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ORIGINAL ARTICLE

Smad3 signaling in the regenerating liver: implications for the regulation of IL-6 expression

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Abstract

Liver regeneration is vital for graft survival and adequate organ function. Smad activation regulates hepatocyte proliferation and macrophage function. The aim of the current study was to evaluate the impact of Smad3 signaling during liver regeneration in the mouse. Male C57Bl/6 wild-type (wt) mice or mice deficient in Smad3 ($Smad3^{-/-}$) were subjected to a 70% partial hepatectomy (pHx) or sham surgery and sacrificed 24, 42, or 48 h later. Tissue was analyzed for TGF-β signaling, the mitogenic cytokine response [i.e., tumor necrosis factor alpha, TNF-α; interleukin (IL)-6], and liver regeneration. Partial hepatectomy stimulated a strong regenerative response measured by proliferating cell nuclear antigen-positive hepatocytes 42 and 48 h post-pHx in conjunction with an increased expression of IL-6, TNF-α, and Smad2/3 phosphorylation 24 h post-pHx in both hepatocytes and nonparenchymal cells. Surprisingly, Smad3 deficiency led to reduced hepatocyte proliferation 42 h post-pHx which recovered by 48 h, a process that correlated with and was preceded by significant reductions in IL-6 expression and signal transducer and activator of transcription 3 phosphorylation, and cyclin D1 induction 24 h post-pHx. Loss of Smad3 signaling suppresses the expression of key mitogenic cytokines and delays hepatocellular regeneration. Therapies directed at finely regulating Smad3 activation early within the regenerating liver may prove useful in promoting liver cell proliferation and restoration of liver mass.

Introduction

Liver transplantation remains the primary treatment option for patients suffering from chronic liver pathologies including cirrhosis and severe acute liver failure. Limited donor organs have necessitated the use of marginal livers as well as small for size liver grafts including living donor-related transplantation. Transplantation of marginal or reduced size liver grafts requires regeneration of tissue [1]. Limited or impaired regeneration is a major cause of organ failure following transplantation in these patients [2]. Fortunately, the healthy liver possesses the ability to regenerate as much as 70% of its tissue mass within 14 days although a variety of factors have been associated with the inhibition of this response [3]. Indeed, inflammation associated with ischemic tissue injury, viral infection, and immune-mediated tissue rejection are known to significantly disrupt the regenerative response [4–6]. Experimentally, the mechanisms of the regenerative response have been evaluated and it is clear that a number of soluble factors contribute significantly to hepatocyte proliferation and liver regeneration. For example, inhibited hepatocyte growth factor signaling [7], disrupted early hepatocyte proliferation [8], and delayed restoration of organ mass increased the susceptibility of organs to failure. Likewise, loss of normally pro-inflammatory cytokines including IL-6 and TNF-α significantly suppressed early hepatocyte proliferation and delayed organ re-growth. Downstream activation of signal

transducer and activator of transcription (Stat) 3 and nuclear factor kappa B by IL-6 and TNF-α, respectively, are critical to the induction of hepatocyte proliferation and survival following tissue loss [9,10]. Together, liver regeneration represents an important and highly regulated mechanism of tissue repair and critical feature for small for size liver grafts.

Counterbalancing the early pro-proliferative genes is the expression and action of transforming growth factor beta (TGF-β) [11]. TGF-β-mediated activation of Smad proteins, particularly Smad2, 3, and 4 (i.e., the inhibitory Smads), upregulates several inhibitors of cell proliferation including $p27^{kip}$ limiting cellular regeneration in a number of cell lines including hepatocytes [12]. Recent studies have highlighted Smad3 as key intracellular signaling molecule activated by the TGF-β receptor upon ligation with active TGF-β [13]. TGF-β and associated Smad signaling are known to suppress immune cell function in a number of experimental models including inflammatory bowel disease [14]. Within the liver, Smad3 activation promotes hepatic stellate cell activation during severe chemical-induced liver injury and fibrogenesis while also supporting T-cell-mediated liver injury through the promotion of hepatocellular apoptosis [15,16]. During liver regeneration, TGF-β induction is thought to suppress the proliferation of hepatocytes and limit or terminate the regenerative response [10,17]. The importance of Smad3 in this process has not been investigated although it was hypothesized that Smad3 activation would limit hepatocyte regeneration and promote hepatocellular apoptosis following a significant tissue loss. To test this hypothesis, wild-type mice or mice deficient in Smad3 were subjected to a partial (70%) hepatectomy and allowed to recover for up to 48 h. Surprisingly, absence of Smad3 was associated with the decreased early hepatocyte proliferation in conjunction with reduced IL-6 production and Stat3 activation. These data highlight, for the first time, the ability of Smad3 signaling to positively regulate the proliferative response through the regulation of important pro-proliferative cytokine production.

Materials and methods

Animals and treatment

All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals'. The conduct of the study was approved by the University Institutional Animal Care and Use Committee. Animals were housed in pathogen-free barrier facilities and had free access to water and diet. Male 10- to 14-week-old wild-type (wt) C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) and mice deficient in Smad3 ($Smad3^{-/-}$) on a C57Bl/6 background (kindly provided by Dr. Xiao-Fan Wang, Duke University Medical Center [13]) were subject to a 70% partial hepatectomy (pHx) or sham surgery for control as published previously [4]. After 24, 42, or 48 h, mice were sacrificed and tissue was collected to assess liver damage, cell proliferation, gene expression, and transcription factor activity. In addition, to evaluate the cytokine response in wild-type or $Smad3^{-/-}$ mice, lipopolysaccharide was injected i.p. at a dose of 2.5 mg/kg 6 h prior to sacrifice. Again, serum and tissue were collected to assess liver injury and the hepatic cytokine response.

Pathologic evaluation/terminal UTP nick-end labeling (TUNEL) staining

Livers were fixed in 4% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin to assess histopathology. To assess liver cell death, deparaffinized sections were stained for DNA fragmentation using a commercially available Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations and as previously described [15]. Slides were viewed by fluorescent microscopy and images captured with a digital camera (Olympus DP-70, Center Valley, PA, USA).

Immunohistochemistry

Immunohistochemistry was performed with minor modifications as reported previously [18]. Deparaffinized liver sections were stained for phosphorylated Smad2 and 3 (pSmad2/3; Santa Cruz, Santa Cruz, CA, USA). In brief, after proteinase K incubation (20 μg/ml) for 5 min with consecutive blocking for 20 min using 1% bovine serum albumin diluted in phosphate-buffered saline (PBS–1% BSA), sections were incubated with the primary antibody (1:250) for 1 h. After washing in PBS, slides were treated with anti-rabbit HRP-conjugated secondary antibody (Amersham, Little Chalfent, UK) at a concentration of 1:250 in PBS–1% BSA. Proliferating cell nuclear antigen (PCNA) and BrdU staining were completed as previously described [4,19]. Immunocomplexes were visualized using diaminobenzidine (Dako, Carpenteria, CA, USA) and signals quantified using IMAGE J (NIH image, www.nih.gov). PCNA quantitation is presented as positive hepatocyte nuclei per 1000 total hepatocytes, indicated as PCNA labeling index.

Total RNA isolation and real-time reverse transcription PCR

Total RNA was isolated from the whole liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out using Superscript II reverse

transcription reagents (Invitrogen) and random hexamers according to the manufacturer's direction. Polymerase chain reaction was performed using a 170-9740 MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. An optimal PCR reaction for all investigated genes was established using the LightCycler–DNA Master SYBR Green I Kit (Roche). Results were normalized to β-actin using the comparative cT method. The data are presented as mean ± SEM with the sequences of the primers used listed in Table 1.

Western blotting

Cellular proteins were extracted as described previously [20] from the whole liver and quantified using a Bio-Rad protein assay (Bio-Rad). Cellular proteins (100 μg) were separated by electrophoresis on 8–16% Tris-glycine gels by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Amersham). The membranes were blocked using 5% BSA in TTBS (0.1% Tween-20 in 100 mM Tris–HCl pH 7.5, 0.9% NaCl), followed by an incubation with anti-pSmad3, total Smad3, pStat3 and total Stat3, cyclin D1, and cyclin-dependent kinase 4 (CDK4), respectively (all from Cell Signaling Technologies, Burlingham, UK), at 4 °C overnight at a dilution of 1:1000. After washing, membranes were treated with appropriate HRP-conjugated secondary antibody (1:10 000) for 1 h at room temperature. Signals were detected using ECL Western Detection Reagent (Amersham). Western blots were quantified using IMAGE J (www.nih.gov) and corrected for their proper control, respectively.

Statistics

Data are presented as the mean ± SEM. The statistical significance of differences between Smad3-deficient mice and wt control groups was determined by comparison of the mean using the independent-samples *t*-test. A p value of <0.05 was considered as statistically significant. Statistical analyses were performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

Smad3 is activated in the regenerating murine liver

To test our hypothesis that Smad3 is activated and therefore might play a role in the regenerating liver, wt mice were subject to a standardized 70% partial hepatectomy or sham surgery. Western blotting showed an increase in pSmad 3 24 h post-pHx as shown in Fig. 1a and quantification of band density (Fig. 1b). Additionally, immunohistochemistry of pSmad 2/3 24 h after pHx confirmed the findings of increased phosphorylation of Smad3 which localized to both the nuclei of hepatocytes and in nonparenchymal cells in the regenerating liver (Fig. 1c). These data highlight the activation of Smad3 within the regenerating liver and implicate it in the function of both hepatocytes and nonparenchymal cell populations.

Smad3 promotes early pHx-induced liver regeneration

TGF-β/Smad activation promotes senescence of hepatocytes late in the regenerative process. The function of Smad3 specifically in this process has not been evaluated. It was hypothesized that Smad3 plays a critical intermediary role for connecting TGF-β receptor binding and inhibition of hepatocellular proliferation. pHx promoted a significant hepatocellular proliferation as early as 24 h post-pHx which continued to increase at 42 and 48 h post-pHx as measured by proliferating cell nuclear antigen-positive hepatocyte nuclei (Fig. 2a and b). Interestingly, and in contrast to our original hypothesis, Smad3 deficiency reduced hepatocellular proliferation at 42 h post-pHx as assessed by $PCNA^+$ hepatocyte nuclei (Fig. 2a–b). This was further confirmed by reduced bromodeoxyuridine incorporation, a specific indicator of DNA replication in these cells at 42 h, but not 48 h, post-pHx (Fig. 2c).

To further evaluate the proliferative response, and confirm immunohistochemical findings, total tissue protein was isolated from regenerating liver samples and probed for cyclin D1, CDK4, and PCNA. As shown in Fig. 2d, pHx in wild-type mice induced cyclin D1 expression at 24 h

Table 1. Primer list for quantitative PCR measurements.

Gene	Forward primer	Reverse primer
TNF-α	5′-AGCCCACGTAGCAAACCACCAA-3′	5′-ACACCCATTCCCTTCACAGAGCAAT-3′
IL-6	5′-GAGGATACCACTCCCAACAGACC-3′	5′-AAGTGCATCATCGTTGTTCATACA-3′
TGF-β	5′-TGACGTCACTGGAGTTGTACGG-3′	5′-GGTTCATGTCATGGATGGTGC-3′
SOCS3	5′-CACAGCAAGTTTCCCGCCGCC-3′	5′-GTGCACCAGCTTGAGTACACA-3′
PIAS3	5′-CTGTCACCTGGGGCAACTAT-3′	5′-AGATGAGGGACACTCGCACT-3′
CD14	5′-AGGGTACAGCTGCAAGGACT-3′	5′-CTTCAGCCCAGTGAAAGACA-3′
β-Actin	5′-AGGTGTGCACCTTTTATTGGTCTCAA-3′	5′-TGTAGTAAGGTTTGGTCTCCCT-3′

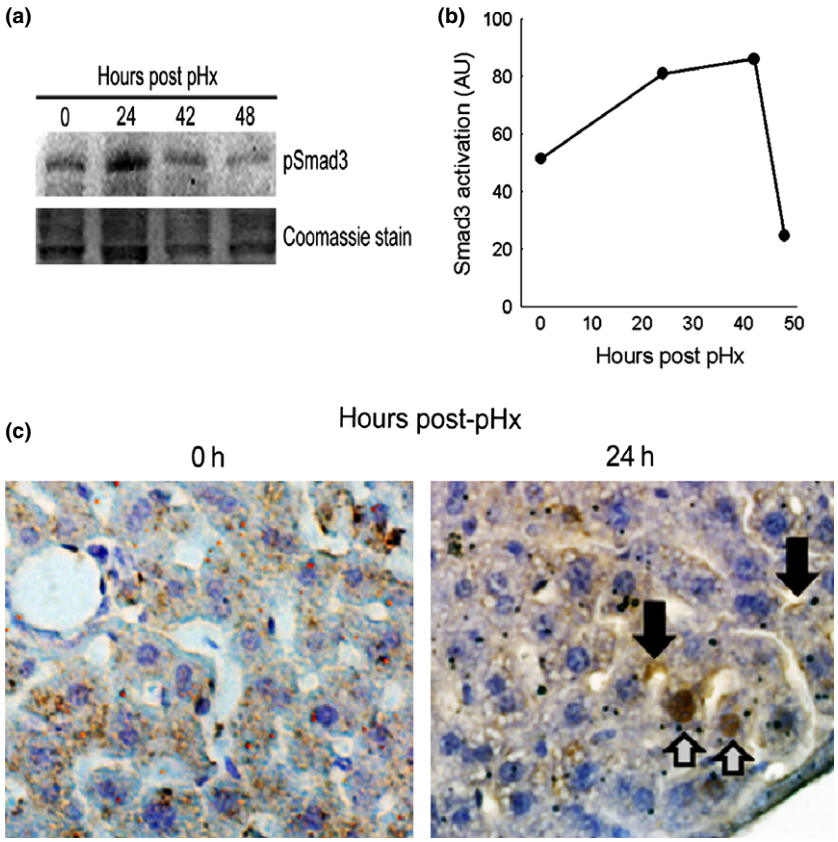

Figure 1 Smad3 is activated early within the regenerating liver. Wild-type mice were subjected to partial (70%) hepatectomy. Phosphorylated Smad3 was then examined by (a) Western blot with (b) quantification of band density and (c) immunohistochemistry for pSmad3 at the indicated time-points. Representative data are presented from 4 to 6 animals per group.

post-pHx and remained increased through 48 h post-pHx. Similarly, CDK4, a key activator of the regenerative process in hepatocytes, was also induced at 24 h post-pHx and remained increased through 48 h post-pHx [21,22]. In the absence of Smad3, induction of CDK4 and cyclin D1 was delayed out to 42 and 48 h, respectively. This inhibition of cyclin D1 expression was further confirmed by immunohistochemistry at 24 h post-pHx in Smad3-deficient livers when compared to their wild-type pHx-treated controls (Fig. 2e).

In addition to potentially regulating the regenerative response, TGF/Smad signaling may also promote hepatocellular apoptosis. Indeed, previous studies by our laboratory demonstrated the profound importance of Smad3 in regulating hepatocellular apoptosis following toxin administration [15]. To this end, liver sections were evaluated by terminal UTP nick-end labeling (TUNEL) staining to evaluate the apoptotic response in this model of murine liver regeneration. pHx did not induce a significant hepatocellular apoptosis at any time-point examined in either wild-type or Smad3-deficient mice (Fig. S1).

Together, these data highlight the importance of Smad3 signaling in the early regenerative response following major tissue loss in the mouse.

Smad3 promotes pHx-induced cytokine production

To investigate the underlying mechanism by which Smad3 signaling might influence the proliferative response in hepatocytes, cytokine expression was assessed by quantitative PCR for TNF-α and IL-6, two key pro-inflammatory yet mitogenic cytokines. pHx resulted in significant increases in both TNF-α and IL-6, but not TGF-β, at 24 and 42 h postsurgery when compared to pre-pHx levels (Fig. 3). This is consistent with previous studies by our laboratory demonstrating enhanced mRNA expression of IL-6 and TNF-α post-pHx [4]. Loss of Smad3 led to a significant reduction in IL-6 gene expression 24 h post-pHx and TNF-α expression 42 h post-pHx when compared to similarly treated wt mice. While these data are limited to the analysis of gene expression for these factors, they do suggest that Smad3 signaling interacts with promitogenic cytokine production in the regenerating murine liver and highlight a

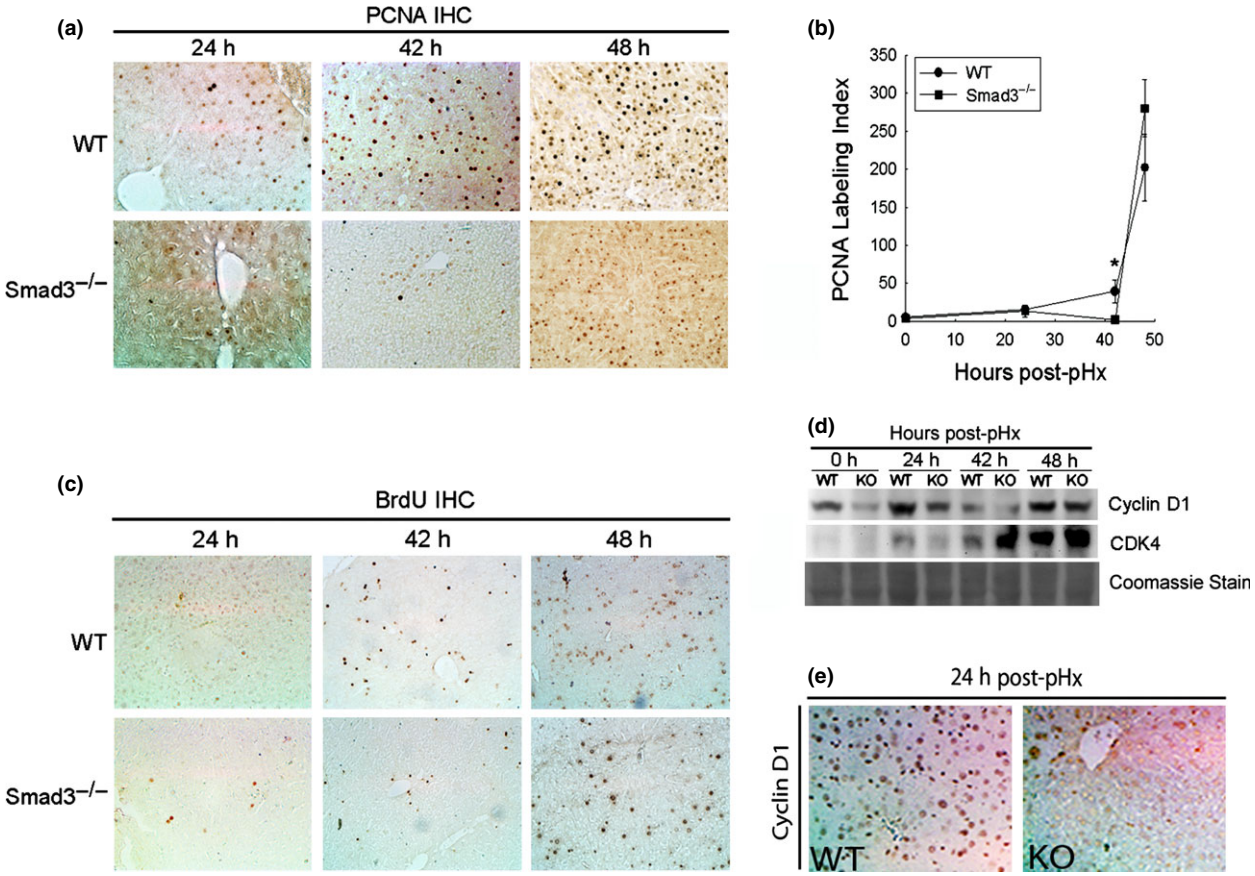

Figure 2 Smad3 signaling promotes early proliferation within the murine liver. Wild-type (WT) or Smad3-deficient mice were subjected to partial (70%) hepatectomy and allowed to recover for 24, 42, or 48 h. (a) PCNA immunohistochemical analysis of liver sections from various time-points post-pHx. Representative photomicrographs at 200× magnification from indicated time-points. (b) Quantitation of positively stained cells from indicated time-points. Data presented as PCNA labeling index, quantified as positive hepatocyte nuclei per 1000 hepatocytes. (c) Bromodeoxyuridine incorporation 24, 42, or 48 h post-pHx in WT or Smad3$^{-/-}$ mice. (d) Western blot analysis of cyclin D1 and CDK4 protein expression 0, 24, 42, or 48 h post-pHx with representative immunoblot presented. Coomassie-stained gel provided to demonstrate loading equality. (e) Cyclin D1 immunohistochemistry 24 h following pHx in WT or Smad3-deficient liver sections. Specific staining in (a), (c), and (e) appears as brown to black. *$P < 0.05$ vs. time-matched wild-type (WT) mouse with 3–6 mice per group.

potential mechanism for the impaired regenerative response observed.

Role of Stat3 in the regenerating liver

To better define the potential impact of disrupted promitogenic cytokine production on the regenerative response, we focused on the downstream targets of these cytokines, particularly IL-6. Stat3 is a key target and regulator of IL-6 signaling in regenerating cells including hepatocytes [6]. Consistently, loss of Stat3 suppresses the regenerative response of hepatocytes following major tissue loss [23]. Given that IL-6 expression was reduced, we sought to determine the impact of this reduction on Stat3 activation. As shown in Fig. 4, Stat3 is activated early during regeneration in the wt liver as indicated by its level of phosphorylation (24 h post-pHx; Fig. 4a) and remains increased through 48 h post-pHx. Consistent with reduced IL-6 production in Smad3-deficient mice, Stat3 phosphorylation is impaired in these mice 24 h post-pHx, a time-point preceding the reductions in liver regenerative response. Stat3 activation recovers, however, at 42 and 48 h to levels at or above those seen in similarly treated wt mice.

To further define the interactions of Smad3 signaling and Stat3 regulation, two additional regulators of the response were quantified. Suppressor of cytokine signaling 3 (SOCS3) and protein inhibitor of activated stat 3 (PIAS-3) are key regulators of Stat3 activation and downstream targets of Stat3 activation as well [24,25]. Indeed, both SOCS3 and PIAS-3 are induced by Stat3 activation and act as negative feedback regulators to suppress

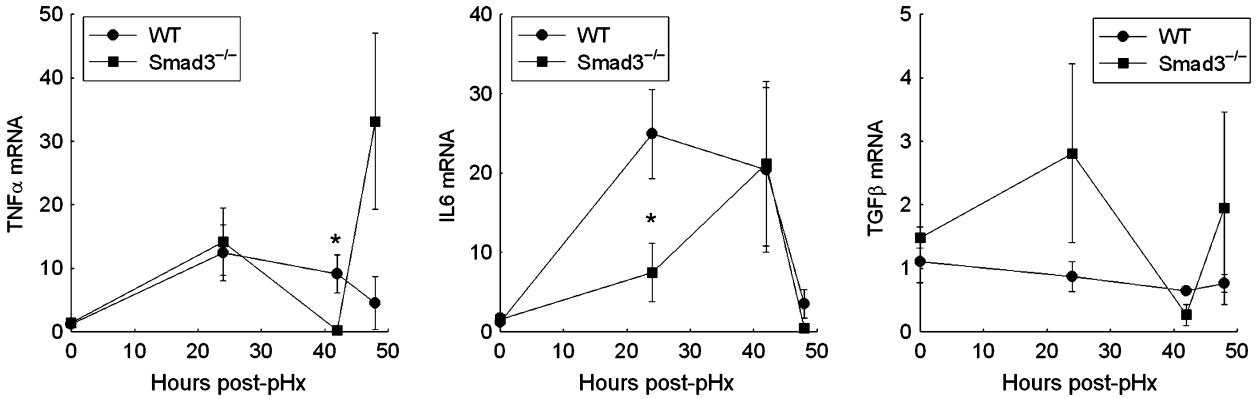

Figure 3 Smad3 signaling promotes IL-6 expression within the regenerating liver. Wild-type or Smad3-deficient mice were subjected to partial (70%) hepatectomy for up to 48 h. Gene expression for tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and transforming growth factor beta (TGF-β) was then quantified at indicated time-points by real-time PCR. $n = 4$–6 animals per group. *$P < 0.05$ vs. time-matched wild-type (WT) control.

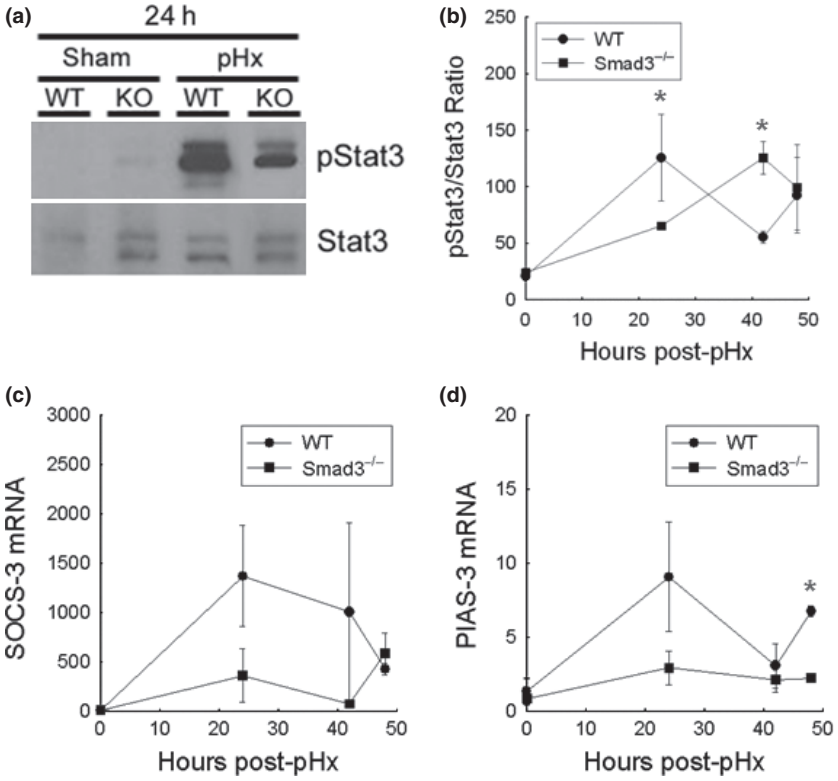

Figure 4 Absence of Smad3 signaling inhibits Stat3 activation within the regenerating liver. Wild-type or Smad3-deficient mice were subjected to partial (70%) hepatectomy and Stat3 activation evaluated at 0, 24, 42 or 48 h postsurgery. (a) Representative blot of phosphorylated Stat3 (pStat3) and total Stat3 24 h following hepatectomy in wild-type and Smad3-deficient mice. (b) Quantification of Stat3 activation 0, 24, 42, or 48 h following hepatectomy. (c) Hepatic PIAS3 and (d) SOCS3 gene expression 0, 24, 42, or 48 h post-pHx as assessed by quantitative PCR. $n = 3$ animals per group.

Stat3 function. As shown in Fig. 4c, SOCS3 expression is strongly increased in wt mice 24 h post-pHx and remains increased through 48 h post-pHx, consistent with the continued activation state of Stat3 in these livers. Figure 4d further confirms the induction of a negative regulator of Stat3 activation, PIAS-3, at 24 h post-pHx in wt mice when compared to pre-pHx levels. Interestingly, and consistent with impaired Stat3 activation, both SOCS3

and PIAS-3 were not induced in Smad3-deficient livers when compared to similarly treated wt mice at 24 or 42 h post-pHx. Together, these data demonstrate reduced Stat3 activation in the Smad3-deficient liver, a process occurring independent of the induction of potential negative regulators of the response and further suggesting an important connection between Smad3 and IL-6 production in the regenerating liver.

Smad3 promotes endotoxin-induced hepatic IL-6 production

It is clear from these studies that Smad3 signaling affects key promitogenic cytokine production, Stat3 activation, and hepatocellular regeneration early during the regenerative response to pHx. Interestingly, immunohistochemistry reveals Smad3 activation, as assessed by its phosphorylation, both in parenchymal and in nonparenchymal cells during this early period of regeneration. Kupffer cells (KC), the hepatic macrophage, contribute to inflammatory cytokine production in acute and chronic liver injuries [26]. KCs also play a substantive role in liver regeneration, as their loss prolongs the time to restoration of tissue mass [27]. The influence of Smad3 activation on hepatic KC responses has not been thoroughly investigated although it is thought that TGF-β-mediated activation of Smad3 likely suppresses their function. As KCs may be a significant contributor to IL-6 production in the liver, we sought to address the function of Smad3 in their production of this important pro-proliferative cytokine [27]. As shown in Fig. 5, lipopolysaccharide (LPS) administration 6 h prior to sacrifice does not induce a significant TGF-β mRNA synthesis but promotes strong IL-6 and TNF-α production in conjunction with CD14 expression consistent with numerous previous reports [28]. Loss of Smad3 does not affect CD14 induction or alter the TGF-β response but completely blocks IL-6 production and limits TNF-α mRNA synthesis in response to LPS, suggesting that KCs are impaired in their synthesis of key pro-inflammatory but also pro-proliferative mediators in response to a common endogenous activator. These data implicate Smad3 signaling in the regulation of IL-6 production by liver, and likely by KCs, and may suggest a potential mechanism by which Smad3 signaling positively regulates early regenerative responses in the hepatectomized animals.

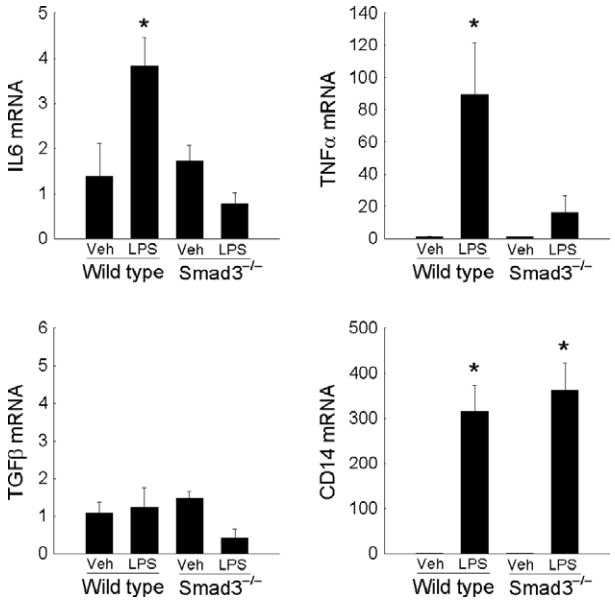

Figure 5 Loss of Smad3 reduces endotoxin-elicited IL-6 and TNF-α production, but not CD14 expression in the murine liver. Wild-type or Smad3$^{-/-}$ mice were administered lipopolysaccharide (LPS; 2.5 mg/kg) or saline by intraperitoneal injection 6 h prior to sacrifice. Tissue expression for IL-6, TNF-α, TGF-β, and CD14 was determined by quantitative PCR. n = 4–6 animals per group.

Discussion

The balance between cell proliferation and apoptosis is vital for tissue homeostasis but also regeneration that depends on several growth factors and cytokines [9]. Upon binding of TGF-β to its type I and II surface receptor complexes, intracellular Smad2 and 3 are phosphorylated and form a complex with Smad4, which translocates to the nucleus to modulate the transcriptional response by interacting with a variety of factors regulating the expression of target genes [29]. Intriguingly, while Smad2 and 4 are required for development, Smad3 function appears dispensable given the survival and health of rodents deficient in its expression [30]. Accumulating data would suggest that this factor may be more centrally responsible for regulation of the inflammatory response with redundant developmental support provided by other inhibitor Smads [31–33]. This led us to investigate its function in the regenerating liver, where both cellular regeneration and inflammatory responses converge to promote a regulated and balanced restoration of liver mass. Surprisingly, and in contrast to our original hypothesis, global deletion of Smad3 impaired hepatocellular regeneration and interrupted key early IL-6 induction and Stat3 activation within the murine liver.

TGF-β and hepatocyte regeneration

TGF-β and Smad signaling is associated with cell cycle arrest and is also known to be an inducer of apoptosis [34]. TGF-β is significantly increased in regenerating human transplanted livers and likely contributes to cessation of liver growth. Experimentally, overexpression of TGF-β inhibits the proliferative response of hepatocytes following major tissue loss, while the loss of TGF-β receptor 2

promotes early mitogenic reflexes within hepatocytes. Studies by Zhong *et al.* [19] have confirmed this relationship demonstrating an enhancement in liver regeneration characterized by increased graft size and BrdU incorporation in transplanted rat livers overexpressing Smad7 in conjunction with reduced nuclear Smad2/3 translocation. It was therefore hypothesized that loss of Smad3, a key signaling element within the TGF-β cascade, would also promote hepatocyte regeneration following partial hepatectomy similar to that seen in TGF-βR2-deficient or Smad7-overexpressing livers. Surprisingly, loss of Smad3 reduced early replicative responses in regenerating hepatocytes within the murine liver. Analysis of both $PCNA^+$ hepatocytes and BrdU incorporation revealed significantly reduced proliferation at 42 h post-pHx in conjunction with impaired cyclin D1 and CDK4 expression at 24 h post-pHx. Intriguingly, hepatocellular proliferation rebounded and overshot the wild-type response by 48 h post-pHx, consistent with previous studies characterizing the TGF-β effect and suggesting apparent dueling effects of TGF-Smad3 signaling in the context of liver regeneration, possibly through differential effects on different hepatic cell populations. Indeed, our studies stop short of defining a cell-specific effect of TGF-β-Smad3 signaling although separate approaches do define a potential disruption in KC in Smad3-deficient mice.

TGF-β signaling and Kupffer cell function

It is clear that TGF-β plays an important role in nonparenchymal cell function within the liver. Interruption of TGF-β signaling within lymphocytes promotes hepatocellular injury and autoimmune-like hepatitis, while this same signaling pathway promotes stellate cell activation and expression of type I collagen [16,35]. TGF-β has also been shown to inhibit pro-inflammatory cytokine production in Kupffer cells in conjunction with their transition toward a more regulatory phenotype [36]. Within the current study, loss of Smad3 signaling suppressed IL-6 and TNF-α production following stimulation with LPS *in vivo,* suggesting a potential defect in this response as a mechanism for the delayed regenerative response observed. Studies in our laboratory support a similar role for TGF-β and Smad3 in the induction of TNF-α production by hepatic macrophages (Son and Hines, unpublished observation). Administration of TGF-β to hepatic macrophages promoted TNF-α production in response to Tlr4 engagement, while the loss of Smad3 or overexpression of Smad7 suppressed this response. The connection between Smad3 and IL-6 production remains unclear. Nath *et al.* [37] recently demonstrated reduced IL-6 production in ischemic kidney injury in Smad3-deficient mice. IL-6 is regulated by a number of factors including nuclear factor kappa B (NFκB), NF-IL-6, Fos-Jun, and CAAT/enhancer-binding protein (C/EBP) [38]. Likewise, soluble mediators including TNF-α and IL-1 have also been shown to promote its transcription. TGF-β is known to interact with a number of these pathways with its effects being at least partly cell specific. Aoki and others demonstrated the ability of pancreatic stellate cells to produce IL-6 in response to TGF-β stimulation *in vitro*, and the coordinate upregulation of TGF-β in response to IL-6 treatment suggesting autocrine loop to promote the expression of both [39]. Further investigation in bronchial epithelial cells revealed the ability of TGF-β to promote IL-6 production, a process that was related to c-Jun N terminal kinase (JNK) activation [40]. Activation of NFκB by TGF-β also promotes epithelial-to-mesenchymal transition (EMT) defining a connection for these signaling pathways [41]. Within macrophages, TGF-β signaling in part through the co-receptor endoglin promotes their recruitment and cytokine production, specifically IL-1β and IL-6, in radiation-induced kidney damage [42]. Similarly, in osteoclasts, TGF-β-mediated activation of TGF-activated kinase 1 (TAK1) promoted Akt activation and subsequent NFκB induction leading to cell survival [43]. Thus, it appears that TGF-β-mediated responses may promote inflammatory transcription factor activation and support IL-6 production among other cytokines and have potentially pro-proliferative roles in the regenerating murine liver.

TGF-β-Smad3 and the regenerative response – Is the IL-6-Stat3 axis the connection?

Within the current study, it is clear that Smad3 signaling promotes IL-6 production in the regenerating liver, possibly from hepatic KCs, and this induction correlates with hepatic Stat3 activation and cell proliferation. In the liver, IL-6 is a major regulator of the acute-phase response and promotes early liver regeneration [10,44]. Fausto and others demonstrated increased IL-6 early during the regenerative response, a process that was preceded by and associated with TNF-α induction [10,45]. Additional experiments using IL-6- and TNF-α receptor 1 (TNF-α R1)-knockout mice after pHx demonstrated an impaired S phase progression with defects in hepatocyte proliferation and high mortality rate. In both IL-6- and TNF-α-R1-knockout mice, the blunted regenerative response could be rescued by treating the animals with IL-6 [44]. Conversely, overexpression of IL-6 has been shown to inhibit the proliferative capacity of hepatocytes within the regenerating liver likely owing to its ability to act as a potent inflammatory mediator and highlighting the importance of a fine-tuned pro-proliferative cytokine response during the restoration of lost liver mass [46]. The current study has demonstrated an interaction

between Smad3 signaling and pro-proliferative cytokine production IL-6 and the downstream activation of Stat3. Stat3 signaling is a known regulator of cell survival and proliferation [6]. Upon activation by a number of factors including IL-6, Stat3 promotes cell survival including the induction of antiapoptotic factors while also promoting cell cycle progression and liver regeneration [23,47]. In support of this notion are studies which show that loss of Stat3 in hepatocytes reduced their proliferation early during regeneration following partial hepatectomy and to a similar degree following carbon tetrachloride-induced liver damage [6,23,47]. Connectivity between Stat3 and Smad3 has also been shown previously. Wierenga *et al.* [48] showed a reduction in IL-6-mediated Stat3 activation in the presence of TGF-β in leukemia cells, a process that involved interruption of JAK activation and induction of cellular apoptosis. In contrast, induction of protein inhibitor of activated Stat3 (PIAS3), a factor induced by Stat3 activation, significantly increased TGF-β-mediated Smad3 activation, suggesting that TGF-β-Smad signaling could be a negative feedback loop to inhibit Stat3-mediated proliferative signals [49]. Within the current study, a new connection can be drawn between TGF-β-Smad3 signaling, IL-6 production, downstream Stat3 activation, and early hepatocellular regeneration in the hepatectomized liver. It is likely that the net effect of loss of Smad3 early during regeneration is interruption of IL-6 production independent of interactions between Smad3 and Stat3 signaling pathways. However, TGF-β-mediated Smad3 activation could limit later Stat3 activation in hepatocytes independent of alterations in IL-6 induction as Stat3 activation is strongly and significantly increased at 42 h post-pHx in $Smad3^{-/-}$ livers when compared to similarly treated wild-type mice. The current study is limited in this regard with respect to understanding the cell-specific effects of a loss of Smad3 throughout the regenerative response although it is clear that TGF-β-mediated Smad3 activation provides both pro-proliferative and potentially inhibitor signals during liver regeneration. In sum, however, the current studies do draw a connection between Smad3, IL-6 production, and early hepatocellular regeneration following major tissue loss in the mouse but do remain limited in directly defining the cell-specific impact of this signaling molecule on IL-6 production and liver regeneration.

Conclusion

Understanding the mechanisms of tissue repair and regeneration, particularly following major tissue loss, is critical to defining new treatments to extend or promote organ survival clinically. Early regenerative efficiency, especially within the first 48 h postsurgery, is critical for graft and recipient survival. Great strides have been made to better define characteristics favorable for organ survival, both with the organ itself and the recipient. The current study highlights Smad3 signaling as a potential, and surprising, positive regulator of IL-6 production and hepatocellular proliferation during this critical early period of liver regeneration. These studies are no doubt limited, however, in that the cell-specific effects of TGF-β and Smad activation remain unresolved. Recent generation of floxed alleles associated with TGF-β and TGF-β-associated signaling will provide the technical framework to assess these processes more specifically. Nevertheless, the current results implicate components of TGF-β signaling in the induction of pro-proliferative responses and provide potential new targets for the regulation of hepatocellular regeneration.

Authorship

MK, INH and GS: participated in research design, performance of the research, data analysis, and writing of the paper. KZ: participated in performance of the research. SM: participated in performance of research and data analysis. GM: participated in data analysis and writing of the paper. MDW: participated in research design, data analysis, and writing of the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hepatocellular apoptosis is not induced within the regenerating liver.

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