

Supporting Information

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SI Materials and Methods

Animal Models. C57Bl/6 mice, 6 weeks, or 19 months of age, were purchased from Harlan (UK), all mice are 6 weeks of age unless otherwise stated. Fibroblast growth factor-inducible immediate-early response protein 14 (Fn14)^{-/-} (1) and TNF-like weak inducer of apoptosis (TWEAK)^{-/-} mice (2) were generously provided by Biogen Idec. TgTP6.3 tauGFP (3) and CD11b-Diphtheria Toxin (DT) Receptor (DTR) - (4) and their respective WT littermates were from in house colonies. BrdU (50mg/g, Amersham, UK) was administered by i.p. injection. Murine recombinant-(r)TWEAK (R and D Systems) was reconstituted in sterile PBS and 0.4μg (1, 5) was administered by tail vein injection daily for 7 days.

Donor bone marrow (BM) cells (BMCs) were extracted from the femurs and tibias of mice with PBS and a single cell suspension prepared by passing the cells through a 21 gauge needle prior to a 40mm filter (BD Falcon), after which the cells were centrifuged at 400g for 5 minutes, washed with PBS, and re-suspended in PBS for injection at 5x10⁷ cells/ml. For irradiated cell controls, cells were exposed to 30 gray from a GammaCell 40E (MDS Nordian, Fleuvus, Belgium) with a Caesium 137 source.

DT was prepared to working concentration by dissolving in DMSO and administered at 10ng/g via i.p. injection on days 0, 3, and 6 as previously described (4) to WT mice following transfer of 10⁷ BMC from CD11b-DTR donors. Controls received volume matched DT-free DMSO diluted in PBS.

Blood was collected by cardiac puncture and serum prepared for analysis using commercial kits for: Alanine transaminase (ALT; Alpha Laboratories Ltd, UK), Albumin (Olympus Diagnostics Lt, UK), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (both Randox Laboratories, UK) according to manufacturer's instructions.

Macrophage Differentiation and Purification in Vitro. Isolated BMCs (2x10⁷) were cultured for 7 days in Teflon pots (Roland Vetter Labs) in DMEM containing penicillin and streptomycin, 10% FCS and 20% L929 conditioned media, as previously described (6). Purity was assessed by morphology, CD45 and F4/80 expression and was consistently >99% (Figure S3A-S3C). In preparation for injection, macrophages were washed twice and re-suspended in PBS at 5x10⁷ cells/ml. Irradiation was performed as described above.

Harvest of Transferred Cells. Animals were sacrificed by CO₂ inhalation and their livers digested *in situ* using a modified retrograde perfusion technique (7) consisting of: 1) Liver Perfusion Medium (Gibco) for 5 minutes; 2) Liver Digest Medium (Gibco) for 10 minutes. The liver was then excised and the capsule disrupted to yield a cell suspension which was collected in Liver Perfusion Medium (Gibco) and passed through a 50mm filter (BD Biosciences). Liver disruption for FACS based HPC isolation used a gentleMAX Dissociator with C tubes (Miltenyi). Following red cell lysis (5 minutes in 160mM NH₄Cl 10mM KHCO₃ containing 0.01% EDTA), cells were re-suspended in DMEM (Gibco) containing 50% HamsF-10 nutrient mix (Gibco), 50ml of 10mg/ml insulin (Sigma, UK) and 50ml of 50mg/ml hydrocortisone (Sigma, UK), 500ml gentamicin (Gibco), 5ml 50mg/ml Sodium Pyruvate and underlayered with an equal volume of 20% and 50% (v/v) Percoll (Sigma, UK) in PBS, respectively. Following centrifugation at 1400g for 20 minutes at 4°C, the fraction lying between the 20% and 50% Percoll layers (enriched for non-parenchymal cells) was collected, washed twice and re-suspended in PBS.

In Vitro Colony Formation Assay. For clonal analysis, FACS sorted cells were plated on Rat Tail Collagen Coated plates (1mg/ml, Sigma) at clonal density of 500 cells/cm² (8). Cells were cultured in Williams media containing 17.6mM NaHCO₃, 20mM HEPES pH 7.5, 10mM Nicotinamide, 1x ITS, 100nM Dexamethasone, 0.2mM Ascorbic Acid, 1mM Na Pruvate, 14mM Glucose, 10ng/ml Il-6, 10ng/ml Hepatocyte Growth Factor (HGF), 10ng/ml Epidermal Growth Factor (EGF), with either 10% FCS (first 24hrs) or 5% FCS (subsequently). Assessment of colonies was made daily upto 56 days.

Flow Cytometry. Analysis and sorting was performed using a FACS Vantage, equipped with a Coherent INNOVA Enterprise II laser (Becton and Dickinson, UK). Propidium Iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) (Sigma, UK) was added to the cells prior to sorting at a final concentration of 2mg/ml to distinguish and exclude dead cells in the BM population. Cells were also immunophenotyped using phycoerythrin (PE) conjugated lineage antibodies, anti-CD45, anti CD31, and anti-Ter119 (Clones 30-F11, 390 and Ter119 respectively, eBiosciences), or the HPC markers PE-cyanine 7 conjugated anti-CD24 (9) (Clone M1/69, Biolegend) or Allophycocyanin conjugated anti-Epithelial Cell Adhesion Molecule (EpCAM) (10) (Clone G8.8 eBiosciences). Living cells were gated using 7 Aminoactinomycin-D (biollegend) and compensation was performed using BD CompBeads (BD Biosciences, UK). GFP⁺ and CD24⁺/EpCAM⁺ cells were gated as shown (Figures S2C and 4G respectively).

Immunohistochemistry. Three mm thick paraffin sections were stained for the accepted murine HPC markers: pan-cytokeratin (panCK) (11) (Z0622, Dako), Delta Like Homolog 1 (Dlk1) (12), EpCAM (10) (AB21682 and AB2392 respectively, Abcam, UK), sex determining region Y (SRY) box (Sox)9 (13) (AB5535, Millipore UK), and MIC1-1C3 (14, 15) (Novus Biologicals) or for the alternate antigens: CD45 (Mab114 R&D systems, UK), Hepatocyte Nuclear Factor (HNF)4α (6556 Santa Cruz), Ki67 (M7249, Dako), BrdU, F4/80, Fn14 and GFP (AB6326, Ab6640, Ab85089 and AB13970 respectively, AbCam, UK). Cytological smears were fixed in methanol at 4°C for 5 minutes prior to staining. Species isotype (Santa Cruz) staining controls were routinely performed. Detection was performed with DAB (DAKO) followed by counterstaining with Harry's Haematoxylin or alternately with Alexa 488 and 555 (A21206 and A21434 or S32355 respectively; Invitrogen, UK) with a DAPI containing Vectashield mounting media (Vector, UK). All scale bars denote 50mm.

Fluorescence In Situ Hybridization (FISH). Male cells were detected by FISH for the Y chromosome (STARFISH 1189-YMF; Cambio, UK) exactly as previously described (4). Co-staining of FISH stained sections with F4/80 or panCK was performed by three-step immunohistochemistry with 1:200 Streptavidin-Alkaline Phosphatase as the tertiary reagent and VectorRed (Vector, UK) for visualization. Slides were mounted in Vectorshield Hard Set with DAPI (Vector, UK).

Real-Time PCR and Gene Expression Analysis. Genomic (g)DNA was extracted from perfused whole liver using DNA Blood Mini Kit (Qiagen, UK). Total RNA was extracted from whole liver and purified cell populations using a combination of TRIzol[®] reagent (Invitrogen) and Qiagen RNeasy Mini system according to manufacturer's instructions (Qiagen, UK). FACS sorted hepatic progenitor cells (HPCs) and colony mRNA was amplified using QuantiTect Whole Transcriptome Kit (Qiagen, UK). Reverse

Transcription (including gDNA decontamination) and real time PCR was performed using reagents and primers (Quantifast and Quantitect respectively, Qiagen, UK) on a ABI Prism 7500 cycler. Y chromosome analysis used SRY primers and β -actin control primers (ABI). Data were normalized to β -actin and quantified against a standardized serial dilution series of murine male gDNA into female gDNA. Data were analysed using the LightCycler system following normalization to the housekeeping gene peptidylprolyl isomerase A (PPIA) or β -Actin (SRY gPCR only). All samples were run in triplicate alongside positive controls of either spleen, thymus or embryonic day 15 liver.

Statistical Analysis. Prism software (GraphPad Software, Inc) was used for all statistical analysis. Mean HPCs/ductular reactions (DRs) per x200 magnification field from 30 fields for each mouse were compared. For comparison of parametric data sets the one

tailed Student's t test was used when results were concordant between experiments.

Microscopy and Cell Counting. Images were obtained on a Zeiss Axiovert 200 microscope using a Zeiss Axiocam MRc camera. Cell counts were performed manually on blinded slides. For each antigen, 30 consecutive non overlapping fields were counted at x200 magnification. HPCs were defined as previously described and interlobular bile ducts were excluded from quantification (16). Following F4/80 immunohistochemistry, macrophage quantification was performed from 30 captured images and individual cells counted using ImageJ software (NIH). Confocal image analysis was performed using a Leica SP5 system with the pinhole set to 1 airy unit. DAPI, Alexafluor 488 and VectorRed were detected using band paths of 433-469, 495-540 and 561-682nm for 405, 488 543nm lasers respectively.

- Jakubowski A, et al. (2005) TWEAK induces liver progenitor cell proliferation. *J Clin Invest* 115(9):2330–2340.
- Campbell S, et al. (2006) Proinflammatory effects of TWEAK/Fn14 interactions in glomerular mesangial cells. *J Immunol* 176(3):1889–1898.
- MacKay GE, et al. (2005) Evaluation of the mouse TgTP6.3 tauGFP transgene as a lineage marker in chimeras. *J Anat* 206(1):79–92.
- Duffield JS, et al. (2005) Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 115(1):56–65.
- Tirnitz-Parker JE, et al. (2010) Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. *Hepatology* 52(1):291–302.
- Boltz-Nitulescu G, et al. (1987) Differentiation of rat bone marrow cells into macrophages under the influence of mouse L929 cell supernatant. *J Leukoc Biol* 41(1):83–91.
- Tirnitz-Parker JE, Tonkin JN, Knight B, Olynyk JK, Yeoh GC (2007) Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. *Int J Biochem Cell Biol* 39(12):2226–2239.
- Suzuki A, et al. (2008) Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. *Hepatology* 48(6):1964–1978.
- Qiu Q, Hernandez JC, Dean AM, Rao PH, Darlington GJ (2011) CD24-positive cells from normal adult mouse liver are hepatocyte progenitor cells. *Stem Cells Dev* 20(12):2177–2188.
- Okabe M, et al. (2009) Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. *Development* 136(11):1951–1960.
- Kofman AV, et al. (2005) Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury. *Hepatology* 41(6):1252–1261.
- Jelnes P, et al. (2007) Remarkable heterogeneity displayed by oval cells in rat and mouse models of stem cell-mediated liver regeneration. *Hepatology* 45(6):1462–1470.
- Furuyama K, et al. (2011) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nat Genet* 43(1):34–41.
- Dorrell C, et al. (2008) Surface markers for the murine oval cell response. *Hepatology* 48(4):1282–1291.
- Dorrell C, et al. (2011) Prospective isolation of a bipotent clonogenic liver progenitor cell in adult mice. *Genes Dev* 25(11):1193–1203.
- Oben JA, et al. (2003) Sympathetic nervous system inhibition increases hepatic progenitors and reduces liver injury. *Hepatology* 38(3):664–673.

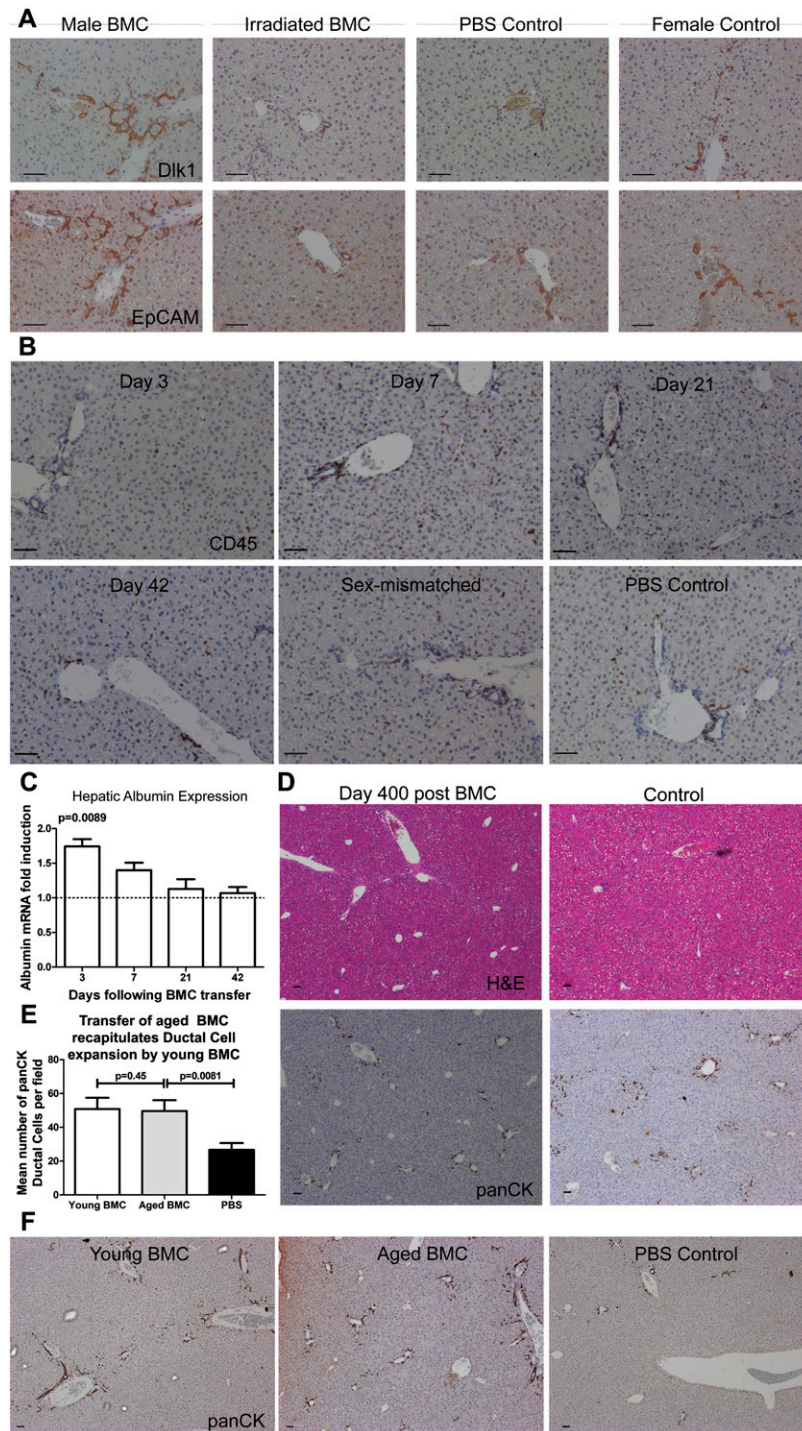


Fig. S1. (A) Expansion of EpCAM⁺/Dlk1⁺ ductular reactions (DRs) in female mouse liver 42 d following tail vein injection of 10⁷ bone marrow cells (BMCs). (B) No evidence of generalized inflammatory infiltrate (CD45⁺) is seen following BMC transfer either with syngenic BMCs (days 3–42) or sex-mismatched BMCs. (C) Transient increase in whole liver albumin mRNA after syngenic BMC transfer, values normalized to PBS control, $n = 5$ each group. (D) Four hundred days following 10⁷ BMC transfer, normal hepatic architecture and absence of DR activation. (E) A total of 10⁷ BMCs from aged male mice (19 mo) were transferred into healthy young female recipient mice and panCK⁺ DR expansion assessed 42 d following BM transfer (F). Data are presented as mean number of cells per field \pm SEM; $n = 5$ each group. All P values denote one-tailed Student t test.

