 1% Penicillin Streptomycin. Cells were allowed to adhere for four hours before washing with PBS. Cells were then cultured for 48 hours in a low-serum version of the plating medium, containing only 0.5% Fetal Bovine

Serum. The β8 integrin subunit blocking antibody or non-binding control antibody were added at 20µg/mL. Growth factors (Thermo Fisher Scientific, hepatocyte growth factor (HGF), PHG0254, epidermal growth factor (EGF), PMG8044) were added at 40ng/mL. Culture medium, antibodies, and growth factors were refreshed at 24 hours, at which time 10µM EdU (5-ethynyl-2'-deoxyuridine, Thermo Fisher Scientific, C10640) was added. After the 48-hour culture period, cells were washed with PBS-BSA (PBS supplemented with 1% Bovine Serum Albumin (Sigma-Aldrich, A8806)) and then fixed using 4% paraformaldehyde in PBS for 15 minutes at room temperature.

Proliferating hepatocytes were detected using the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific, C10640). Briefly, fixed cells were washed with PBS-BSA and incubated in 0.5% Triton X-100 (Sigma-Aldrich, T8787) in PBS for 20 minutes at room temperature. Following washing, the Click-iT Plus reaction cocktail was added and cells incubated for 30 minutes at room temperature and protected from light. The cells were washed again and then incubated in 5µg/mL Hoechst 33342 for 30 minutes at room temperature and protected from light. Finally, cells were washed with PBS and imaged. Imaging was performed using an LSM780 confocal microscope system (Carl Zeiss Ltd, Cambridge, UK). Tiled images were acquired, with three non-overlapping areas of 18µm^2 imaged per well. Imaris (version 8.4.1, Bitplane AG, Zurich, Switzerland) was used to identify the total (Hoechst-positive) nuclei number and the number of EdU-positive nuclei, and the percentage of proliferating nuclei was calculated.

Whole liver microarray

Sample preparation, labeling, and array hybridizations were performed using the Agilent GE 4x44 Mouse microarray platform (Agilent Technologies (Palo Alto, CA); https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL7202; last accessed 13th September 2018). Total RNA quality was assessed using a Pico Chip on an Agilent 2100 Bioanalyzer, and RNA was amplified and labeled with Cy3-CTP using the Agilent low RNA input fluorescent linear amplification kits, following the manufacturer's protocol. Labeled cRNA was assessed using the Nanodrop ND-100 (Nanodrop Technologies, Inc., Wilmington DE), and equal amounts of Cy3-labeled target were hybridized to Agilent whole mouse genome 4x44K Ink-jet arrays (Agilent Technologies, G4122F). Hybridizations were performed for 14 hours, according to the manufacturer's protocol. Arrays were scanned using the Agilent microarray scanner and raw signal intensities were extracted with Agilent Feature Extraction v10.5 software. Raw data are accessible at the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE111591; last accessed 13th September, 2018).

Human liver tissue

De-identified sections of uninjured and fibrotic human liver tissue were provided by the Lothian NRS Bioresource with approval from Tissue Governance. Samples of acetaminophen-injured human liver tissue were obtained as part of the Pathophysiology of Acute Liver Injury study. This study was approved by the Scotland A Research Ethics Committee and NHS Lothian Research and Development.

Statistics

The statistical significance of differences between groups was calculated with a 2-tailed Student's *t* test or Mann Whitney test as appropriate. Differences with a *P*-value of less than 0.05 were considered statistically significant. PCR data obtained for individual genes were log-transformed prior to analysis and a Bonferroni correction was applied to account for multiple testing. PCR array data were standardized as previously reported,[37] to identify genes in test samples with a 95% confidence interval for standardized relative fold change that did not overlap 1 (the value assigned to the fold change for the same gene in control samples).

For microarray analysis, differential gene expression was examined with the R package limma (version 3.32.7).[38] Quality control was performed by identifying outliers in the log2 intensity between arrays and comparison of multidimensional scaling of distances between microarray expression profiles. Background correction was conducted according to the *normexp* method and the data were normalized using the *quantile* normalization method.[39,40] A two-way ANOVA linear model was fitted to the comparison to estimate the mean M values and calculate moderated t-statistic, B statistic, false discovery rate, and *P*-value for each gene for the comparison of interest. Adjusted *P*-values were produced by the method proposed by Holm.[41] Gene Ontology (GO) analysis was performed with the R package topGO (version 2.28.0),[42] and the *elim* algorithm, combined with the Fisher exact test, was used to calculate the enrichment scores for each of the GO terms.

RESULTS

Depletion of hepatocyte integrin αvβ8 leads to increased hepatocyte proliferation and accelerated liver regeneration

To deplete integrin αvβ8 in hepatocytes, *Itgb8$^{flox/flox}$;Alb-Cre* mice were generated.[32,33] Primary hepatocytes were isolated from *Itgb8$^{flox/flox}$;Alb-Cre* mice and Cre-negative littermate controls. Quantitative PCR for *Itgb8* confirmed expression in control hepatocytes and successful depletion in hepatocytes isolated from *Itgb8$^{flox/flox}$;Alb-Cre* mice (Fig 1A). Assessment of hepatocyte proliferation following two-thirds partial hepatectomy showed significantly increased proliferation in *Itgb8$^{flox/flox}$;Alb-Cre* mice at 36, 48, and 72 hours following liver injury compared to controls (Fig 1B,C). This increased hepatocyte proliferation was not followed by an increase in hepatocyte apoptosis at day five post partial hepatectomy, when liver regeneration was nearing completion in the *Itgb8$^{flox/flox}$;Alb-Cre* mouse (Supplemental Fig S1). Interestingly, the proportion of hepatocyte mitoses (identified morphologically) was decreased in *Itgb8$^{flox/flox}$;Alb-Cre* mice at 72 hours following liver injury compared to controls (Fig 1D). However, liver-to-body weight ratio was significantly increased in *Itgb8$^{flox/flox}$;Alb-Cre* mice at 72 and 96 hours after partial hepatectomy, demonstrating that the increase in hepatocyte proliferation in *Itgb8$^{flox/flox}$;Alb-Cre* mice detected by BrdU immunohistochemistry resulted in accelerated restoration of liver mass compared to control (Fig 1E).

Depletion of hepatocyte integrin αvβ8 does not alter baseline hepatocyte proliferation or subsequent inflammatory phenotype

As integrin αvβ8 is able to activate TGFβ, a well-characterized suppressor of epithelial proliferation, it was assessed whether genetic depletion of hepatocyte αvβ8 alters baseline hepatocyte proliferation or liver-to-body weight ratio. Hepatocyte BrdU incorporation and

mitoses, and liver-to-body weight ratio was measured in uninjured *Itgb8$^{flox/flox}$;Alb-Cre* mice and controls (Fig 1B, D, E), and no difference was found in any of these variables between groups. Furthermore, there was no difference in baseline liver biochemistry, hepatic morphology, or resident non-parenchymal cell populations (Kupffer cells and HSCs) between uninjured *Itgb8$^{flox/flox}$;Alb-Cre* mice and controls (Fig 1F-H, Supplemental Fig S2). Following partial hepatectomy, there was also no difference in hepatic inflammation (Kupffer cells or neutrophils), or HSC immunostaining (Fig 2A-C). This suggests that the increased liver regeneration observed following partial hepatectomy in *Itgb8$^{flox/flox}$;Alb-Cre* mice was not due to differences in degree of initial injury or the subsequent inflammatory response.

Depletion of HSC integrin αvβ8 does not lead to increased hepatocyte proliferation

Integrin αvβ8 is also expressed on HSCs.[21] As HSCs have been shown to play an important regulatory role in liver regeneration,[43,44] mice in which integrin αvβ8 had been depleted from HSCs (*Itgb8$^{flox/flox}$;Pdgfrb-Cre*) were used to examine the role of HSC integrin αvβ8 during liver regeneration. Following two-thirds partial hepatectomy, there was no significant difference in hepatocyte proliferation between *Itgb8$^{flox/flox}$;Pdgfrb-Cre* mice and controls (Fig 2D). Liver sinusoidal endothelial cells have also been shown to play a key role in liver regeneration.[45-47] However, integrin αvβ8 expression was not observed in liver sinusoidal endothelial cells by qPCR.

Assessment of hepatic cell cycle genes following depletion of hepatocyte integrin αvβ8 and partial hepatectomy

To examine whether depletion of hepatocyte integrin αvβ8 might have a direct effect on the cell cycle, the expression of genes with key roles in cell cycle regulation was measured at

multiple time points following partial hepatectomy. Overall, partial hepatectomy resulted in expected changes in gene expression in whole liver from both *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls (Fig 3A, B, Supplemental Fig S3). There was a trend towards increased expression of *Ccna2* and *Ccnb1* in *Itgb8*$^{flox/flox}$*;Alb-Cre* mice compared to controls; however, this did not reach statistical significance at any time point (Fig 3A, B). Analysis of other cell cycle–related genes (*Ccnd1*, *Ccne1*, *Cdkn1a*, *Cdkn1b*) showed no difference between *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls (Supplemental Fig S3).

Depletion of integrin αvβ8 on hepatocytes does not alter adhesion to multiple matrix proteins present in normal and regenerating liver

Integrin αvβ8 binds extracellular matrix ligands such as vitronectin, fibronectin, collagen IV, and fibronectin.[48,49] To test whether depletion of integrin αvβ8 on hepatocytes alters adhesion to cell matrix proteins present in normal and regenerating liver, an *in vitro* cell adhesion assay with multiple different matrix substrates was used. No difference was found in adhesion between *Itgb8*$^{flox/flox}$*;Alb-Cre* and control hepatocytes across all seven matrix proteins tested (Fig 3C), suggesting that altered hepatocyte adhesion is not responsible for the pro-regenerative phenotype observed in *Itgb8*$^{flox/flox}$*;Alb-Cre* mice.

Inhibition of integrin αvβ8 modulates TGFβ-responsive genes in hepatocytes

Integrin αvβ8 has previously been shown to play a key role in the activation of latent TGFβ,[25-31] a potent inhibitor of hepatocyte proliferation.[5-7] Therefore, we hypothesized that depletion of hepatocyte integrin αvβ8 might promote hepatocyte proliferation through modulation of TGFβ signaling pathways. The time course of hepatic *Itgb8* expression following partial hepatectomy supports a role for integrin αvβ8 as a suppressor of

hepatocyte proliferation during liver regeneration (Fig 3D). Hepatic *Itgb8* expression falls markedly in the 24 hours immediately following partial hepatectomy, and this down-regulation appears to be permissive for hepatocyte proliferation. As the liver approaches full restoration of its functional mass at five days post partial hepatectomy, hepatocyte *Itgb8* expression peaks at 10 times baseline expression, consistent with a role for integrin αvβ8 as a brake on hepatocyte proliferation (Fig 3D).

Detecting the modulation of TGFβ activation in the hepatocyte regenerative niche is very challenging, as it is not possible to measure the levels of active TGFβ in tissue directly. Therefore, an experiment was designed to examine how inhibition of integrin αvβ8 might modulate TGFβ-responsive genes in primary mouse hepatocytes (Fig 3E). Firstly, a custom qPCR array was designed, containing 87 genes either shown to be responsive to TGFβ signaling in hepatocytes[50] or comprising components of the TGFβ pathway (Supplemental Table S1). Primary murine hepatocytes were isolated from wild-type mice and plated onto collagen in the presence of either a β8 integrin subunit blocking antibody or a non-binding control antibody.[26] After incubation for 24 hours, hepatocytes were lyzed, RNA was extracted, and gene expression quantified using the custom qPCR array. Data were then standardized as previously described by Willems et al[37] Following culture with β8 integrin subunit blocking antibody, 20 genes in the qPCR array were found to have a detectable change in expression with a 95% confidence interval which did not overlap control values (Fig 3F). Of these, 12 genes showed greater than 10% up- or down-regulation when compared to controls, with 10 out of 12 responding as predicted. *Plat*, encoding tissue plasminogen activator (tPA), showed the greatest up-regulation. Hepatocyte expression of *Plat* has been shown to decrease in the presence of TGFβ,[50] whereas expression increased

three-fold in wild-type hepatocytes treated with β8 integrin subunit blocking antibody. Increased expression of *Plat* was not observed when hepatocytes from *Itgb8*$^{flox/flox}$*;Alb-Cre* mice were treated with β8 integrin subunit blocking antibody, suggesting the observed response is specific to β8 integrin subunit inhibition (Supplemental Fig S4). Conversely, hepatocyte expression of the TGFβ-responsive gene *Hmox1* (heme oxygenase 1) was down-regulated in the presence of β8 integrin subunit blocking antibody. These data demonstrate that inhibition of integrin αvβ8 modulates TGFβ-responsive genes in hepatocytes, suggesting a possible mechanism through which integrin αvβ8 depletion promotes hepatocyte proliferation.

Inhibition of hepatocyte integrin αvβ8 does not alter the proliferative response to mitogenic growth factors

Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are key drivers of liver regeneration.[8] To investigate whether integrin αvβ8 might have a role in regulating the hepatocyte response to these direct mitogens, the effect of β8 integrin subunit inhibition during *in vitro* proliferation of primary hepatocytes in response to EGF and HGF was examined. A robust increase in hepatocyte proliferation was achieved with addition of either, or both, EGF and HGF, compared to standard culture medium (Fig 3G). However, inhibition of integrin αvβ8 had no effect on the degree of *in vitro* hepatocyte proliferation. This suggests that the accelerated liver regeneration observed following hepatocyte integrin αvβ8 depletion does not occur via modulation of HGF or EGF downstream signaling pathways.

Microarray analysis of whole liver from control and *Itgb8*$^{flox/flox}$*;Alb-Cre* mice

To explore further the potential mechanisms by which inhibition of integrin αvβ8 increases hepatocyte proliferation and accelerates liver regeneration, global gene expression changes were examined by microarray analysis of whole liver. Samples were obtained from control and *Itgb8$^{flox/flox}$;Alb-Cre* mice prior to, and at 24 hours post, partial hepatectomy. From 26,136 transcripts, 1,080 showed statistically significant differential expression following partial hepatectomy. Three-hundred and thirty of these occurred only in the *Itgb8$^{flox/flox}$;Alb-Cre* mouse (Fig 4A). Gene ontology (GO) analysis was performed on the differentially expressed genes. The dominant GO terms enriched in genes up-regulated exclusively in *Itgb8$^{flox/flox}$;Alb-Cre* mice following partial hepatectomy are shown in Figure 4B. The majority of these terms relate to cytoskeletal organization and cellular adhesion. Similarly, the dominant GO terms enriched in genes down-regulated exclusively in *Itgb8$^{flox/flox}$;Alb-Cre* mice following partial hepatectomy are shown in Figure 4C. These terms were relatively non-specific, relating to a range of intracellular metabolic processes.

Given the increased hepatocyte proliferation observed following partial hepatectomy in *Itgb8$^{flox/flox}$;Alb-Cre* mice, changes in expression of genes known to regulate the cell cycle were specifically studied. However, when comparing post partial hepatectomy samples from *Itgb8$^{flox/flox}$;Alb-Cre* mice and controls, no significant difference in the expression of cell cycle genes was detected by microarray. This mirrors the findings of both our examination of cell cycle gene expression in whole liver pre and post partial hepatectomy (Fig 3A, B and Supplemental Fig S3) and the qPCR array in cultured hepatocytes treated with β8 integrin subunit blocking antibody, in which no consistent changes in gene expression were noted in the 10 cell-cycle and proliferation genes included in the array (Supplemental Table S1).

Depletion of hepatocyte integrin αvβ8 does not increase tumor formation in a mouse model of HCC

In addition to promoting hepatocyte proliferation, disruption of TGFβ signaling can accelerate the development of HCC in mice following DEN administration.[12] As depletion of integrin αvβ8 on hepatocytes increased hepatocyte proliferation and accelerated liver regeneration following injury, and blockade of hepatocyte integrin αvβ8 *in vitro* modulated TGFβ-responsive genes, the possibility that this pro-proliferative phenotype might increase the risk of HCC development was also assessed. *Itgb8*$^{flox/flox}$*;Alb-Cre* and control mice were injected with DEN at 12 to 14 days of age to induce HCC (Fig 5A). Following sacrifice at forty weeks, the number and size of tumors in each liver was quantified (Fig 5B). There was no difference in either tumor number or median tumor size between *Itgb8*$^{flox/flox}$*;Alb-Cre* and control mice (Fig 5C, D). This demonstrates that depletion of hepatocyte integrin αvβ8 does not predispose to increased tumor formation in this mouse model of HCC.

Human hepatocytes express integrin αvβ8 and represent a viable therapeutic target to promote liver regeneration in patients with liver disease

To assess the potential utility of integrin αvβ8 as a therapeutic target to promote hepatocyte proliferation and liver regeneration in patients with liver disease, the expression of integrin αvβ8 was assessed in samples of human liver. Uninjured liver tissue and tissue obtained from patients with acute liver failure secondary to acetaminophen overdose or from patients with cirrhosis was stained for the β8 integrin subunit. Widespread expression in hepatocytes was detected in all samples, demonstrating that integrin αvβ8 is a viable potential therapeutic target in patients with a broad range of liver diseases (Fig 5E).

DISCUSSION

We show that depletion of hepatocyte integrin αvβ8 leads to increased hepatocyte proliferation and accelerated liver regeneration following partial hepatectomy in mice. The time course of hepatic *Itgb8* expression following partial hepatectomy, namely a rapid down-regulation followed by rebound up-regulation as the liver returns to its normal size, is consistent with a role for integrin αvβ8 as a brake on hepatocyte proliferation. This anti-proliferative role for integrin αvβ8 appears to be mediated via TGFβ, rather than altered hepatocyte adhesion, since blocking integrin αvβ8 on hepatocytes alters TGFβ-responsive gene expression. Importantly, the augmentation in hepatocyte proliferation in *Itgb8*$^{flox/flox}$*;Alb-Cre* mice was not accompanied by increased susceptibility to hepatocellular tumor formation. Finally, human hepatocytes also express integrin αvβ8 in both acute and chronic liver disease, and therefore integrin αvβ8 represents a viable therapeutic target to promote liver regeneration in patients with a broad range of liver diseases.

Integrin αvβ8 has previously been shown to have a key regulatory role in the activation of latent TGFβ.[25-31] The inhibitory effect of active TGFβ on hepatocyte proliferation is well-established, including evidence demonstrating tonic inhibition of hepatocyte proliferation in the uninjured liver.[5-7] The rapid down-regulation of hepatic *Itgb8* expression observed following partial hepatectomy is in line with the hypothesis that a reduction in integrin αvβ8–mediated activation of TGFβ is permissive for a pro-regenerative environment in the liver. Demonstrating subtle changes in activation status of TGFβ within the hepatic regenerative niche is very challenging, given the magnitude and localized nature of these changes, and also the small amount of remnant tissue present following two-thirds partial

hepatectomy. However, inhibition of hepatocyte integrin αvβ8 *in vitro*, using a β8 integrin subunit blocking antibody, resulted in changes in expression of multiple TGFβ-responsive genes such as *Plat* and *Hmox1*. Tissue plasminogen activator, encoded by *Plat*, can activate HGF[51] and has been shown to play a role in liver lobule reorganization following acute injury.[52] Knockout of tPA in mice worsens injury following bile duct ligation, but this phenotype is reversed by administration of HGF.[53] These data suggest that the regulatory role of integrin αvβ8 during hepatocyte proliferation is, at least in part, mediated via TGFβ signaling, and that integrin αvβ8 depletion or inhibition may drive hepatocyte proliferation through tPA-mediated activation of HGF. Inhibition of hepatocyte integrin αvβ8 did not alter the proliferative response to stimulation with EGF and HGF *in vitro*, suggesting that the accelerated liver regeneration observed following hepatocyte integrin αvβ8 depletion does not occur via modulation of HGF or EGF downstream signaling pathways.

Detectable changes in the expression of genes regulating the cell cycle were identified following partial hepatectomy, similar to those previously reported,[54] but did not differ between *Itgb8$^{flox/flox}$;Alb-Cre* mice and controls. Inhibiting integrin αvβ8 *in vitro* also had no effect on expression of cell cycle genes, as measured by qPCR gene array. This would suggest that depletion of integrin αvβ8 on hepatocytes does not appear to change the kinetics of cell cycle regulation in the individual cell. Instead, depletion of hepatocyte integrin αvβ8 may promote liver regeneration by permitting a greater number of hepatocytes to escape the anti-proliferative effects of active TGFβ. The observation of a reduction in the proportion of hepatic mitoses in *Itgb8$^{flox/flox}$;Alb-Cre* mice compared to controls at 72 hours post partial hepatectomy is difficult to reconcile with the increase in BrdU incorporation and the accelerated restoration of total liver mass. Although no change

was detected in cell cycle kinetics, if the overall time in M phase were reduced in hepatocytes of *Itgb8*$^{flox/flox}$*;Alb-Cre* mice, this could decrease the proportion of mitotic hepatocytes at any single point in time. Previous work has demonstrated a strong effect of circadian rhythm on hepatocyte entry into M phase following partial hepatectomy in mice, an effect not seen when assessing BrdU incorporation.[55] However, in this study, partial hepatectomy was always performed in the morning and experimental order randomized, so this should not account for differences between *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls.

To screen for additional pathways that might be regulated by integrin αvβ8, gene expression was assessed using microarray in whole liver samples from *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls, comparing gene expression in uninjured liver and 24 hours post partial hepatectomy. Despite observing a large number of changes in gene expression (330) following partial hepatectomy that were restricted to *Itgb8*$^{flox/flox}$*;Alb-Cre* mice, there were again no differences in expression of genes regulating the cell cycle or proliferation, when compared to control mice. The failure to detect changes in expression of genes regulating the cell cycle may also reflect limitations in sensitivity, particularly of the microarray technique, when applied to whole liver lysates from *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls. Even following partial hepatectomy, only a minority of hepatocytes will be proliferating at any one time and the presence of non-parenchymal cell mRNA in the whole liver lysates that were analyzed will further reduce the signal-to-noise ratio.

Gene ontology analysis of genes up-regulated only in *Itgb8*$^{flox/flox}$*;Alb-Cre* mice following partial hepatectomy returned multiple terms relating to cytoskeletal organization and extracellular adhesion. Integrins are well-known for their role in extracellular adhesion and

their cytoplasmic domains can bind the cytoskeleton.[14] However, it has previously been suggested that the cytoplasmic domain of the β8 subunit does not bind the cytoskeleton.[48] Furthermore, no difference was found in the ability of hepatocytes isolated from *Itgb8*$^{flox/flox}$*;Alb-Cre* mice and controls to adhere to multiple extracellular matrix proteins found in both normal and regenerating liver.

Targeting of TGFβ pathways has been a major focus of research across several fields, particularly in the context of inflammation, wound healing, and oncogenesis. Unfortunately, global inhibition of TGFβ signaling can be associated with serious, undesirable effects, including excessive inflammation and development of neoplasia.[10-12] This is highly likely to be due to the pleiotropic, context-dependent functions of TGFβ. Selective targeting of TGFβ activation by inhibition of integrin αvβ8 in the hepatic regenerative niche may potentially avoid many of the adverse effects noted with pan-TGFβ blockade, while still promoting the desired effects on hepatocyte proliferation and liver regeneration. Importantly, these results did not demonstrate an increase in either hepatic inflammation or carcinogenesis in mice following depletion of hepatocyte integrin αvβ8.

Human hepatocytes express integrin αvβ8 in uninjured liver, following acute hepatic injury secondary to acetaminophen overdose, and also in cirrhosis. Therefore, hepatocyte integrin αvβ8 appears to be a viable translational target. There are potentially multiple clinical scenarios to which integrin αvβ8 inhibition could be applied. For example, using αvβ8 inhibition as a pro-regenerative therapy in the setting of acute liver failure may obviate the requirement for, or buy more time prior to, liver transplantation. Furthermore, combination with anti-fibrotic therapies could permit the restoration of functional, parenchymal liver

mass in tandem with a reduction in fibrosis in patients with chronic liver disease. It might also allow more patients with primary or metastatic liver cancer to be treated successfully.

In summary, depletion of integrin αvβ8 on murine hepatocytes leads to increased hepatocyte proliferation and accelerated liver regeneration. Targeting integrin αvβ8 may therefore represent a promising therapeutic strategy to drive liver regeneration in patients with a broad range of liver diseases.

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Figure Legends

Figure 1 Genetic depletion of hepatocyte integrin αvβ8 accelerates liver regeneration. **A:** qPCR of *Itgb8* expression in hepatocytes isolated from control and *Itgb8$^{flox/flox}$;Alb-Cre* (β8-AlbCre) mice (n=3). **B:** Quantitation of BrdU^{+} hepatocyte nuclei in control and β8-AlbCre mice after partial hepatectomy (n=3 to 6). **C:** Representative images from BrdU immunostaining of liver sections from control and β8-AlbCre mice at 0 and 48 hours after partial hepatectomy. Quantitation of hepatocyte mitoses (**D**) and liver-to-body weight ratio (**E**) in control and β8-AlbCre mice after partial hepatectomy (n=3 to 6). **F:** Serum biochemistry (total bilirubin, alanine transaminase (ALT), alkaline phosphatase (ALP), albumin) from uninjured control and β8-AlbCre mice (n=6). **G** and **H:** Quantification and representative images from F4/80 (Kupffer cell, **G**) and PDGFRβ (HSC, **H**) immunostaining of liver tissue from uninjured control and β8-AlbCre mice (n=6). All data presented as mean; error bars – SEM. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. Scale bars 100μm.

Figure 2 Depletion of hepatocyte integrin αvβ8 does not alter inflammatory phenotype following partial hepatectomy. **A-C:** Quantification and representative images from F4/80 (Kupffer cell, **A**), GR1 (neutrophil, **B**), and PDGFRβ (HSC, **C**) immunostaining of liver tissue from control and *Itgb8$^{flox/flox}$;Alb-Cre* (β8-AlbCre) mice at 48 hours post partial hepatectomy (n=5). **D:** Quantitation of BrdU^{+} hepatocyte nuclei and representative images of BrdU immunostaining of liver tissue from control and *Itgb8$^{flox/flox}$;Pdgfrb-Cre* (β8-PdgfrbCre) mice at 48 hours post partial hepatectomy (n=4 and 8). All data presented as mean; error bars – SEM. Scale bar 100μm.

Figure 3 Investigation of the mechanisms mediating the pro-regenerative effect of hepatocyte integrin αvβ8 depletion. **A, B:** Whole liver expression of cell cycle genes *Ccna2* (**A**) and *Ccnb1* (**B**) from control and *Itgb8*$^{flox/flox}$*;Alb-Cre* (β8-AlbCre) mice following partial hepatectomy (n=4 to 6 per time point). **C:** Isolated hepatocytes from control and β8-AlbCre mice (n=4) were tested in a colorimetric extracellular matrix adhesion assay (BSA, bovine serum albumin; Col, collagen). **D:** Whole liver expression of *Itgb8* following partial hepatectomy (n=3 to 6 per time point). **E:** Schematic of experimental design to test the effect of a β8 integrin subunit blocking antibody on hepatocyte expression of transforming groath factor (TGF)-β–responsive genes. **F:** Fold regulation of genes from the qPCR array with a detectable change in hepatocyte expression following culture with β8 integrin subunit blocking antibody (n=3). **G:** Proliferation of primary hepatocytes cultured for 48 hours with β8 integrin subunit blocking antibody or control antibody in culture medium (Control) and with addition of either epidermal growth factor (EGF), hepatocyte growth factor (HGF), or both (n=3). All data presented as mean; error bars – SEM.

Figure 4 Microarray analysis of whole liver (uninjured and 24 hours post partial hepatectomy) from control and *Itgb8*$^{flox/flox}$*;Alb-Cre* (β8-AlbCre) mice. **A:** Summary of the number of transcripts with significant changes in expression following partial hepatectomy in β8-AlbCre mice and controls (n = 4 per group per time point). **B** and **C:** The top 10 GO terms enriched in those genes either up-regulated (**B**) or down-regulated (**C**) exclusively in β8-AlbCre mice following partial hepatectomy.

Figure 5 Depletion of hepatocyte integrin αvβ8 does not increase HCC in mice, whereas human hepatocytes express integrin αvβ8 in acute and chronic liver disease. **A:** Schematic of mouse model of HCC (i.p., intraperitoneal). **B:** Representative images of livers from control and *Itgb8*$^{flox/flox}$*;Alb-Cre* (β8-AlbCre) mice at harvest. **C** and **D:** Quantification of tumor number (**C**) and median tumor size (**D**) in control and β8-AlbCre mice at 40 weeks (horizontal bar indicates mean, n=16 and 14). **E:** Representative low- and high-power images of β8 integrin subunit immunostaining in uninjured human liver tissue (n=5), following acetaminophen overdose (n=5), or in cirrhosis (n=6). Scale bars 250µm (upper) and 100µm (lower).

Figure 1

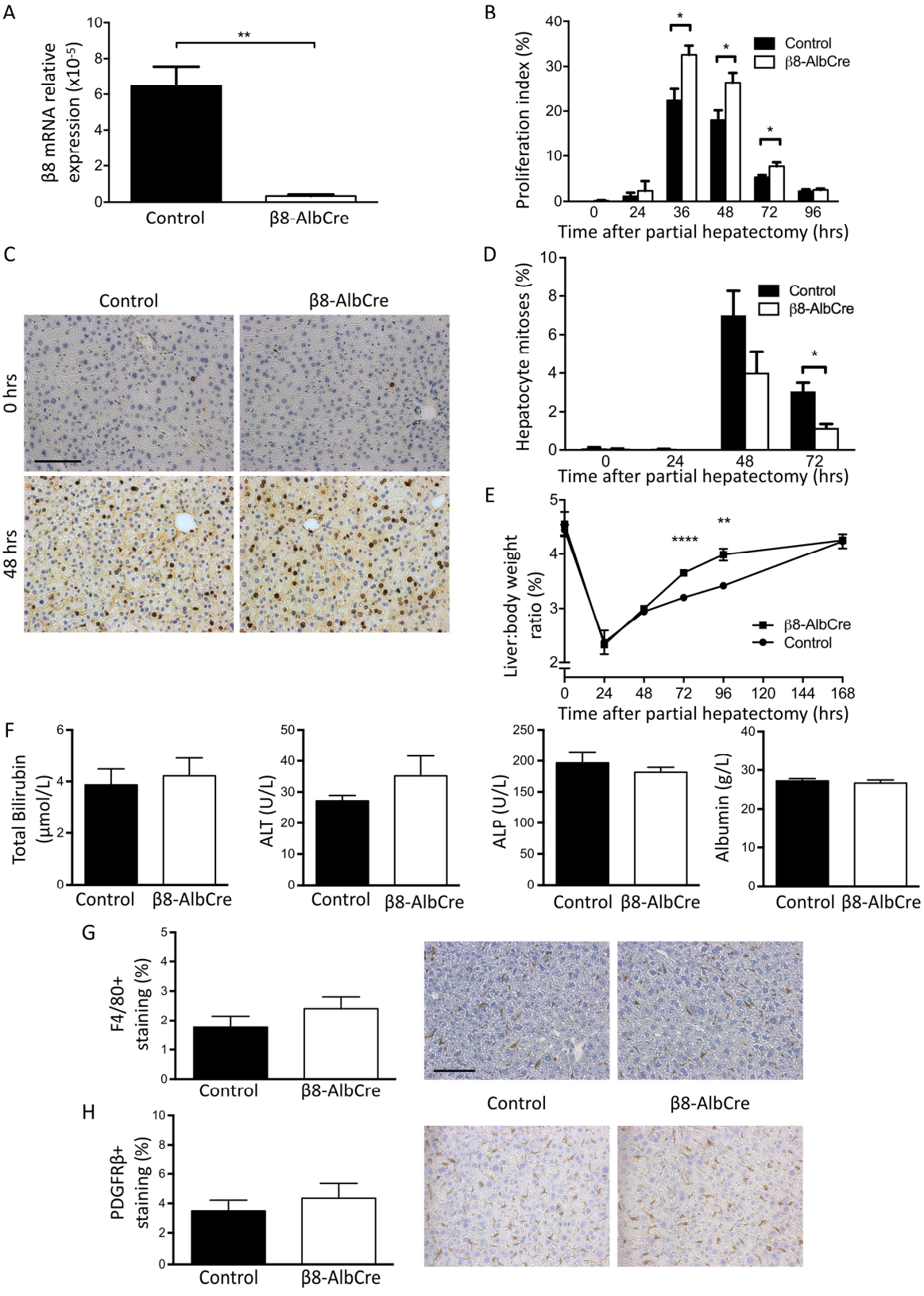

A
β8 mRNA relative expression (x10-5)
Control
β8-AlbCre
B
Proliferation index (%)
Control
β8-AlbCre
Time after partial hepatectomy (hrs)
C
Control
β8-AlbCre
0 hrs
48 hrs
D
Hepatocyte mitoses (%)
Control
β8-AlbCre
Time after partial hepatectomy (hrs)
E
Liver:body weight ratio (%)
β8-AlbCre
Control
Time after partial hepatectomy (hrs)
F
Total Bilirubin (µmol/L)
ALT (U/L)
ALP (U/L)
Albumin (g/L)
Control
β8-AlbCre
G
F4/80+ staining (%)
H
PDGFRβ+ staining (%)

Figure 2

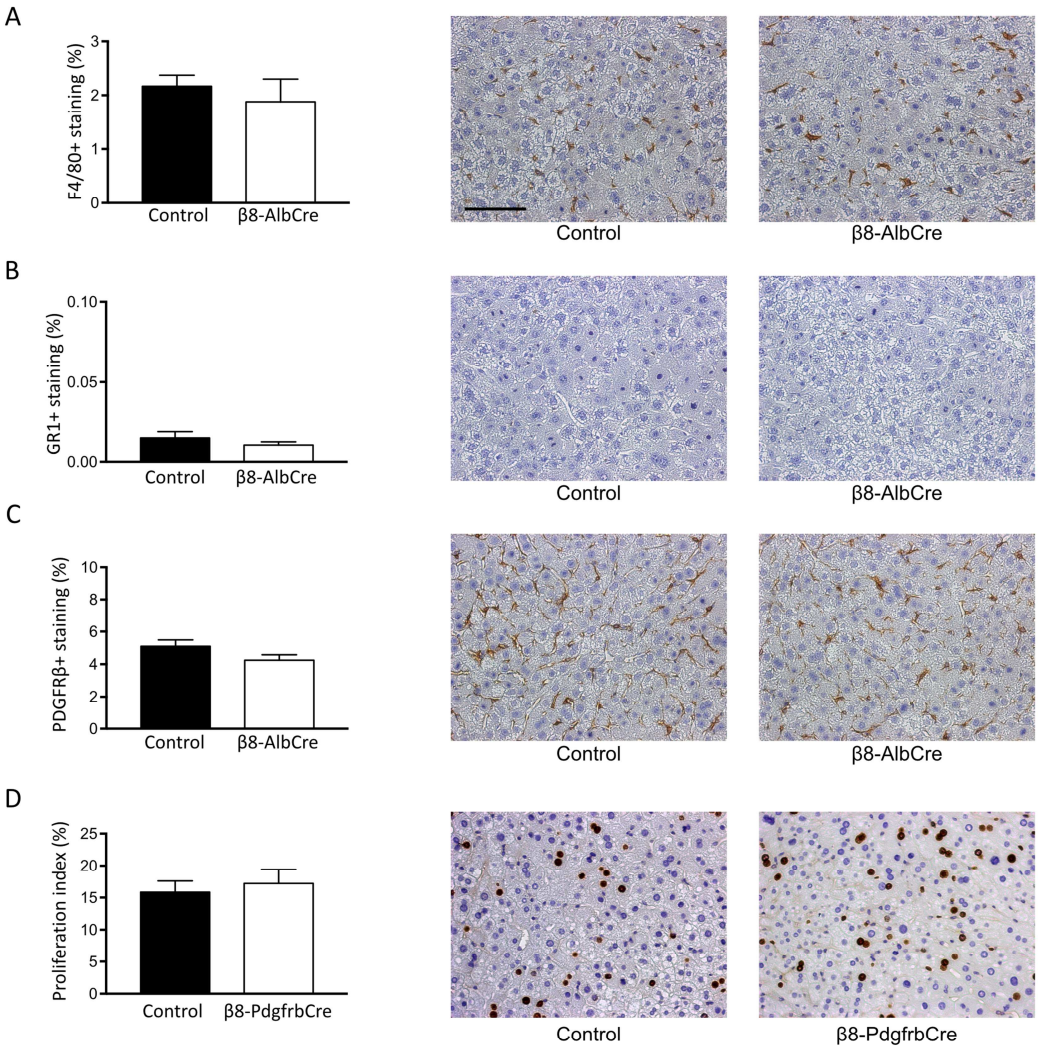

A
F4/80+ staining (%)
3
2
1
0
Control
β8-AlbCre
Control
β8-AlbCre
B
GR1+ staining (%)
0.10
0.05
0.00
Control
β8-AlbCre
Control
β8-AlbCre
C
PDGFRβ+ staining (%)
10
8
6
4
2
0
Control
β8-AlbCre
Control
β8-AlbCre
D
Proliferation index (%)
25
20
15
10
5
0
Control
β8-PdgfrbCre
Control
β8-PdgfrbCre

Figure 3

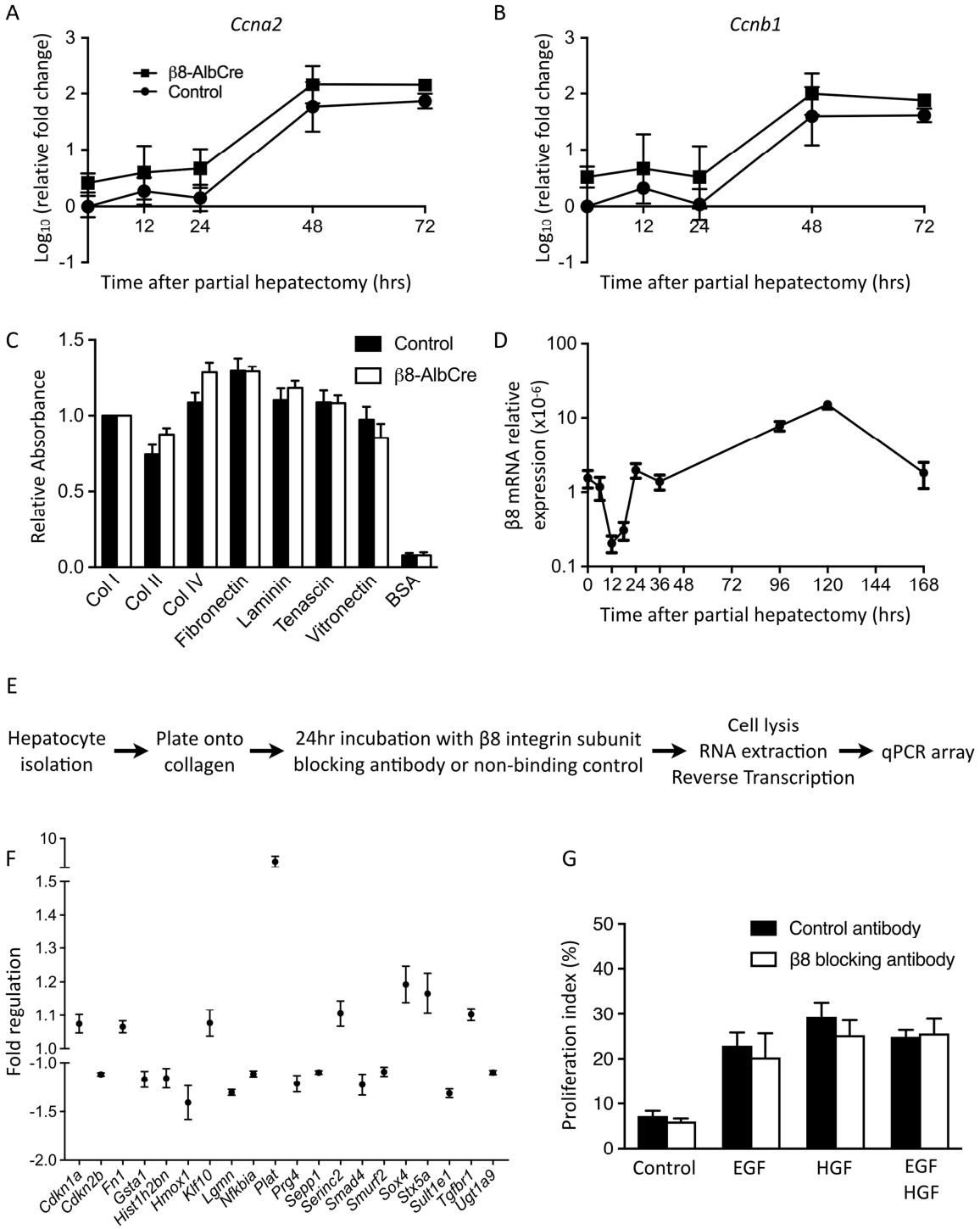

A
Ccna2
β8-AlbCre
Control
Log10 (relative fold change)
Time after partial hepatectomy (hrs)
B
Ccnb1
Log10 (relative fold change)
Time after partial hepatectomy (hrs)
C
Control
β8-AlbCre
Relative Absorbance
Col I
Col II
Col IV
Fibronectin
Laminin
Tenascin
Vitronectin
BSA
D
β8 mRNA relative expression (x10-6)
Time after partial hepatectomy (hrs)
E
Hepatocyte isolation
Plate onto collagen
24hr incubation with β8 integrin subunit blocking antibody or non-binding control
Cell lysis
RNA extraction
Reverse Transcription
qPCR array
F
Fold regulation
Cdkn1a
Cdkn2b
Fn1
Gsta1
Hist1h2bn
Hmox1
Klf10
Lgmn
Nfkbia
Plat
Prg4
Sepp1
Serinc2
Smad4
Smurf2
Sox4
Stx6a
Sulf1e1
Tgfbr1
Ugt1a9
G
Control antibody
β8 blocking antibody
Proliferation index (%)
Control
EGF
HGF
EGF HGF

Figure 4

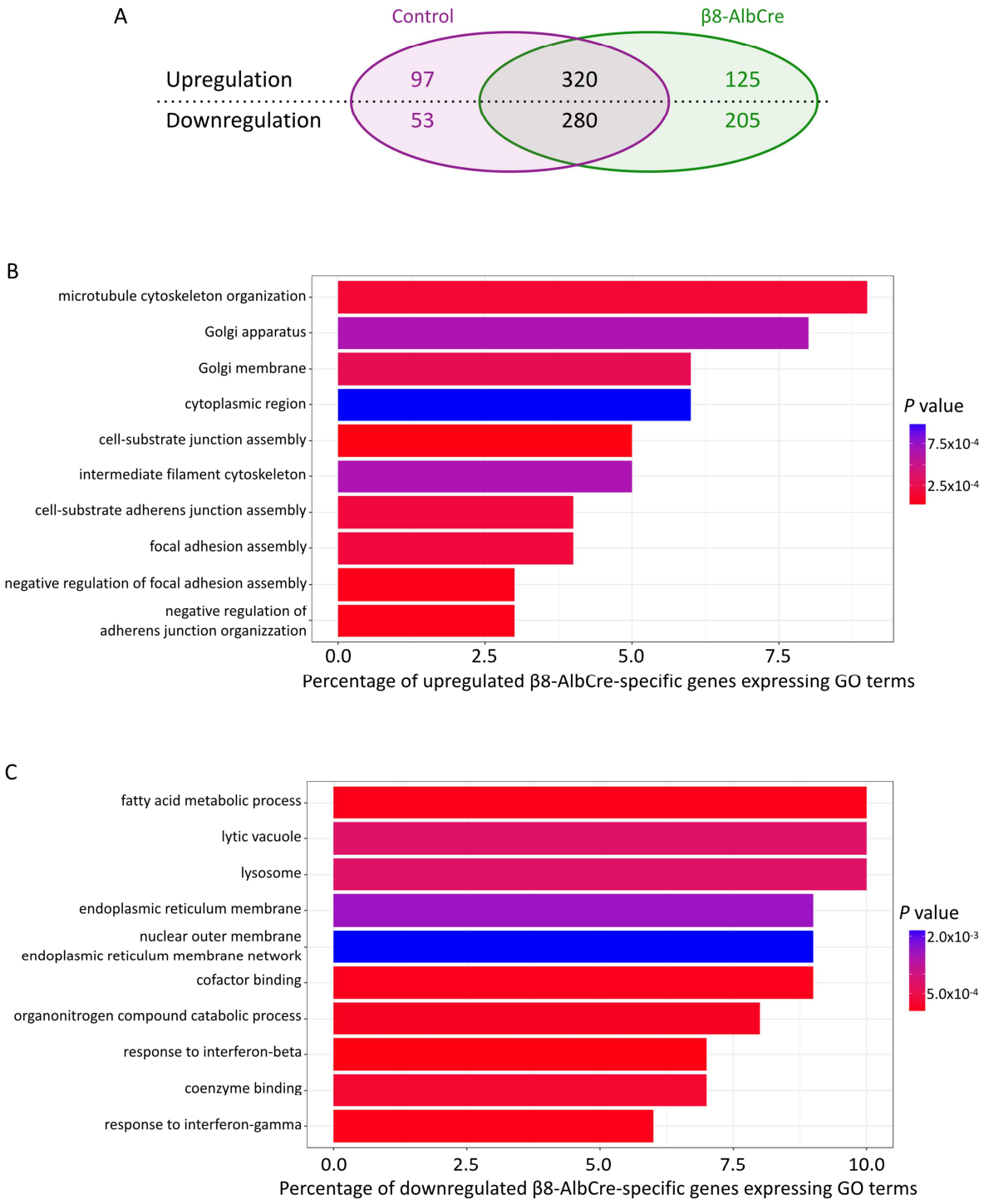
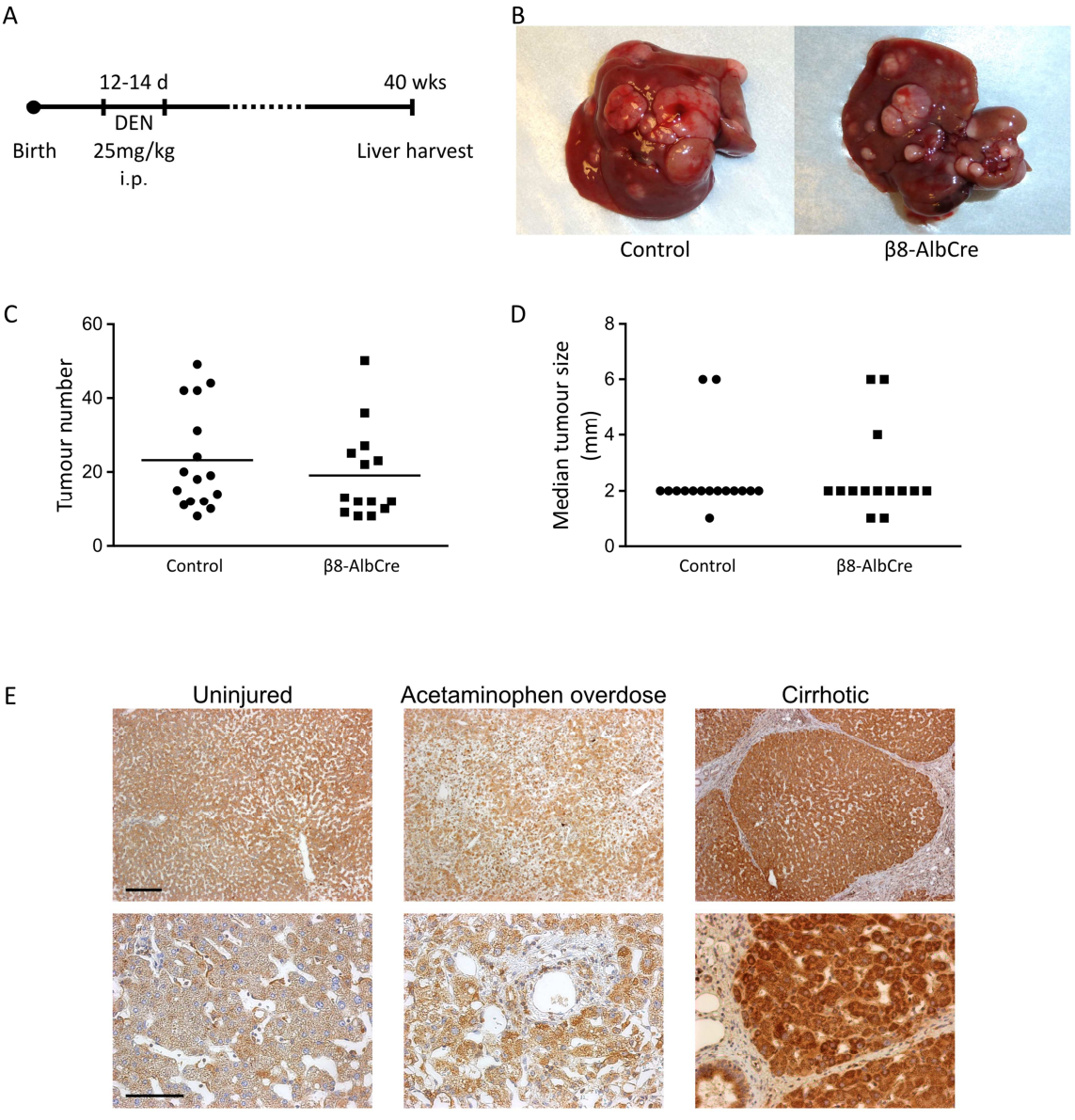

A
Control
β8-AlbCre
Upregulation
Downregulation
97
320
125
53
280
205
B
microtubule cytoskeleton organization
Golgi apparatus
Golgi membrane
cytoplasmic region
cell-substrate junction assembly
intermediate filament cytoskeleton
cell-substrate adherens junction assembly
focal adhesion assembly
negative regulation of focal adhesion assembly
negative regulation of adherens junction organizzation
0.0
2.5
5.0
7.5
Percentage of upregulated β8-AlbCre-specific genes expressing GO terms
P value
7.5x10-4
2.5x10-4
C
fatty acid metabolic process
lytic vacuole
lysosome
endoplasmic reticulum membrane
nuclear outer membrane endoplasmic reticulum membrane network
cofactor binding
organonitrogen compound catabolic process
response to interferon-beta
coenzyme binding
response to interferon-gamma
0.0
2.5
5.0
7.5
10.0
Percentage of downregulated β8-AlbCre-specific genes expressing GO terms
P value
2.0x10-3
5.0x10-4

Figure 5


A
12-14 d
40 wks
DEN
Birth
25mg/kg
i.p.
Liver harvest
B
Control
β8-AlbCre
C
Tumour number
60
40
20
0
Control
β8-AlbCre
D
Median tumour size (mm)
8
6
4
2
0
Control
β8-AlbCre
E
Uninjured
Acetaminophen overdose
Cirrhotic