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TGFβ inhibition restores a regenerative response in acute liver injury by suppressing paracrine senescence

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Title: TGFβ inhibition restores a regenerative response in acute liver injury by suppressing paracrine senescence

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1

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2

One Sentence Summary: We report that inhibiting injury induced senescence, which propagates via TGFβ signalling within the regenerative epithelium, improves liver regeneration.

Abstract: Liver injury results in rapid regeneration through hepatocyte proliferation and hypertrophy. However, after acute severe injury, such as acetaminophen poisoning, effective regeneration may fail. We investigated how senescence underlies this regenerative failure. In human acute liver disease, and murine models, p21-dependent hepatocellular senescence was proportionate to disease severity and was associated with impaired regeneration. In an acetaminophen injury model a transcriptional signature associated with the induction of paracrine senescence is observed within twenty four hours, and is followed by one of impaired proliferation. In genetic models of hepatocyte injury and senescence we observed transmission of senescence to local uninjured hepatocytes. Spread of senescence depended upon macrophage derived TGFβ1 ligand. In acetaminophen poisoning inhibition of TGFβ receptor 1 (TGFβR1) improved survival. TGFβR1 inhibition reduced senescence and enhanced liver regeneration even when delivered after the current therapeutic window. This mechanism, in which injury induced senescence impairs regeneration, is an attractive therapeutic target for acute liver failure.

[Main Text:]

Introduction

After moderate liver injury or resection, the liver regenerates efficiently through hepatocyte proliferation (*1, 2*). However, following severe acute liver injury (ALI) there is a failure of

3

regeneration and acute liver failure (ALF) may follow. ALF can be caused by a variety of insults including viruses, toxins and medical therapy, with the most common single agent in the Western world being acetaminophen (paracetamol) (*3*). There are approximately 2000 patients affected annually in the USA. Despite its relative rarity ALF is clinically important, due to its high morbidity and mortality in previously healthy individuals. Outcomes in ALF have improved modestly with advances in supportive care (*4*). However, once ALF of a defined clinical severity is established no specific medical therapies exist, recovery is unlikely and unless liver transplantation occurs death usually ensues (*5*). Novel therapies are needed for the potential treatment window during this progression from ALI to the most severe forms of ALF.

Cells may enter growth arrest in response to stress, termed senescence when permanent. Senescence is associated with changes in morphology, lysosomal activity including senescence-associated β-galactosidase (SA-βGal) expression, DNA damage response (DDR) related alterations in chromatin structure (e.g. γH2Ax expression) and the activation of a dynamic pro-inflammatory senescence-associated secretory phenotype (SASP) including expression of IL1α and TGFβ (*6, 7*). When senescence results from oncogenic stress it reinforces cell cycle arrest in an autocrine manner (*8, 9*), activates immune surveillance (*10-13*) and induces paracrine senescence via the SASP (*14, 15*). SASP may also modulate fibrosis and regeneration in response to acute tissue injury (*11, 16, 17*). Hepatocyte senescence involves the induction of p53 (*TRP53*), p21 (*WAF1*) and p16 (*INK4A*) (*18*). It is described in both chronic diseases (*19*) and steatosis (*20*), but not in acute liver disease. Fibroblast senescence occurs in the dermis following acute wounding (*21*) and acute myocardial infarction (*22*). However, acute epithelial senescence in response to liver injury is not described to date.

4

Here we show that acute injury is associated with a suite of senescence markers in previously uninjured hepatocytes. Senescence is transmitted between hepatocytes in a feedback loop dependent upon TGFβ derived from macrophages. Importantly, targeting TGFβ signalling after injury reduces senescence development and improves both regeneration and survival.

5

Results

Acute liver injury results in proportionate acute hepatocellular senescence

Human liver specimens resected at the time of liver transplantation from patients with hyper-acute fulminant hepatic failure (less than 1 week from jaundice to encephalopathy, with no prior liver disease) showed expression of various senescence related markers: p21 (**Fig. 1A**), DcR2, γH2Ax and SA-βGal (**Fig. S1**). Thus, in liver disease's most severe form a previously healthy liver develops widespread markers of hepatocellular senescence within days of acute insult. To examine a potential relationship between disease severity and senescence induction we then analysed a case series of human diagnostic biopsy samples from patients with sub-massive hepatic necrosis. Here, we observed a direct association between hepatic necrosis and hepatocyte senescence as well as an indirect association between necrosis and hepatocellular proliferation (**Fig. 1B** and **S1**). Therefore, worsening acute liver injury in humans results in a proportional expression of senescent markers by hepatocytes, associated with reduced regeneration.

To investigate the functional impact of senescence in acute liver injury we examined the established murine models of carbon tetrachloride (CCl_4) (*23*) and acetaminophen (*24*) induced acute liver injury. Both injury models result in expression of senescence markers (p21, SA-βGal and p16) by hepatocytes, demonstrating features of growth arrest (absence of BrdU or Ki67), DDR (γH2Ax), senescence associated heterochromatic foci (HMGA2) and the SASP (IL1α) (**Fig. 2A-C** and **Figs. S2** and **S3**). We also observed senescence marker expression by hepatocytes in dietary acute liver injury models (**Fig. S4**). In both CCl_4 and acetaminophen induced injury expression of senescence markers is maximal two days following initiation and is lost following hepatocellular recovery (**Fig. 2D** and **Figs. S2** and **S3**). In acetaminophen injury, using unbiased transcriptomics, we confirmed a senescence

6

associated signature 24 hours following injury (**Fig. 2E** and **Tables S1** and **S2**). At 48 hours we observed p21 expression particularly focused to hepatocytes surrounding the area of receding necrosis at this time of resolving injury (**Fig. 2F** and **S3C**). In acute injury-induced senescence the principle target population is the hepatocyte, however p21 expression by non-parenchymal cells also occurs (**Fig. S2**) consistent with the previous report by Krizhanovsky *et al.* (*11*).

To assess the necessity for p21 in the formation of injury induced senescence we performed acetaminophen induced injury in WT and p21^{KO} mice (**Fig. 2G**). Here, we measured the proliferative response within the perinecrotic area, where p21 was expressed by WT hepatocytes. Injury was equivalent between WT and p21^{KO} mice (**Fig. S3**). However, perinecrotic hepatocellular regeneration was increased in p21^{KO} mice compared to WT mice (**Fig. 2H**). Furthermore, whilst a negative correlation between injury and regeneration existed in WT mice, a positive correlation was observed in p21^{KO} mice animals (**Fig. 2I**) indicating that in the absence of p21 injury no longer impedes the regenerative response. Together these data show that hepatocytes can enter a p21-dependent senescent state following acute injury and that this is associated with impaired local regeneration.

TGFβ-dependent senescence transmission between hepatocytes in vivo.

Rapid entry of the liver parenchyma into senescence following injury may represent a precursor to cell death. However, as we observed increasing expression of senescence markers by hepatocytes during the time of necrosis recession, we explored the hypothesis that senescence may be a cellular response to local injury and senescence. We, and others, have shown that oncogene-induced senescence is not only cell-autonomous but also spreads via paracrine factors (*7, 15*). Furthermore transcriptomic analysis revealed a transition between a

7

signatures associated with SASP induced senescence and one of growth arrest/senescence during the induction of senescence following paracetamol (**Fig. 3A**). Thus, we studied the spread of senescence in two independent genetic models of senescence.

Murine Double Minute 2 (*Mdm2*) is a key negative regulator of p53 and the p53/p21 pathway is central to senescence induction. We up-regulated p53 in hepatocytes by hepatocyte-specific deletion of *Mdm2* (Δ*Mdm2*[Hep]) via β-naphthoflavone (βNF) activation of AhCre (*25*). This resulted in hepatocellular injury as we have previously reported (*26*). In the Δ*Mdm2*[Hep] model we observed a rapid expression of a suite of senescence markers (**Fig. S5**). Using a mitogen cocktail, HGF and T3 (*27*), we attempted to promote proliferation in senescent hepatocytes in the Δ*Mdm2*[Hep] model; but were unable to do so, unlike in WT hepatocytes (**Fig. S5**). Therefore, these cells are in a state of functional senescence. Next, we tested p21 dependence of growth arrest in this model. When *Mdm2* was deleted in hepatocytes of *p21*[KO] mice, we observed rescue of the growth arrest (**Fig. S5**). In summary, the Δ*Mdm2*[Hep] model induces acute p21-dependent hepatocellular senescence.

To examine spread of senescence we utilised reduced titration of βNF to delete *Mdm2* in a subpopulation of hepatocytes (partial Δ*Mdm2*[Hep]*)*. By doing so we aimed to distinguish cell-autonomous and non-cell-autonomous senescence induction in hepatocytes (**Fig. 3B**). We define cell-autonomous senescence as being caused through genetic manipulation in that cell (e.g. through *Mdm2* deletion), whereas we consider cell non-cell-autonomous senescence an indirect response to environmental senescence and injury in a genetically un-manipulated cell. In Δ*Mdm2*[Hep]*,* a cell-autonomous senescence was observed with activation of p21/p16 in association with p53 overexpression within a subgroup of the total hepatocyte population (**Fig. 3C** and **Fig. S6**). Non-cell-autonomous expression of p21/p16 occurred in a distinct

8

hepatocyte subpopulation in the absence of p53 overexpression. These cells had atypical morphology and more pronounced p21 expression than their p21[+]/p53[+] *Mdm2*-deleted neighbours.

Next, we examined a potential geographical relationship between non-cell-autonomous p21 expression and regional p53[+] hepatocytes using a further titrated genetic induction. Here, we observed a lower frequency of non-cell-autonomous p21 expression and geographical clustering of non-cell-autonomous p21 expression with p53 overexpressing hepatocytes (**Fig. S6**).

We then aimed to study senescence transmission in different models dependent upon *Mdm2* deletion. To exclude any extrahepatic effects of AhCre mediated recombination (including the intestinal epithelium (*28*)), we utilised a hepatocyte-specific induction regime using an adeno-associated viral vector (AAV8-thyroxine binding globulin [TBG]) to induce hepatocyte-specific deletion of *Mdm2* (*29*). As predicted, AAV8-TBG-Cre induced deletion of *Mdm2* in a subpopulation of hepatocytes (**Fig. S6**). In this model non-cell-autonomous p21 expression was also observed (**Fig. 3D**). We did observe a very low (0.3% hepatocytes) cre-independent expression of p21 as a result of transfection with the control AAV8 vector (**Fig. S6**). To test transmission of senescence to WT hepatocytes we utilised a large scale hepatocyte repopulation model. Here, using iterative βNF dosing, the livers of AhCre *Mdm2*^{fl/fl} mice were repopulated hepatocytes derived from transplanted WT GFP-tagged cells (*26*). Following final βNF dosing and p53 expression by native hepatocytes, transplanted WT hepatocytes expressed p21; particularly at the margins of the engrafted nodules (**Fig. 3E**).

9

In the $\Delta Mdm2^{\text{Hep}}$ model we observed activation of the TGFβ pathway (**Fig. S5**). TGFβR1 was

expressed by hepatocytes, in addition to non-epithelial cells. The TGFβR1, ~~whilst its~~ ligand,

TGFβ1, was expressed both by non-parenchymal cells and to a lesser degree by hepatocytes.

As p21 is a canonical TGFβ signalling target gene and the TGFβ signalling pathway has a

role in oncogene-induced paracrine senescence (14, 15), we hypothesised that the TGFβ1

ligand plays a mechanistic role in non-cell-autonomous p21 expression. To test the functional

role of TGFβR1 and TGFβ1 ligand in transmitted senescence we utilised a model of

hepatocyte-specific TGFβ signal pathway activation using LSL-TGFβR1-CA mice, which

possess a genetically inducible constitutively active TGFβR1 (*30*). This was activated using

hepatocyte-targeted recombination (AAV8-TBG-Cre). In this model we observed TGFβ

pathway activation and acute senescence marker induction in hepatocytes. This was

accompanied by liver injury and increased paracrine TGFβ1 ligand production (**Fig. 3F** and

S7 and **S8**). Using this as a further model of senescence induction *in vivo*, we investigated

non-cell-autonomous senescence driven specifically by the TGFβ pathway as a model distinct

from *Mdm2* deletion. Using lower titrations of AAV8-TBG-Cre we induced TGFβ pathway

activation and a R26-LSL-tdTomato reporter in approximately 5% of hepatocytes. Here we

observed evidence of non-cell-autonomous spread of senescence to adjacent hepatocytes in

response to cell-autonomous TGFβ pathway activation (**Fig. 3G**). Thus, we observed non-

cell-autonomous senescence in various models of acute hepatocellular senescence *in vivo*;

demonstrating that senescence can spread within the epithelium.

To test the necessity of TGFβ signalling for the transmission of senescence we returned to the

$\Delta Mdm2^{\text{Hep}}$ model. Using SB525334, a small molecule inhibitor of TGFβR1, in the partial

$\Delta Mdm2^{\text{Hep}}$ model we observed reduced hepatocellular pSMAD3 without affects upon

hepatocyte $\Delta Mdm2^{\text{Hep}}$ efficiency (**Fig. S6**). SB525334 in partial $\Delta Mdm2^{\text{Hep}}$ resulted in

10

reduced non-cell-autonomous expression of p21 (**Fig. 3H**), demonstrating that TGFβ signalling was required for the paracrine induction of non-cell-autonomous p21 in this model.

Senescence induced by acute injury is dependent upon macrophage derived TGFβ.

Clinically relevant TGFβ inhibitors are currently available (*31*). Given our findings of TGFβ-dependent transmitted senescence in the genetic models, we examined the functional role of TGFβR1 signalling (**Fig. 4A**) in senescence formation in acute liver injury. In human fulminant liver failure, senescent hepatocytes show TGFβ pathway activity (**Fig. 4B**). Acetaminophen induced liver injury in mice is accompanied by elevated TGFβ1 ligand (**Fig. 4C** and **D**). SMAD7 (a TGFβ pathway target gene) is expressed upon injury and up-regulated particularly by perinecrotic hepatocytes (**Fig. 4E**). These perinecrotic hepatocytes express both TGFβR1 and senescence markers adjacent to local TGFβ expression (**Fig. 4F** and **Fig. S10**). Therefore, we observed evidence of active TGFβ signalling in senescent hepatocytes adjacent to necrosis following acute injury.

Macrophages are a known source of TGFβ ligands, particularly in the context of tissue injury (*32*). Perinecrotic macrophages in murine acetaminophen-induced liver injury express TGFβ1 ligand (**Fig. 5A**). As both TGFβ and CCL2 (a macrophage chemokine and known SASP component) are associated with severe human acute liver disease (*33*), we proceeded to examine the functional role of macrophages recruitment and TGFβ ligand expression in senescence induction in the injury models. A SASP related pro-migratory chemokine axis develops in partial Δ*Mdm2*^Hep with expression of both chemokine ligands and receptors (**Fig. S11**). CCL2 is expressed by non-parenchymal cells in and around the areas of hepatocellular necrosis in acetaminophen injury (**Fig. 5B**) prior to peripheral monocytosis (**Fig. 5C**) and then local macrophage accumulation (**Fig. 5D**).

11

To examine the role of macrophages in non-cell-autonomous senescence we returned to the $\Delta Mdm2^{\text{Hep}}$ model. Inhibition of leukocyte recruitment via CCL2 blockade in the $\Delta Mdm2^{\text{Hep}}$ model reduced non-cell-autonomous p21 expression and improved hepatocellular regeneration (**Fig. 5E** and **S11**). Next we next performed macrophage ablation using liposomal clodronate in the partial $\Delta Mdm2^{\text{Hep}}$ model. This reduced hepatic *TGFβ1* expression by 87% (**Fig. 5F** and **S11**), implying that macrophages are the principal source of the TGFβ1 ligand. Consistent with this hypothesis both *p21* gene expression and non-cell-autonomous p21 expression were reduced when macrophages were depleted in the $\Delta Mdm2^{\text{Hep}}$ model. To functionally test the role of macrophage derived TGFβ1 ligand in liver injury we used myeloid specific *TGFβ1* deletion in the acetaminophen model (**Fig. 5G** and **S11**). This resulted in equivalent injury but improved regeneration. Therefore, macrophage-derived TGFβ1 ligand is required for optimal induction of paracrine senescence following acute liver injury.

Inhibition of TGFβR1 signalling impairs senescence induction and improves regeneration function and outcome in acute liver injury.

Given the finding of TGFβ-dependent paracrine senescence in the genetic models, we tested whether this effect was also observed in widely accepted clinically relevant models of liver injury - the CCL4 and acetaminophen models. We examined the effect of TGFβ signalling disruption in both acute and chronic CCl4 models. In the acute model we used the TGFβR1 inhibitor AZ12601011 (AstraZeneca) administered twelve hours after administration of 1μl/g CCl4. (**Fig. S12**). This resulted in reduced senescence induction, improved regeneration and reduced jaundice. In a chronic model CCl4 was given repeatedly over eight weeks in

combination with a genetic depletion approach targeting hepatocellular TGFβR1 ($\Delta TGF\beta R1^{\text{Hep}}$; **Fig. S12**). Here, we also observed reduced hepatocellular p21 expression and increased hepatocellular proliferation. Next, we examined $\Delta TGF\beta R1^{\text{Hep}}$ in acute acetaminophen-induced injury (**Fig. S13**). Here we observed early necrosis equivalent to controls but reduced hepatocellular p21 expression by perinecrotic hepatocytes. There was also an altered distribution of hepatocellular regeneration, with marked proliferation by perinecrotic hepatocytes. Accelerated resolution of necrosis was observed in mice lacking hepatocellular *TGFβR1*.

To test the clinical utility of TGFβR1 inhibition we administered AZ12601011 at the time of a lethal acetaminophen dose (**Fig. 6A**). TGFβR1 inhibition resulted in marked clinical improvement from 6-16 hours and permitted survival following 525mg/kg acetaminophen dosing (**Fig. 6B**). At endpoint, vehicle treated mice showed worsened jaundice compared to AZ12601011 treated counterparts (**Fig. 6C**).

Conventional treatment of acetaminophen toxicity in humans involves N-acetylcysteine therapy which, to be effective, must be given within eight hours or four hours in man and mice respectively (*34*). Many patients present to medical services too late for this to be effective (*35*). In order to model delayed therapy after this limited treatment window we used small molecule TGFβR1 inhibition with therapeutic intent in 'delayed presentation' acetaminophen poisoning; commencing either SB525334 (Tocris) or AZ12601011 (AstraZeneca) twelve hours after acetaminophen administration (**Fig. 6D**). Additionally, as injury peaks prior to treatment administration, this strategy was designed to test whether the improvements in clinical outcome were distinct from the reduced hepatocellular injury we observed with synchronous toxin and therapy administration. Using SB525334, downstream

13

signalling through TGFβR1 was inhibited and necrosis was unchanged (**Fig. S14**). Liver injury was improved upon TGFβR1 inhibition (**Fig. S14**), along with a resolution of jaundice (**Fig. 6E**). Hepatocellular senescence was reduced by TGFβR1 inhibition (**Fig. 6F** and **Fig. S14**) and hepatocellular proliferation increased, both overall and specifically within the perinecrotic area (**Fig. 6G**). During acetaminophen-induced injury alone an apparent inverse relationship between severity of hepatocellular injury and hepatocellular regeneration was once again observed (**Fig. 6H** and **Fig. 2I**). Therefore, severe liver injury in the mouse recapitulates the negative correlation between injury and regeneration observed in severe human disease (**Fig. 1**). In the mouse this relationship was reversed upon inhibition of TGFβR1, restoring a proportional regenerative response to injury, mimicking genetic deletion of *p21*. Using a heightened dose of acetaminophen to induce injury we tested the second clinical compound AZ12601011 in the 'delayed presentation' model. Here, jaundice was once again improved and associated with an inhibition of hepatocellular senescence (**Fig. 6I**). These effects were accompanied by reduced local TGFβ pathway activation in perinecrotic hepatocytes (**Fig. S14**). Therefore, inhibition of TGFβ signalling following acute liver injury reduces hepatocellular senescence and improves regeneration and clinical recovery from injury.

Discussion

In contrast to minor forms of acute liver injury, where regeneration occurs efficiently, increasingly severe liver injury exhibits regenerative failure and poorer prognosis (*4*). Validated clinical scoring systems predict the outcomes of patients who will survive versus those in whom regeneration will ultimately fail (*36*), suggesting a tipping-point beyond which recovery is unlikely. The pathophysiological mechanism underlying this remains poorly understood and is a barrier to therapeutic development. Our finding that senescence inhibits

regeneration may underpin this tipping point. This data contrasts with previous reports in which senescence during injury can improve regeneration and limit fibrosis (*37*).

With these data we provide a mechanistic model whereby injury induced senescence is amplified by macrophage-dependent paracrine TGFβ signalling (**Fig. S15**). In our *in vivo* models we observe the expression of local TGFβ ligand in response to cell intrinsic TGFβR pathway activation. This, therefore, represents a paracrine positive feedback loop reinforcing and amplifying local TGFβ signalling and downstream senescence.

Senescence is challenging to define and study *in vivo*. The ΔMdm2^{Hep} model shows functional senescence *in vivo*, expression of ~~together with~~ a suite of senescence markers (*38*), markers of the DNA damage response and~~,~~ growth arrest and the SASP ~~that are seen in acute liver injury~~. ~~Similarly~~Likewise, the acetaminophen model displays senescence marker expression, together with ~~the~~ a transcriptomic signature matching ~~match of *in vivo* tissue from the acetaminophen model to~~ that of a~~then~~ *in vitro* Oncogene Induced Senescence (OIS) model (IMR90 ER:RAS (*15*)). Therefore we conclude that these models demonstrate ~~support a definition of~~ senescence *in vivo*. A similarly ~~The~~ rapid ~~establishment of a~~ senescence programme (including TGFβ, direct Notch targets and p16) has been observed in OIS models within 48 hours (*7, 39*). This ~~and~~ is mirrored by our *in vivo* observations, and is accompanied by ~~of~~ an early inflammatory signature (e.g. TGFβ, IL6 and NFκB) within 24 hours post acetaminophen which evolves into one of cell cycle arrest. In our model, SASP components TGFβ and IL6 are expressed concurrently within 12 hours, however Hoare *et al.* have shown that they appear sequentially in senescence *in vitro* (*7*). ~~—~~The~~is~~ acute injury-induced senescence described here~~in~~ may represent a generic response to severe tissue injury whereby regional regeneration is inhibited. Our study does not dissect ~~out~~ the mechanisms

15

which link hepatocellular injury to senescence initiation. Whether the recently described cGAS-STING driven senescence pathway detecting cytoplasmic chromatin (*40*) plays a central role in liver injury induced senescence remains to be studied. Furthermore, the rapid resolution of senescent hepatocytes in these models justifies future investigation, as the mechanism of their clearance is unknown.

We have recently reported in an *in vivo* model of hepatocyte growth arrest that cholangiocytes may act as facultative stem cells to derived hepatocytes (*29*) in which replacement of hepatocytes occurs over weeks to months. In comparison, resolution of injury and architecture in both acetaminophen and CCl_4 models is complete within a week and is not accompanied by a ductular reaction. Future studies are required to test whether inhibition of senescence in chronic injury models (e.g. TGFβ inhibition) may affect regeneration from both the hepatocyte and facultative stem cell pools, however our data in genetic models suggest that when senescence formation is impaired the ductular expansion, which include facultative stem cells, is also impaired (Fig. S16).

In the chronic setting, iterative CCl_4-induced fibrosis has been associated with non-parenchymal (myofibroblast) senescence and an impaired fibrotic response to injury (*11*). Consistent with this previous report we observed senescence marker expression by non-parenchymal cells both in acute and chronic liver injury. However, following acute injury, the predominant population expressing senescence markers are hepatocytes. Our observations are consistent with a requirement for chronic or iterative injury in order to observe persistent populations of non-parenchymal cells with senescence marker expression.

In this study we have investigated TGFβ as a tractable target to interrupt paracrine induced non-cell-autonomous p21 expression by hepatocytes. However, other SASP components are also worth future investigation (*15, 41, 42*). In ALF TGFβ is produced in the injured liver (*43*). TGFβ tonically inhibits hepatocellular regeneration during health and changes both in TGFβ ligand and receptor sensitivity facilitate regeneration following partial hepatectomy (*44, 45*). However, TGFβ is believed to restrict hepatocellular regeneration rather than acting as a break during the termination phase of liver regeneration (*1, 46*). Trials of TGFβR1 or TGFβ inhibitory compounds in humans are currently underway (e.g. NCT02452008, NCT02581787 respectively; see also (*31*)). TGFβR2, by acting as a co-receptor of TGFβR1, may serve as a further potential target. Long term therapy using TGFβ inhibition raises potential concerns such as carcinogenesis, autoimmunity or cardiac valvulopathies (*47*); however these may prove to be less relevant for the short periods of therapy (<one week) required for acute liver failure; a condition in which the prognosis is otherwise grave. None the less a potential physiological role of TGFβ in acute injury may be to mechanically stabilize the local environment. We did not observe haemorrhagic transformation following TGFβR1 inhibition in our treated mice, therefore further studies to ensure efficacy and safety in humans are required.

SASP components including chemokines (e.g. CCL2/CCR2 and CX3CL1/CX3CR1), which promote macrophage recruitment and local TGFβ expression within areas of necrosis, are well described in human fulminant hepatic failure (*33, 48*). Macrophage recruitment in injury shares similarities to the p21-dependent recruitment observed during hepatic oncogene-induced senescence (*40*) and the clearance of early hepatocellular carcinoma (*13*).

Overall, severe acute hepatic necrosis induces the spread of senescence to remaining viable hepatocytes which impairs hepatocyte-mediated regeneration. This process is therapeutically modifiable providing the possibility of additional future therapy for patients with this devastating condition.

Materials and Methods

Study design

This study was designed to examine the role of injury induced senescence in the mammalian liver. With consent and ethical approval, we used archival human tissue retrieved as part of routine clinical care. Murine *in vivo* models were used for mechanistic dissection and preclinical compound testing. The n for murine models was based on the predicted variance in the model and was powered to detect 0.05 significance of 30% magnitude; in the event that no predicted variance was inferable from previous work preliminary experiments were performed using n= 3. Animals were randomly assigned to experimental groups prior to experimental readings, no animals were excluded from analysis (two mice in Fig. 6G did not receive BrdU). No blinding was performed during animal experimental administration; vehicle controls were used and no bias was applied during husbandry or during tissue harvesting. Histological sections were assigned a randomized blinded code prior to quantification by a separate researcher and the randomization decoded at the time of final data analysis.

Human tissue

Human liver biopsies from a clinical series of cases of sub-massive hepatic necrosis (but not necessarily progressing to acute liver failure; n= 74; viral hepatitis n= 13, drug-induced hepatitis n= 21 and cryptogenic hepatitis n= 40) were assessed histologically using H&E,

CK19, p16 and Ki67 staining and evaluated by an expert pathologist (TR), who also performed cellular quantification using x400 magnification fields. Diagnoses were based on clinical and radiological data and confirmed by histology. Control human tissue was obtained from the Brain Bank, University of Edinburgh, comprising cases of sudden unexpected death. These cases were reviewed by a pathologist prior to their inclusion as normal control tissue. The use of human tissues for this study was approved by the Local Commission for Medical Ethics of the University of Leuven and the University of Edinburgh.

Animal Models

Animal welfare conditions have been previously described (*26*). Briefly, male and female animals were housed in a specific pathogen free environment and kept under standard conditions with a twelve hour day/night cycle and access to food and water *ad libitum*. Eight week old male C57BL/6J mice were purchased from Charles River UK. All animal experiments were carried out under procedural guidelines, severity protocols and within the UK with ethical permission from the Animal Welfare and Ethical Review Body (AWERB) and the Home Office (UK); or in CNIO Spain performed according to protocols approved 193 by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CEIyBA) and 194 the Autonomous Community of Madrid. As described previously (*26*), AhCre$^{+/WT}$ mice were crossed with both *Mdm2*$^{fl/fl}$ and *Mdm2*$^{fl/+}$ mice to generate AhCre^{+} *Mdm2*$^{fl/fl}$ and AhCre^{WT} *Mdm2*$^{fl/fl}$ and AhCre^{+} *Mdm2*$^{fl/+}$ controls and then subsequently crossed with *p21*^{KO} (*49*) and *TGFβR1*$^{fl/fl}$ (*50*) animals. LysMCre mice were crossed with *TGFβ1*$^{fl/fl}$ animals. Litters from *LysMCre*^{het} *TGFβ1*$^{fl/fl}$ x *LysMCre*^{WT} *TGFβ1*$^{fl/fl}$ crosses were used for experimental and control animals. Power calculations were not routinely performed, however, animal numbers were chosen to reflect the expected magnitude of response taking

into account the variability observed in previous experiments. Genotyping, BrdU administration and i.p. injection of β-Napthoflavone (βNF, Sigma UK) at 20-80mg/kg were performed as previously described (*26, 51*), with BrdU given two hours prior to tissue harvest. AAV8 recombination was performed as previously described (*52*). Briefly, viral particles ($6.4x10^8$, $2x10^{11}$ or $2.5x10^{11}$ genetic copies (GC)/mouse as specified) of AAV8.TBG.PI.Cre.rBG (UPenn Vector Core, Catalogue number: AV-8-PV1091) were injected via tail vein in 100μL PBS into male AhCre[WT] Mdm2^{fl/fl}, LSL-TGFβR1-CA[Hom] (*30, 53*) or WT mice. Control male AhCre[WT] *Mdm2*^{fl/fl} or LSL-TGFβR1-CA[Hom] mice received equal AAV8.TBG.PI.Null.bGH (UPenn Vector Core, Catalogue number: AV-8-PV0148) injection. Cell transplantation was performed as previously described (*26*), AhCre *Mdm2*^{fl/fl} recipient mice received 10mg/kg i.p. βNF 4 days prior to cell transplant of $5x10^6$ GFP expressing cells suspended in 200μL of PBS and injected intrasplenically after laparotomy. Transplanted 7-AAD^{-}CD31^{-}CD45^{-}Ter119^{-}EpCAM^{+}CD24^{+}CD133^{+} hepatic progenitor cells from WT mice fed the choline deficient ethionine supplemented diet were transfected using 1μg of vector with a puromycin resistant CAG-GFP prior to transplantation. The transplantation control group received 200μL PBS only. Recipient mice received intraperitoneal injections of 20mg/kg βNF every ten days after transplantation to induce persistent liver injury. Mice were sacrificed and the livers were harvested twelve weeks after cell transplantation. HGF (250μg/kg, R and D technologies) was administered via tail vein injection. Triodothyronine (T3, Sigma) was dissolved in solution (0.01M NaOH, 0.9M NaCl) at 0.4g/l. This solution was then neutralized with 2M HCl upto just prior to T3 precipitation and stored at -20°C. T3 was administered at 4mg/kg to mice via subcutaneous injection. Choline Deficient Ethionine supplemented (CDE) and DDC dietary protocols were as previously described (*54*). 200μl of clodronate liposomes or control PBS were injected i.v. as previously described (*54*). TGFβR1 antagonists; SB525334 (Tocris) was given at 10mg/kg

twice daily in 10% polyethylene glycol, 5% DMSO, 85% saline vehicle by gavage; AZ12601011 (AstraZeneca) (*47*) was given at 50mg/kg twice daily in 0.5% HPMC / 0.1% Tween vehicle by gavage. Acetaminophen was prepared as previously described (*8*) and delivered at 350mg/kg or 450mg/kg by single i.p. injection of 20µl/g following a ten hour fast. 525mg/kg acetaminophen was administered by injection of 30µl/g. CCl_4 was delivered by weekly i.p. injection for eight weeks at 0.75ml/kg or by single dose at 1ml/kg 1:3 in corn oil. CCL2 inhibitory antibody (#AF-4679-NA, R and D Systems) was administered (10µg per injection) daily for four days by. tail vein injection of a 100µg/ml stock diluted in PBS.

Animal tissue harvesting and serum analysis

Mice were killed by CO_2 inhalation or cervical dislocation and blood harvested by cardiac puncture. Organs were harvested and stored in paraffin blocks following fixation in 10% formalin (in PBS) for 18 hours prior to embedding. Blood hematology was performed using an IDEXX ProCyte Dx analyzer on blood collected in EDTA. Serum analysis used commercial kits according to manufacturer's instructions for Alanine transaminase (ALT; Alpha Laboratories Ltd, UK), microalbumin (Olympus Diagnostics Lt, UK), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (Alk Phos; both Randox Laboratories, UK).

Immunohistochemistry (IHC) and in situ hybridization (ISH).

Three µm thick paraffin sections were stained for BrdU, p16, HMGA2, γH2Ax, DcR2 and pSMAD3 (AB6326 – clone BU1/75, AB54210 – clone 2D9A12, AB52039, AB81299 – clone EP854(2)Y, AB108421 – clone EPR3588(2) and AB52903 – clone EP823Y respectively Abcam, UK), p53 (VP-P956-clone CM5, Vectorn), p21 (Clones BMK-2202, Santa Cruz and HUGO 291H), Ki67 (M7249 - clone TEC-3 Dako), pSMAD2/3, pSMAD2

21

(Cell Signalling #8828 – clone D26F4, #3101), CYP2D6 (Generous gift from Prof R Wolfe, University of Dundee) and the ductular cell marker panCK (Z0622 Dako), Species isotype (Santa Cruz) staining controls were routinely performed. Detection was performed with DAB (DAKO) followed by counterstaining with Haematoxylin or alternately with Alexa 488, 555 or 650 (A21206, A21434A21436/S32355 and A21448 respectively; Invitrogen, UK) with a DAPI containing Vectashield mounting media (Vector, UK). Histochemical detection of senescence-associated β-galactosidase (SA-βG) was performed as previously described (*55*). In situ mRNA hybridization was performed using RNAscope LS probes for *TGFβ1*, *TGFβR1*, *CCL2*, *SMAD7* and *PPIB* control (407758, 406208, 469608, 429418 and 313918); Advanced Cell Diagnostics) as per the manufacturer's instructions.

ELISA for murine TGFβ1 ligand was performed using Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (R and D system) according to the manufacturer's protocol. Whole liver tissue samples were homogenized in RIPA-Buffer (50mM Tris, 150 mM NaCl, 1% Triton x-100, 0.5% Deoxycholate, 0.1% SDS) supplemented with NaF, protease- and phosphatase-inhibitors and cleared by centrifugation. Protein concentration was determined by BCA Assay (Thermo Scientific #23225). The samples were diluted at 1:4. To allow TGFβ activation 20ul of 1M HCL and 20ul of 1.2M NaOH/0,5M HEPES were added to each 100ul sample. Optical density was measured using Safire II microplate reader (TECAN) at 450 nm (reference wavelength 540nm).

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 and consecutive non-overlapping fields at x200 magnification. Perinecrotic hepatocytes were defined as those

22

contacting the area of necrosis. Confocal image analysis was performed using a Leica SP5 system with the pinhole set to 1 airy unit. DAPI, Alexafluor 488 and 555 were detected using band paths of 415-480, 495-540 and 561-682nm for 405, 488 543nm lasers respectively. Serial sections were aligned manually in Adobe Photoshop CS5; images were colour deconvoluted using imageJ using haematoxylin/DAB settings (version 1.5). For RNAscope and quantification of necrosis slides were scanned on an SCN400F slide scanner (Leica, Milton Keynes, UK) and the files analysed using Halo v2.0 Image Analysis Software (Indica Labs, Corrales, NM, USA) as previously described. For perinecrotic SMAD7 quantification, perinecrotic and uninjured pericentral areas were manually defined by drawing a ring (200μm radius from vein or necrosis) around ten centrilobular structures per sample. Results are expressed as probe copies/area for RNAscope. Necrosis was defined after validation of classifier definition of healthy liver, haemorrhagic necrosis and non-haemorrhagic necrosis with results expressed as % area necrosis. All scale bars = 50μm.

Real time PCR and gene expression analysis

Total RNA was extracted from 30-50mg tissue samples previously stored in RNAlater at -80°C, using a combination of TRIzol™ reagent (Invitrogen) and Qiagen RNeasy Mini system (Qiagen, UK) according to both manufacturers' instructions. gDNA decontamination, reverse transcription and real time PCR were performed using reagents and primers (Quantifast and Quantitect respectively, Qiagen, UK) on an ABI Prism 7500 cycler, except for chemokine/chemokine receptor analysis which was performed as previously described (*56*). Data were collected using the LightCycler system following normalization to the housekeeping gene peptidylprolyl isomerase A (*Ppia*); or *Gapdh* for chemokine/chemokine receptor data. All samples were run in triplicates.

23

RNAseq analysis

Total RNA was extracted from 30-50mg tissue samples as described above. Purified RNA was tested on an Agilent 2200 Tapestation using RNA screentape. Libraries for cluster generation and DNA sequencing were prepared following an adapted method from Fisher et al.(*57*) using Illumina TruSeq Stranded mRNA LT Kit, Quality and quantity of the DNA libraries was assessed on a Agilent 2200 Tapestation (D1000 screentape) and Qubit (Thermo Fisher Scientific) respectively. The libraries were run on the Illumina Next Seq 500 using the High Output 75 cycles kit (2x36cycles, paired end reads, single index). Quality checks on the raw RNASeq data files were done using fastqc version 0.10.1 and fastq_screen version 0.4.2. RNASeq reads were aligned to the GRCm38 (*58*) version of the mouse genome using tophat2 version 2.1.0 (*59*) with Bowtie version 2.2.6.0 (*60*). Expression values were determined and statistically analysed by a combination of HTSeq version 0.5.4p3, the R 3.4.2 environment, utilizing packages from the Bioconductor data analysis suite and differential gene expression analysis based on the negative binomial distribution using the DESeq2 (*61*). Gene set enrichment analysis was performed using the Broad Institute Online Platform. An OIS signature was defined by the top 100 upregulated gene in the IMR90 ER:RAS model (*15*).

Statistical analysis

Prism software (GraphPad Software, Inc) was used for all statistical analyses; T tests were used from normally distributed samples (D'Agostino and Pearson omnibus test was used to assess Gaussian distribution) with Welch's correction if variances differed (F test). One or two way ANOVA was used to compare multiple (>2) samples or groups respectively. Mean HPCs per x200 magnification field from 30 fields for each mouse were compared. Data are presented as mean +/- SEM throughout; n refers to biological replicates in all instances unless otherwise stated.

Supplementary Materials

Fig. S1. Senescence markers in human acute liver disease.

Fig. S2. Senescence in acute carbon tetrachloride model.

Fig. S3. Senescence in acute acetaminophen model.

Fig. S4. Senescence in acute dietary models of liver injury.

Fig. S5. Hepatocyte *Mdm2* deletion model.

Fig. S6. Non-cell-autonomous senescence marker induction.

Fig. S7. Hepatocyte TGFβ pathway activation model.

Fig. S8. Hepatocyte TGFβ pathway promotes hepatic TGFβ ligand production.

Fig. S9. TGFβ pathway activity in acetaminophen model.

Fig. S10. Serial sections of TGFβ pathway and senescence hepatocytes.

Fig.S11. Macrophage recruitment and TGFβ secretion and induced senescence.

Fig. S12. TGFβR1 inhibition in acute and chronic carbon tetrachloride models.

Fig. S13. Genetic deletion of hepatocyte TGFβR1 in acetaminophen model.

Fig. S14. Therapeutic TGFβR1 inhibition in acetaminophen model.

Fig. S15. Schematic representation of paracrine induced senescence in acute liver injury.

Fig. S16. Ductular reaction responses in murine models and human disease.

Table S1. RNAseq Gene: Hallmarks

Table S2. RNAseq Gene Set Enrichment Analysis: Selected Ranked Hallmarks and OIS signature

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27

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29

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Figures:

Fig. 1. **Human liver necrosis causes acute hepatocellular senescence.**

(**A**) Explanted human livers following liver transplantation for severe acetaminophen overdose (n= 8) show expression of senescence marker p21 by IHC detection in residual hepatocytes surrounding areas of necrosis (necrosis interface = dashed white line, CV = central vein and * = area of necrosis). As control liver with normal histology was used (n= 50). Scale bars = 50μm. (**B**) A cases series of human sub-massive necrosis divided into subgroups by extent of hepatocellular necrosis (<25% n= 8, 25-50% n= 16, 50-75% n= 22, >75% n= 28) and quantified for hepatocellular senescence markers by IHC (p16) and proliferation (Ki67); * = $p < 0.05$. Mean ± SEM.

Fig. 2. Toxin-mediated liver injury models cause p21-dependent hepatocellular senescence.

Murine acute liver injury models treated with carbon tetrachloride (CCl$_4$, **A**) or acetaminophen (**B**) result in pericentral necrosis two days post administration. IHC for senescence (p21) plus proliferation (BrdU) or hepatocyte (HNF4α) markers is shown. (**C**) IHC for Ki67 two days following acetaminophen shows proliferation away from but not next to necrosis. (**D**) Quantification of p21$^+$ hepatocytes post injury; n≥3 each time point, p values = two way ANOVA. (**E**) Gene Set Enrichment Analysis (GSEA) plot showing enrichment of the early (24 hours) acetaminophen injury signature for oncogene induced senescence (OIS) signature from an IMR90 ER:RAS model. ES=enrichment score, NES=normalised enrichment score. (**F**) Perinecrotic hepatocytes were quantified for p21 expression (74.9% mean of total perinecrotic hepatocytes were p21$^+$ in n= 8 mice). (**G**) IHC for Ki67 in p21^{KO} mice two days post acetaminophen induced injury shows proliferative hepatocytes in the perinecrotic area. Perinecrotic hepatocytes were quantified (**H**) and total Ki67$^+$ hepatocytes related to serum Alanine Transaminase (ALT; U/l) as a marker of liver injury (**I**). n= 5 vs. 8, 20 high power fields quantified each. p value = two-tailed T test. Linear regression for WT and p21^{KO}: R^2 0.54 and 0.92, with slope 95% confidence intervals -0.10 to -0.0045 and 0.082 to 0.28 and probability slope≠0 p = 0.037 and 0.010 respectively. Scale bars = 50μm. CV = central vein, dashed white lines = necrosis interface and * = area of necrosis.

Fig. 3. Non-cell-autonomous senescence in hepatocyte-specific senescence models.

(**A**) GSEA plotting Normalised Enrichment Scores (NES) over time in the paracetamol model; unbiased top 15 rank Hallmarks are shown together with the OIS signature from IMR90 ER:RAS, black borders highlight p values <0.05; raw data is shown in Tables S1 and S2. Top and bottom panel present inflammatory and cell cycle arrest signatures. (**B**) Scheme showing the use of genetic induction of transgenes in hepatocytes to induce cell-autonomous senescence and assessment by a combination of markers for genetic recombination and senescence. Presence of senescence markers in the absence of markers of genetic recombination identifies non-cell-autonomous senescence. (**C**) p53 accumulates in a subpopulation of hepatocytes in the partial $\Delta Mdm2^{\text{Hep}}$ model. Dual IHC for p21/p53 and p53/p16$^{\text{INK4As}}$ assessed by confocal microscopy. (**D**) Dual IHC for p53/p21 after deletion of *Mdm2* using AAV8-TBG-Cre (2.5×10^{11} GC/mouse) with confocal microscopic analysis. (**E**) Confocal analysis of dual IHC for GFP/p21 in hepatocyte transplant model 94 days post transplantation. AhCre$^+$ Mdm2$^{\text{fl/fl}}$ recipients were given WT donor cells tagged with GFP and iterative doses of βNF. White dotted line = boarder of the engrafted cells. Magnified area is shown in individual channels. (**F**) IHC for p21 following hepatocellular TGFβR1 activation by AAV8-TBG-Cre in LSL-TGFβR1-CA mice. (**G**) Dual IHC for p21/RFP analysed by confocal microscopy after reduced dosing of AAV8-TBG-Cre in LSL-TGFβR1-CA R26-LSL-tdTomato mice. (**H**) Following partial $\Delta Mdm2^{\text{Hep}}$, mice were given TGFβR1 inhibitor (SB525334) or vehicle control. Dual IHC for p53/p21 with confocal microscopic assessment and quantification of non-cell-autonomous p21 expression; p = two tailed Mann-Whitney; n = 6 vs. 7. Mean ± SEM. Scale bars = 50μm. Open arrow = cell-autonomous senescence, closed arrow = non-cell-autonomous senescence and arrowhead = unaffected.

Fig. 4. TGFβ signalling is activated in acetaminophen-induced hepatocellular senescence.

(**A**) Scheme demonstrating TGFβ ligands binding to heterodimeric TGFβ receptors, activation of downstream signalling through phosphorylation of SMAD2 and SMAD3 and transcription of target genes (e.g. SMAD7). (**B**) IHC detection of p21 and pSMAD2/3 in healthy human liver and patients with fulminant hepatic failure (FHF) secondary to acetaminophen; arrows = senescent hepatocytes. (**C**) ISH for TGFβ1 ligand in livers of acetaminophen treated (350mg/kg) and untreated C57BL/6J mice. TGFβ1 ligand is expressed by non-parenchymal cells with a monocyte-like appearance. Scale bars = 50µm. CV = central vein and * = area of necrosis. (**D**) ELISA of liver TGFβ1 ligand in untreated and acetaminophen treated mice (12 hours); n= 6 vs. 7, p value = two tailed Mann-Whitney. Mean ± SEM. (**E**) Quantification of perinecrotic SMAD7 expression by ISH (two days following acetaminophen); one-tailed Mann-Whitney test. (**F**) Liver serial sections assessed for SMAD7, TGFβR1 and TGFβ1 ligand and p21 by ISH and IHC respectively (twelve hours post acetaminophen).

Fig. 5. Macrophage recruitment and TGFβ1 production drives hepatocellular senescence and impairs hepatocellular regeneration.

(**A**) Serial liver sections assessed for hepatic TGFβ1 ligand production and F4/80$^+$ macrophages by ISH and F4/80 IHC respectively (350mg/kg acetaminophen). (**B**) CCL2 chemokine expression detected by ISH. (**C**) Dual IHC detection of F4/80$^+$ macrophages and p21$^+$ hepatocytes. (**D**) Peripheral monocyte quantification following acetaminophen versus fasted baseline (dashed line); n = 5 each time point, p value = one way ANOVA with Dunnett's multiple comparison baseline vs. day one. (**E**) Dual IHC for p53/p21 or IHC for BrdU, non-cell-autonomous hepatocyte p21 expression and proliferation were quantified following partial Δ*Mdm2*$^{\text{Hep}}$ and antibody mediated CCL2 inhibition; p= 0.05, Mann-Whitney, n= 3 vs. 3 mice. (**F**) Liposomal clodronate depletion of macrophages three days following partial Δ*Mdm2*$^{\text{Hep}}$ vs. PBS control. *TGFβ* ligand and *p21* expression in whole liver by qRT-PCR; p values = T test, n= 4 mice each group. Non-cell-autonomous p21$^+$ hepatocytes were quantified following dual p53/p21 IHC; p values = T test; n= 4 mice each group. (**G**) Acetaminophen 350mg/kg was administered to *LysMCre*$^+$ *TGFβ*$^{\text{fl/fl}}$ and *LysMCre*$^{\text{WT}}$ *TGFβ*$^{\text{fl/fl}}$ littermates and proliferation was assessed by BrdU IHC; p value = two tailed T test; n= 10 vs. 8 mice. Data presented as mean ± SEM. CV = central vein, dashed white line = necrotic interface and * = area of necrosis. Scale bars = 50μm.

Fig. 6. Pharmacological inhibition TGFβR1 signaling reduces hepatocellular senescence and restores proportional regenerative response after acetaminophen.

(**A**) Cohorts of male C57BL/6J mice were given vehicle control or TGFβR1 inhibition (AZ12601011) commencing at the time of 525mg/kg acetaminophen administration and were closely monitored until death or humane endpoint was reached. (**B**) Initially the TGFβR1 inhibitor treated animals (n = 14) were harvested when the control animals reached endpoint irrespective of clinical condition (total biological replicates n = 14 (AZ12601011) and 16 (vehicle) arms; over three separate experiments). A separate survival cohort (n = 10) using TGFβR1 inhibition was compared to retrospective and simultaneous vehicle controls to examine longer term survival; p value = Gehan-Breslow-Wilcoxon test. (**C**) At matched endpoint TGFβR1 inhibitor and vehicle treated controls were compared for serum bilirubin. (**D**) Examining delayed TGFβR1 inhibition (commencing twelve hours after acetaminophen in male C57BL/6J mice), SB525334 or vehicle was given twice daily. (**E**) Serum analysis for bilirubin over time; p values = two way ANOVA with Bonferroni correction, n= 8 each group. (**F**) Hepatocellular p21 IHC was quantified; p= 0.049, T test, 30 fields each in n= 8 vs. 8 mice. (**G**) IHC for hepatocellular BrdU was quantified (representative images from two day time point shown) in both whole liver and perinecrotic hepatocytes (day two only); p values = T test, n= 8 vs. 6 mice. Scale bars = 50μm. (**H**) In individual mice, two days post acetaminophen, serum ALT and BrdU$^+$ hepatocytes were compared and linear regression performed; R^2 0.15 and 0.71, with Slope 95% confidence intervals -0.0094 to 0.0038 and 0.0049 to 0.085 and probability slope $\neq$ 0 p = 0.34 and 0.036 respectively. (**I**) Increased non-fatal dose acetaminophen injury (450mg/kg) was administered to male C57BL/6J mice, followed by treatment with AZ12601011 or vehicle control twelve hours later. Serum bilirubin and p21$^+$ hepatocytes quantified by IHC; p values = two tailed T test, n=9 each group. Data presented as mean ± SEM.