Transforming growth factor $\beta$ (TGF$\beta$) cross-talk with the unfolded protein response is critical for hepatic stellate cell activation

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Transforming growth factor $\beta$ (TGF$\beta$) potently activates hepatic stellate cells (HSCs), which promotes production and secretion of extracellular matrix (ECM) proteins and hepatic fibrogenesis. Increased ECM synthesis and secretion in response to TGF$\beta$ is associated with endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). TGF$\beta$ and UPR signaling pathways are tightly intertwined during HSC activation, but the regulatory mechanism that connects these two pathways is poorly understood. Here, we found that TGF$\beta$ treatment of immortalized HSCs (i.e., LX-2 cells) induces phosphorylation of the UPR sensor inositol-requiring enzyme 1 (IRE1$\alpha$) in a SMAD2/3-procollagen I-dependent manner. We further show that IRE1$\alpha$ mediates HSC activation downstream of TGF$\beta$ and that its role depends on activation of a signaling cascade involving apoptosis signaling kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK). ASK1–JNK signaling promoted phosphorylation of the UPR-associated transcription factor CCAAT/enhancer binding protein $\beta$ (C/EBP$\beta$), which is crucial for TGF$\beta$- or IRE1$\alpha$-mediated LX-2 activation. Pharmacological inhibition of C/EBP$\beta$ expression with the antiviral drug adefovir dipivoxil attenuated TGF$\beta$-mediated activation of LX-2 or primary rat HSCs in vitro and hepatic fibrogenesis in vivo. Finally, we identified a critical relationship between C/EBP$\beta$ and the transcriptional regulator p300 during HSC activation. p300 knockdown disrupted TGF$\beta$- or UPR-induced HSC activation, and pharmacological inhibition of the C/EBP$\beta$–p300 complex decreased TGF$\beta$-induced HSC activation. These results indicate that TGF$\beta$-induced IRE1$\alpha$ signaling is critical for HSC activation through a C/EBP$\beta$–p300–dependent mechanism and suggest C/EBP$\beta$ as a druggable target for managing fibrosis.

Hepatic stellate cell (HSC) activation increases production of extracellular matrix (ECM) proteins, leading to fibrogenesis (1, 2). ECM proteins undergo folding and processing within the endoplasmic reticulum (ER) prior to trafficking through the secretory pathway. Canonicly, increased production of ECM proteins leads to ER stress and activation of the unfolded protein response (UPR), which facilitates their folding and trafficking out of the cell (3, 4). On this basis, several studies show that the UPR is critical for HSC activation. Indeed, stimuli that promote HSC activation, such as TGF$\beta$ are associated with ER stress and UPR induction (5–7). Furthermore, chemical induction of the UPR in HSCs in vitro increased expression of ECM proteins in HSCs, whereas pharmacological inhibition of the UPR attenuated HSC activation (8–11). Despite these observations, the mechanisms by which the UPR regulates HSC activation, and in turn how HSC activation modulates UPR signaling, are unclear.

One of the major ER stress sensors that initiates the UPR is inositol-requiring enzyme 1$\alpha$ (IRE1$\alpha$). IRE1$\alpha$ is critical for UPR signaling to accommodate increased protein folding and trafficking (12–14). Upon sensing increased levels of unfolded proteins in the ER, IRE1$\alpha$ dimerizes and undergoes autotransphosphorylation. This kinase activity is necessary for activation of its endonuclease domain as well as mediating downstream signaling cascades involving apoptosis-signaling kinase 1 (ASK1) (15–19). Both the kinase and endonuclease domains of IRE1$\alpha$ are associated with HSC activation (5, 8, 11), but the mechanisms that regulate and propagate IRE1$\alpha$ signaling during HSC activation and fibrogenesis are not completely understood.

We sought to better understand the mechanisms of IRE1$\alpha$ activation and signaling during fibrogenesis. First, we established that TGF$\beta$ induces IRE1$\alpha$ activation downstream of SMAD2/3 signaling and procollagen I expression. Mutational analysis revealed that IRE1$\alpha$ signaling through its kinase domain, but not its endonuclease domain, is sufficient to pro-

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Results

**TGFB induction of IRE1α signaling is critical for HSC activation**

To better understand the relationship between UPR signaling and TGFB, we first established a model to characterize and understand IRE1α signaling in the presence of TGFB. Pharmacological antagonism of C/EBPβ through adeovir dipivoxil disrupted HSC activation in vitro and fibrogenesis in vivo. Finally, we found that the transcriptional regulator p300 is critical for both TGFB- and UPR-mediated HSC activation and may act through a mechanism involving C/EBPβ. Together, our work shows that TGFB induction of the UPR leads to a feed-forward signaling pathway that acts through C/EBPβ–p300 to promote fibrogenesis and that C/EBPβ can be therapeutically targeted to limit fibrosis in vivo.
downstream of SMAD2/3, we disrupted either SMAD2 or procollagen I expression using RNAi (siSMAD2 or shCollagen 1α). IRE1α phosphorylation in response to TGFβ was attenuated in shCollagen 1α or siSMAD2 cells compared with nontargeting (NT) controls (Fig. 1A and B), quantification adjacent). Furthermore, loss of procollagen Iα1 reduced TGFβ up-regulation of fibronectin, as well as αSMA, a marker of activated HSCs. These observations led us to examine whether IRE1α signaling was critical for TGFβ-induced HSC activation. HSCs were isolated from mice harboring loxP sites within the gene encoding IRE1α and were treated with an adenovirus expressing Cre recombinase (AdCre) to disrupt IRE1α signaling or with LacZ as a control (21, 22). Following TGFβ treatment, protein levels of fibronectin, collagen I, and αSMA were reduced in cells with Cre-mediated IRE1α knockdown (Fig. 1C, quantification adjacent). Similarly, treatment of LX-2 cells with the IRE1α inhibitor 4μ8C (15 μM) effectively blocked TGFβ induction of collagen I and fibronectin protein (Fig. 1D, quantification below). 4μ8C also reduced gene transcription of fibronectin, procollagen 1α1 and 1α2, and αSMA (Fig. 1E). Increased mRNA expression of peroxisome proliferator–activated receptor γ (PPARγ), which is associated with quiescent HSCs, was also observed. Thus, inhibition of IRE1α signaling limits TGFβ activation of HSCs.

We next asked whether IRE1α signaling was sufficient to mediate profibrotic gene expression. A doxycycline-inducible IRE1α construct was stably expressed in LX-2 cells (doxIRE1α), and upon doxycycline treatment (5 μg/ml), increased protein levels of IRE1α, as well as fibronectin and collagen I, were observed (Fig. 2A, quantification adjacent). To elucidate the mechanisms downstream of IRE1α signaling responsible for this effect, we utilized doxycycline-inducible IRE1α mutants that specifically inactivate either the IRE1α kinase domain (K599A) or the endonuclease domain (K907A) (16). Overexpression of the K907A mutant recapitulated the effects of the WT IRE1α construct; however, overexpression of IRE1α K599A failed to up-regulate fibronectin or collagen I (Fig. 2A). This implicates the IRE1α kinase domain in HSC activation independent of IRE1α endonuclease activity. A major down-stream target of the IRE1α kinase domain is ASK1, which in turn phosphorylates and activates signaling cascades through Jun N-terminal kinase (JNK) or p38 MAPK. To explore whether

Figure 2. IRE1α kinase activity promotes HSC activation. A, LX-2 cells were stably infected with doxycycline-inducible IRE1α constructs encoding for WT IRE1α (doxIRE1α WT), a kinase-dead mutant (K599A), or an endonuclease-dead mutant (K907A). Cells were treated with doxycycline (5 μg/ml) or vehicle for 24 h, cells were lysed, and protein levels of fibronectin, collagen I, and αSMA were assessed. HSC70 served as a loading control. Quantification is shown adjacent. B, doxIRE1α cells were treated with GS-444217 (2 μM), SP600125 (10 μM), or SB203580 (0.5 μM) for 1 h to inhibit ASK1, JNK, or p38, respectively, followed by doxycycline treatment for 24 h. Cell lysates were harvested, and IRE1α, fibronectin, collagen I, phosphorylated and total JNK, and phosphorylated and total p38 were assessed by immunoblotting. HSC70 served as a loading control. Quantification is shown adjacent. C, doxIRE1α cells were pretreated with U0126 (5 μM) to inhibit ERK1/2 phosphorylation, followed by doxycycline treatment for 24 h. Cells were lysed and assessed by immunoblotting for IRE1α, fibronectin, collagen I, phosphorylated and total ERK1/2, and HSC70 as a loading control. Quantification is shown adjacent. Statistics were performed using ANOVA followed by Tukey post hoc analysis (*, p < 0.05; **, p < 0.01). n = 3 biological replicates for each experiment. Error bars, S.E.
C/EBPβ mediates HSC activation downstream of the UPR

The role of IRE1α signaling in regulating expression of TGFβ target genes led us to explore the mechanisms responsible for this effect. The ENCODE database showed binding sites for the UPR-associated transcription factor C/EBPβ among several gene promoters, including procollagen 1α1 and fibronectin. C/EBPβ is a b-ZIP domain-containing protein transcribed from a single-exon gene as three different isoforms: liver-activating protein 1 and 2 (LAP1 and LAP2), which are transcriptional co-activators, and liver inhibitory protein (LIP), which acts as a co-repressor (25, 26). To establish a role for C/EBPβ in HSC activation, C/EBPβ protein levels were examined in response to TGFβ and found to be up-regulated; however, this up-regulation was disrupted by loss of collagen 1 (Fig. 3, A and B, quantification below). We also observed translocation of C/EBPβ into the nucleus after TGFβ treatment (4 h) by immunofluorescence (Fig. 3C). We next assessed whether TGFβ-induced C/EBPβ expression required IRE1α signaling. Pretreatment with 4μ8C attenuated TGFβ-induction of C/EBPβ protein expression (Fig. 4A, quantification adjacent). Phosphorylation of C/EBPβ at site Thr-235 (corresponding to Thr-223 on LAP2 and Thr-37 on LIP) is known to mediate its activation; thus, we also assessed whether C/EBPβ phosphorylation was modulated by a TGFβ/IRE1α mechanism (27). Indeed, C/EBPβ phosphorylation increased with TGFβ treatment but was attenuated in the presence of 4μ8C. Due to the increase in both total expression and phosphorylation of C/EBPβ in response to TGFβ, the ratio of phosphorylated/total C/EBPβ was unchanged. We further assessed C/EBPβ expression and phosphorylation in the doxIRE1α cells. Increased expression of C/EBPβ (total LAP1/2 and LIP) was observed with overexpression of WT, K599A, or K907A IRE1α; however, C/EBPβ phosphorylation (p-LAP1/2 or p-LIP) failed to be induced with the K599A mutant, which suggests dependence on an IRE1α-dependent kinase cascade (Fig. 4B, quantification adjacent). Similarly, both the ASK1 and JNK inhibitors blocked C/EBPβ phosphorylation downstream of IRE1α expression (Fig. 4C, quantification adjacent). Interestingly, whereas doxycycline treatment did not alter the ratio of phosphorylated/total C/EBPβ in doxIRE1α WT or K907A cells, phosphorylated/total C/EBPβ was significantly reduced following doxycycline treatment in doxIRE1α K599A cells or with inhibition of ASK1 or JNK. Thus, ASK1/JNK signaling promotes C/EBPβ activity downstream of IRE1α. Together, these data suggest that IRE1α signaling promotes TGFβ-mediated HSC activation through up-regulation of both C/EBPβ protein levels and phosphorylation.

Loss of C/EBPβ disrupts TGFβ-induced HSC activation

To determine whether C/EBPβ is critical for HSC activation, LX-2 cells were infected with a lentivirus expressing an shRNA against C/EBPβ (sh-C/EBPβ), or an NT shRNA. Two clones were selected that exhibited reduced expression of the activating and inhibitory isoforms of C/EBPβ, LAP1/LAP2 (69 and 68% reduction) and LIP (82 and 78% reduction) (Fig. 5A, quantification below). Reduced C/EBPβ expression attenuated the effects of TGFβ on fibronectin and collagen 1 protein levels.
compared with controls in both clones (Fig. 5 (B and C), quantification below). Loss of C/EBPβ expression also attenuated fibronectin, procollagen 1α1, procollagen 1α2, and αSMA mRNA expression in response to TGFβ, indicative of a crucial role for C/EBPβ in TGFβ-mediated gene transcription (Fig. 5D, clone 1). To determine whether C/EBPβ acted downstream of IRE1α, doxIRE1α cells were stably infected with the shRNA targeting C/EBPβ and treated with doxycycline. Despite induction of IRE1α, the associated up-regulation of fibronectin, collagen I, and αSMA was lost in doxIRE1α sh-C/EBPβ cells (Fig. 5E, quantification adjacent). Together, these data highlight a crucial role for C/EBPβ in mediating mRNA and protein expression of ECM proteins and αSMA downstream of TGFβ and IRE1α.

**Adefovir dipivoxil inhibits C/EBPβ expression and blocks HSC activation in vitro and in vivo**

The crucial role of C/EBPβ in HSC activation led us to ask whether C/EBPβ could serve as an antifibrotic target. Recently, the hepatitis B antiviral drug adefovir dipivoxil was shown to antagonize C/EBPβ expression in vitro (28). Indeed, adefovir dipivoxil treatment (10 μM) of LX-2 cells attenuated TGFβ
induction of C/EBPβ in response to TGFβ and blocked the effects of TGFβ on fibronectin and procollagen 1α1 at the mRNA and protein level (Fig. 6A and B), quantification adjacent) despite having no effect on TGFβ induction of SMAD2/3 phosphorylation (Fig. 6C, quantification below). The conserved SMAD2/3 phosphorylation indicates that adefovir dipivoxil is acting downstream of SMAD2/3. We also observed a significant increase in PPARγ mRNA expression. Adefovir dipivoxil similarly reduced collagen I and fibronectin protein levels in isolated rat primary HSCs in response to TGFβ or stiffness-induced activation (Fig. 6D and E, quantification below).

We next tested adefovir dipivoxil as an antifibrotic agent in vivo, injecting 10-week-old, sex-matched C57Bl/6J mice for 6 weeks with CCl4 or vehicle (2 days/week) and 10 mg/kg adefovir dipivoxil (or vehicle) on the remaining 5 days/week. Livers were then harvested and assessed for fibrosis. Mice receiving adefovir dipivoxil in conjunction with CCl4 exhibited significantly less collagen deposition as assessed by sirius red staining (Fig. 7A, quantified below). Hydroxyproline analysis showed a significant increase in collagen content in CCl4-treated mice compared with controls, but no significant difference between Adefovir-CCl4 and Adefovir-olive oil groups (Fig. 7B). Furthermore, adefovir dipivoxil reduced CCl4-induced protein expression of fibronectin and αSMA (Fig. 7C, quantification adjacent) and gene expression of procollagen 1α1, TIMP1, αSMA, and PDGFRA (Fig. 7D). Finally, immunofluorescence was performed on liver sections and showed reduced αSMA, desmin (a marker of HSCs), and collagen I in adefovir-CCl4–treated mice.
Figure 6. Adefovir dipivoxil limits HSC activation through decreasing C/EBPβ expression. A and B, LX-2 cells were pretreated with 10 μM adefovir dipivoxil (ADV) for 1 h, followed by TGFβ (5 ng/ml) for 24 h. Cell lysates or mRNA were harvested and assessed by immunoblotting (A) for FN, collagen I, C/EBPβ isoforms LAP1/2 and LIP, and HSC70 (loading control) or qPCR (B) for fibronectin, procollagen Iα1 and Iα2, or PPARγ expression. Quantification for A is shown in adjacent graphs. C, cells were treated with TGFβ in the presence of adefovir dipivoxil or vehicle for 0, 1, 2, 4, or 24 h. Cell lysates were harvested and assessed for phosphorylated SMAD3 or total SMAD2/3. HSC70 served as a loading control. D, primary HSCs were harvested from rats and infected with a lentivirus expressing an shRNA that targets p300 (sh-p300). UPRTreated sh-p300 or NT cells were treated with TGFβ or Tm treatment increased protein levels of fibronectin, collagen I, and HSC70 served as a loading control. Quantification is shown in the graphs below. E, primary HSCs were harvested from rats, followed by adefovir dipivoxil treatment on days 2, 4, and 6 post-isolation. Cell lysates were harvested on day 7 and analyzed by immunoblotting for fibronectin, collagen I expression, or HSC70 (loading control). Quantification is shown in adjacent graphs. Statistics were performed using ANOVA followed by Tukey post hoc analysis for A–D and paired t test for E (*, p < 0.05; **, p < 0.01; ***, p < 0.001). n ≥ 3 biological replicates for each experiment. Error bars, S.E.

compared with CCl4 alone (Fig. 7E). Assessment of C/EBPβ protein levels in whole-liver tissue revealed that LIP increased in response to CCl4 but was significantly decreased with adefovir dipivoxil treatment (Fig. 7F, quantification below). Alternatively, LAP1/2 protein levels were unaffected. We also analyzed IRE1α phosphorylation in whole liver and observed increased phosphorylation of IRE1α with CCl4 treatment, whereas treatment with adefovir dipivoxil blocked this effect (Fig. 7F). Finally, analysis of the data revealed no differential effect of CCl4 or adefovir dipivoxil between male and female mice (data not shown). Thus, adefovir dipivoxil reduces HSC activation in vitro and fibrogenesis in vivo.

C/EBPβ mediates HSC activation through a mechanism involving p300

With a link between C/EBPβ and HSC activation identified, we questioned the mechanism of C/EBPβ regulation of profibrotic genes. C/EBPβ canonically acts as a homo- or heterodimer to mediate transcription. Analysis of the ENCODE database revealed C/EBPβ-binding sites in close proximity to sites bound by the transcriptional regulator p300 along the promoters of TGFβ-responsive genes, including procollagen Iα1 and fibronectin. p300 has been implicated in HSC activation and is a known C/EBPβ-binding partner (29, 30). In vivo analysis of p300 protein levels showed increased p300 in mice treated with CCl4, but this effect was blocked in mice receiving both CCl4 and adefovir dipivoxil (Fig. 8A, quantification below). This prompted us to study p300 in both TGFβ- and UPR-induced HSC activation. LX-2 cells were infected with a lentivirus expressing an shRNA that targets p300 (sh-p300). sh-p300 or NT cells were treated with TGFβ or tunicamycin (1 μg/ml, a potent inducer of ER stress, for 24 h or vehicle alone. TGFβ or Tm treatment increased protein levels of fibronectin, collagen I, and αSMA, but p300 knockdown attenuated this effect (Fig. 8, B and C). Additionally, p300 knockdown in doxIRE1α cells (doxIRE1α-sh-p300) attenuated IRE1α-mediated up-regulation of fibronectin, collagen I, and αSMA protein levels (Fig. 8D, quantification below).

The similar effects of p300 and C/EBPβ knockdown on TGFβ- and IRE1α-mediated HSC activation suggested that these proteins may act together. Previous studies showed that p300 binding to C/EBPβ led to transcriptional activation in a mechanism that involved C/EBPβ phosphorylation at Thr-235. Thus, we asked whether p300 is responsible for the effects of C/EBPβ during TGFβ-mediated HSC activation (29). To this
end, we utilized helenalin acetate, a sesquiterpene lactone reported to disrupt the interaction between C/EBPβ/H9252 and p300 (31). Co-treatment of LX-2 cells with TGFβ/H9252 and helenalin acetate (1 μM) disrupted TGFβ induction of fibronectin, procollagen 1α1, procollagen 1α2, and αSMA mRNA levels, as well as increased PPARγ mRNA (Fig. 9A). Helenalin acetate also attenuated TGFβ induction of fibronectin in primary HSCs isolated from rats (Fig. 9D, quantification adjacent). Together, these data show that p300 is critical for mediating HSC activation downstream of TGFβ-IRE1α signaling and may act through a mechanism involving C/EBPβ.

Discussion

UPR signaling plays an important role during HSC activation, although the mechanisms that govern the relationship between HSC activation, UPR signaling, and fibrogenesis are not fully understood. Here, we make four key findings that provide mechanistic insight into how IRE1α drives fibrogenesis in response to TGFβ (Fig 10). We show that TGFβ induces the UPR and IRE1α signaling through a SMAD2/3-collagen I-dependent mechanism. Next, we identify C/EBPβ as a crucial mediator of HSC activation that is regulated by IRE1α in an
ASK1/JNK-dependent mechanism. Furthermore, C/EBPβ can be pharmacologically targeted by adefovir dipivoxil to block HSC activation in vitro and fibrogenesis in vivo. Finally, we show that p300 is a crucial mediator of HSC activation downstream of the UPR, which may act together with C/EBPβ in this role. Together, these data map out a UPR-dependent feed-forward mechanism that drives fibrogenesis through C/EBPβ–p300.

UPR signaling in activated HSCs is thought to accommodate increased protein translation, trafficking, and secretion. Chemical induction of the UPR can also drive fibrogenesis independently of canonical activation signals. The relationship between canonical HSC activation mechanisms and the UPR, however, is still unclear. We show here that IRE1α signaling through its kinase domain is crucial for HSC activation. IRE1α kinase activity leads to a signaling cascade involving ASK1 and JNK, which are critical for phosphorylation of C/EBPβ. Phosphorylation of the Thr-235/Thr-223/Thr-37 on C/EBPβ is associated with increased transcriptional activity and is a known target of the MAPK family of kinases, consistent with our data (27). This role of IRE1α is interesting for a few reasons. First, IRE1α signaling has been implicated in HSC fibrogenic gene expression through both its endonuclease domain and through p38 signaling; however, we find that neither of these mechanisms are critical for HSC activation in our inducible IRE1α system or necessary for C/EBPβ phosphorylation. p38 activation was previously associated with SMAD3 phosphorylation and collagen I expression in response to chemically induced ER stress (11), whereas activation of the transcription factor XBP1 downstream of IRE1α endonuclease activity is associated with ER dilation and up-regulation of genes involved in protein trafficking and secretion (5, 6, 8). Furthermore, we show that overexpression of either the K907A or K599A IRE1α mutant still led to up-regulation of total C/EBPβ expression, implicating a kinase- and endonuclease-independent mechanism of C/EBPβ regulation. Thus, IRE1α acts through several mechanisms to contribute to HSC activation. The role of ASK1–JNK signaling in this process is also interesting, as a separate ASK1 inhibitor, selonsertib, is associated with reduced collagen content and liver stiffness when used in a clinical trial to treat patients with non-alcoholic...
Our work suggests that this reduction may be due to inhibition of C/EBPβ phosphorylation, subsequently limiting HSC activation. Finally, the loss of procollagen and fibronectin expression in the absence of IRE1α signaling provides insight into HSC activation mechanisms. We propose here that initial SMAD2/3-dependent up-regulation of ECM proteins leads to UPR signaling and that the UPR is critical for driving and maintaining the fibrogenic phenotype, thus placing procollagen I as both a cause and a consequence of UPR signaling during HSC activation. Alternatively, UPR induction prior to increased protein load has been observed in immune cells (34). Further assessment of UPR induction in response to different activating stimuli may provide insight into the role of the UPR in HSCs.

Our data indicating that C/EBPβ is critical for HSC activation downstream of the UPR may be due to inhibition of C/EBPβ phosphorylation, subsequently limiting HSC activation. Finally, the loss of procollagen and fibronectin expression in the absence of IRE1α signaling provides insight into HSC activation mechanisms. We propose here that initial SMAD2/3-dependent up-regulation of ECM proteins leads to UPR signaling and that the UPR is critical for driving and maintaining the fibrogenic phenotype, thus placing procollagen I as both a cause and a consequence of UPR signaling during HSC activation. Alternatively, UPR induction prior to increased protein load has been observed in immune cells (34). Further assessment of UPR induction in response to different activating stimuli may provide insight into the role of the UPR in HSCs.

Our data indicating that C/EBPβ is critical for HSC activation in a UPR-dependent manner provide some context for the regulation of C/EBPβ and ECM production in HSCs. Previous studies show that C/EBPβ can positively or negatively regulate procollagen 1α1 expression, and these effects are dependent on the stimulus, isoform, and coregulators (35–38). C/EBPβ transcription is unique, in that three isoforms are transcribed from the same gene. The activating isoforms of C/EBPβ (LAP1 and LAP2) bind DNA as well as interact with several transcriptional co-activators, whereas the inhibitory LIP isoform serves as a dominant negative. Thus, the ratio of LAP/LIP greatly influences transcriptional programming. This is evident during the UPR, where LAP1/LAP2 are typically up-regulated during early adaptive UPR, and LIP is up-regulated later and associated with proapoptotic UPR signaling (39). LIP overexpression also inhibits collagen I expression (38). Thus, targeting C/EBPβ or a specific isoform, such as the full-length LAP1, which can bind to p300, could reduce the activated HSC population in vivo (31).

UPR-mediated phosphorylation of C/EBPβ may influence recruitment of transcriptional coactivators during HSC activation. Mutation of mouse C/EBPβ at Thr-188 to alanine (corresponding to Thr-235 in human C/EBPβ) blocks C/EBPβ induction of the c-fos promoter as well as p300 recruitment to the same site (40, 41). A similar mechanism may be responsible during HSC activation, as loss of Thr-235 phosphorylation is associated with attenuated ECM production in response to IRE1α overexpression, and helenalin acetate also limits the effects of TGFβ. The mechanisms and the transcriptional complexes involved in TGFβ- and IRE1α-mediated gene transcription through C/EBPβ merit further study, as both C/EBPβ and p300 can interact with several transcription factors, including SMADs. Of further interest is the requirement for p300 in UPR-induced HSC activation. There is limited evidence of p300...
involvement downstream of the UPR, aside from acetylation of the transcription factor XBP1 (34). p300 may complex with additional UPR-responsive transcription factors to regulate transcriptional activity.

Whereas we have focused here on UPR signaling through IRE1α, signaling through the other two UPR sensors, PERK and ATF6, also likely plays a role in HSC activation (4). In vivo studies show a positive relationship between PERK signaling and HSC activation, and subsequent studies showed that PERK increases SMAD2 expression through a mechanism involving phosphorylation of heterogeneous nuclear ribonucleoprotein A1 and inhibiting the degradation of SMAD2 mRNA (9). Additionally, C/EBPβ is canonically downstream of the PERK pathway and thus may provide mechanisms for further regulation of C/EBPβ in HSCs (39). Less is known regarding ATF6α in HSCs. Inhibition of ATF6α reduced HSC activation in response to chemically induced ER stress, but no direct mechanisms for ATF6α signaling during HSC activation have been identified (11). Further studies are needed to fully understand the integration of IRE1α, PERK, and ATF6α signaling during HSC activation and how cross-talk between activation signals (such as TGFβ) and UPR signaling influences HSC activation and survival.

Adefovir dipivoxil was identified as a pharmacological inhibitor of C/EBPβ during a screen for compounds that preferentially targeted the LIP isoform of C/EBPβ. In the drug screen, adefovir dipivoxil reduced the ratio of LIP/LAP but also impacted expression of the activating isoforms, prompting us to test whether adefovir dipivoxil could limit HSC activation through targeting C/EBPβ. Adefovir dipivoxil effectively reduced protein levels of all C/EBPβ isoforms in LX-2 cells and attenuated TGFβ-stimulated HSC activation, as well as fibrogenesis in vivo. Interestingly, LIP, but not LAP1/2, was significantly reduced in whole liver in vivo. These data suggest a crucial and targetable role for C/EBPβ, and the ratio of LIP to LAP1/2 in fibrosis, further supported by previous findings where mesenchymal C/EBPβ deletion attenuated pulmonary fibrosis (27). Based on the known role of C/EBPβ in both proliferation and apoptosis, we anticipate that adefovir dipivoxil may also limit HSC proliferation or promote HSC apoptosis, both of which are favorable for fibrosis regression and resolution (42–45). Finally, whereas we anticipate that C/EBPβ is one of the major targets of adefovir dipivoxil in HSCs, off-target effects may contribute to the antifibrotic nature of the drug. Another acyclic nucleotide analog used in hepatitis B patients, tenofovir, was associated with reduced sirius red staining in a thioacetamide model of liver injury, but its effects on C/EBPβ or UPR signaling were not assessed (46).

In conclusion, we show that TGFβ induces UPR signaling in HSCs, which acts in a feed-forward mechanism through IRE1α to promote fibrotic gene expression through C/EBPβ−p300.

**Experimental procedures**

**Cell culture**

Immortalized HSCs (LX-2, ATCC) and primary HSCs were cultured in Dulbecco’s modified Eagle’s medium (Gibco) + 10%
fetal bovine serum + 1% penicillin/streptomycin. Compounds used include TGFβ (5 ng/ml; R&D Biosystems), doxycycline (5 µg/ml; Clontech NC0424034), 4µ8C (15 µM; Selleckchem S7272), GS-444217 (2 µM; Gilead Sciences), SB203580 (0.5 µM; Selleckchem S1076), SP600125 (10 µM; Selleckchem S1460), U0126 (5 µM; InvivoGen (San Diego, CA)), adefovir dipivoxil (10 µM in vitro and 10 mg/kg in vivo; Sigma A9730), tunicamycin (1 µg/ml; Sigma 654380), and helenalin acetate (1 µM; Cayman Chemical 17050). For all experiments using compounds in conjunction with TGFβ, cells were serum-starved for 4 h and pretreated with the indicated compound for 1 h, followed by treatment with TGFβ or vehicle. Doxycycline-inducible cell lines were cultured in Dulbecco’s modified Eagle’s medium plus 10% tetacycline-free fetal bovine serum (Clontech) and 1% penicillin/streptomycin. For experiments using ASK1, JNK, or p38 inhibitors in conjunction with doxycycline, the inhibitors were added to the cells for 1 h followed by the addition of doxycycline for 24 h. Transient transfection of LX-2 cells with siRNA against SMAD2 (Qiagen) or a nontargeting control was achieved using Oligofectamine (Invitrogen), and experiments were conducted 48 h post-transfection.

Plasmids and generation of stable cell lines

DNA encoding WT, K599A, or K907A IRE1α was cloned in a pLVX-tet-tomato vector (Clontech) with a hygromycin selection cassette (pLVX-tet-tomato-hygro-IRE1αK599A, pLVX-tet-tomato-hygro-IRE1αK907A). shRNA against procollagen 1α1, C/EBPβ, p300, or a nontargeting shRNA control was purchased from Sigma. To generate lentiviral vectors encoding the aforementioned plasmids, the plasmids were transfected into HEK293T cells as described previously (7). The virus-containing medium was harvested and used to infect LX-2 cells, followed by selection with puromycin (2 µg/ml) for shRNA constructs or hygromycin (2 µg/ml) for pLVX-tet-tomato-hygro constructs.

Primary HSC isolation and infection

HSCs were harvested from IRE1αfl/fl mice (21, 47, 48) or adult female Lewis rats (Charles River Laboratories) (49) as described previously. HSCs isolated from IRE1αfl/fl mice were infected with adenovirus encoding for Ad-Cre-eGFP to induce gene recombination or with an Ad-LacZ control, as described previously (50). All procedures were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee.

In vivo treatment and analysis

Eight-week-old C57Bl/6j mice were purchased from Envigo. Following a 1-week acclimation period, age- and sex-matched mice were separated into four treatment groups: olive oil vehicle (n = 7), CCl4 + vehicle (n = 8), olive oil + adefovir dipivoxil (n = 8), and CCl4 + adefovir dipivoxil (n = 8). CCl4 (0.5 µl/mouse, 0.08 ml/mouse) was administered by intraperitoneal injection twice a week for 6 weeks. Each group consisted of four males and four females, which were housed together according to their treatment group. Mice were housed in transparent polycarbonate cages subjected to 12-h light/dark cycles under a temperature of 21 °C and a relative humidity of 50%. A standard chow diet and freshwater were provided ad libitum. Adefovir dipivoxil (Sigma-Aldrich, SML0240) was dissolved in sterile in citric acid (0.05 M, pH 2.0) and administered at a concentration of 10 mg/kg/day 5 days a week for 6 weeks. Mice were observed daily for distress and tolerated the experimental protocol without adverse effects, aside from one mouse unexpectedly reaching end point criteria (olive oil + vehicle group). After 6 weeks, mice were sacrificed, and livers were harvested for analysis of fibrosis. Assessment of collagen content by sirius red staining or hydroxyproline was performed as described previously (7). For analysis of gene expression, mRNA was harvested using an RNEasy kit (Qiagen), followed by reverse transcription into cDNA and assessment by qPCR. For protein assessment, livers were homogenized in lysis buffer as described previously and analyzed by Western blotting (7). For immunofluorescence, liver samples were snap-frozen in OCT embedding compound and 7-µm frozen sections were obtained using a Leica cryostat. Sections were fixed with 4% paraformaldehyde, followed by incubation with primary antibodies against collagen I, desmin, and αSMA. Anti-goat IgG and anti-rabbit IgG conjugated with Alexa fluorochromes were used as secondary antibodies (Molecular Probes, Inc., Eugene, OR). DAPI was used at 1:2000 for counterstaining. Microscopy was performed by Axio Observer (Carl Zeiss, Thornwood, NY), in which appropriate light and filter combinations were selected according to excitation and emission spectrum features of the Alexa fluorochromes. All procedures were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Antibodies

The following antibodies were used: HSC70 (Santa Cruz Biotechnology, Inc., 7298), Collagen I (Southern Biotech 1310-01), IRE1α (Cell Signaling 3294), phospho-IRE1α (Abcam ab124945), SMAD2/3 (Cell Signaling 3102S), phospho-SMAD3 (Cell Signaling 9520S), fibronectin (for mouse and rat HSCs, mouse tissue, Developmental Studies Hybridoma Bank at the University of Iowa, PCRP-CEBPB-3D10), phospho-C/EBPβ (Thr-235 on LAP1, Thr-223 on LAP, and Thr-37 on LIP; Cell Signaling 3084S), fibronectin (for mouse and rat HSCs, mouse tissue,
**Table 1**

Primer sequences used for qPCR analysis

| Gene name                | Primers                      |
|--------------------------|------------------------------|
| Human fibronectin        | Forward: GTGGCTGCTCTTCAAATCTC Reverse: GTGGTTGCTAAAATCTTCAAT |
| Human procollagen 1α1    | Forward: TGAGGCGCAGCAGTGAAG Reverse: CAGATGACCTGTATATCCACAA |
| Human procollagen 1α2    | Forward: GCCCCCTAAAGTTCTCAAGG Reverse: CACCCTGTTGCTCAACAAACCTC |
| Human αSMA               | Forward: AATGCAGAAGAGATACGCG Reverse: CAGAGCTCCACTATTGATTTT |
| Human pPARγ              | Forward: ACCCTCCACTCTTCCTTGA Reverse: GCAAGCTCCACTTTGAATTTT |
| Human GAPDH              | Forward: CTCTGCTCTTCTCTTGATGA Reverse: TAAAAGCAAGCCTGTTAGAC |
| Mouse 18S                | Forward: GTACAAGGCGCAGCCCTTTATG Reverse: AGGCTCTGATATGCCCTTTAGA |
| Mouse procollagen 1α1    | Forward: GACCAGAGATCTAGTGATCG Reverse: GCCCTTCTTCTTTGCTGCTC |
| Mouse TIMP1              | Forward: CCTTCCAACAAGCTGAGTACA Reverse: AACCAGGATGAGGCTGCTG |
| Mouse αSMA               | Forward: AACACAGATTACCGACGACAG Reverse: GAATGATTTGGAAAGGA |
| Mouse PDGFRα             | Forward: GGCCTGTTGCTCAATTAGAG Reverse: TCAGACCACTCTTGATAGAG |

Abcam ab2413, PDGFRβ (Cell Signaling 28E1), desmin (Abcam 15200), and P300 (Santa Cruz Biotechnology sc-8981).

**qPCR analysis**

Following treatment, cells were harvested using an RNeasy kit (Qiagen). Equal amounts of mRNA were reverse-transcribed, and qPCR was performed. Human GAPDH or mouse 18S primers were used for normalization in experiments using human cells or mouse liver lysates, respectively. Primer sequences used for qPCR analysis are listed in Table 1.

**Statistical analysis**

For all experiments with three or more groups, statistics were performed using analysis of variance (ANOVA) followed by Tukey post hoc analysis (*, p < 0.05; **, p < 0.01; ***, p < 0.001). All statistical analysis comparing two groups utilized paired t tests. n ≥ 3 biological replicates for each experiment.

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