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HuR contributes to Hepatic Stellate Cell activation and liver fibrosis

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Abstract

RNA-binding proteins (RBPs) play a major role in control of mRNA turnover and translation rates. We examined the role of the RBP human antigen R (HuR) during cholestatic liver injury and hepatic stellate cells (HSC) activation. *HuR* silencing attenuated fibrosis development *in vivo* after BDL, reducing liver damage, oxidative stress, inflammation, and collagen and α-SMA (α-smooth muscle actin) expression. HuR expression increased in activated HSC from BDL mice and during HSC activation *in vitro*, and *HuR* silencing markedly reduced HSC activation. HuR regulated platelet-derived growth factor (PDGF)-induced proliferation and migration, and controlled expression of several mRNAs involved in these processes (*Actin*, *MMP9*, *Cyclin D1* and *B1*). These functions of HuR were linked to its abundance and cytoplasmic localisation, controlled by PDGF, via ERK and PI3K activation, and ERK-LKB1 activation respectively. More importantly, we identified the tumor suppressor LKB1 as a novel downstream target of PDGF-induced ERK activation in HSC. HuR also controlled transforming growth factor beta (TGF-β-induced profibrogenic actions by regulating expression of *TGF-β, α-SMA,* and *p21*. This was likely due to an increased cytoplasmic localisation of HuR, controlled by TGF-β-induced p38 MAPK activation. Finally, we found that HuR and LKB1 (Ser428) levels were highly expressed in activated HSC in human cirrhotic samples. Conclusion: Our results show that HuR is important for pathogenesis of liver fibrosis development in the cholestatic injury model, for HSC activation, and for the response of activated HSC to PDGF and TGF-β.

Keywords

TGF-β; PDGF; HSC; LKB1; cirrhosis

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INTRODUCTION

Hepatic fibrosis is the common consequence of chronic liver diseases such as viral and autoimmune hepatitis, alcohol consumption, biliary obstruction, and non-alcoholic fatty liver disease (1). Hepatic stellate cells (HSC) are the major producers of collagen in the damaged liver (2). In healthy liver, HSC have a quiescent phenotype, accumulating retinoids (vitamin A) and expressing markers characteristic of adipocytes (3). After continued liver damage, these quiescent HSC are exposed to apoptotic hepatocytes, ROS, and inflammatory and profibrogenic factors, and undergo a process of activation to a myofibroblastic phenotype. These activated HSC increase proliferation and migration, acquire contractility and pro-inflammatory properties, and express myogenic markers like α-SMA to become the major col1a1-producing cells (4).

In the liver, levels of many mRNAs are regulated in response to fibrosis-inducing injuries (5). RBPs can promote rapid spatiotemporal expression of proteins by binding to U-and AU-rich elements (AREs) in mRNAs (6). HuR, a member of the Hu/Elav family, is a ubiquitously expressed RBP that is predominantly (>90%) localized in the nucleus of most unstimulated cells. In response to proliferative, stress, apoptotic, differentiation, senescence, inflammatory and immune stimuli, HuR is exported to the cytoplasm, increasing the half-life and/or the rate of translation of target mRNAs (6). Several studies have shown that HuR has important functions in hepatocytes, including HGF-induced hepatocyte proliferation (7), differentiation (8) and apoptosis (9), and during hepatocyte malignant transformation (8,10). Also, HuR expression is upregulated in HCC tissue compared to normal tissues (10), suggesting that it could represent a novel target for liver damage research.

The aims of the current work were to study the role of HuR in liver fibrosis and in HSC activation, and examine its role in controlling the functions of two principal mediators of HSC activation, PDGF and TGF-β.

MATERIAL AND METHODS

Reagents

TGF-β and PDGF were from Peprotech. SB203580 and BAY 11-7082 were from Calbiochem, U0126 from Promega and LY 294002 from SIGMA.

Human Samples

Surgically resected liver tumor specimens from 16 cirrhotic patients (hepatitis C, n=7; alcoholic, n= 9) were examined. Informed consent to all clinical investigations, in accordance with the principles in the Declaration of Helsinki, was provided. The institutional review board of the Hospital Clínic de Barcelona approved the protocol.

HSC Isolation

Animals were maintained in the CIC bioGUNE animal facility with appropriate approvals from the institutional review committee on animal use. HSC were isolated from liver of male Sprague-Dawley rats, bile duct ligated (BDL) and sham operated mice as described (11).

Bile duct ligation

BDL was performed in 12-week-old mice by tying the common bile duct using a non-absorbable filament. Mice (n=8) were injected via the tail vein with 200 μl of a 0.75 μg/μl solution of HuR specific Sh RNA (sense 5′-gatgcagagagagcaatca-3′) or control Sh RNA (pSM2c Open Biosystems).

Carbon Tetrachloride (CCl_4) treatment

Rats (n=5) were treated with CCl_4 diluted 1:1 in corn oil (0.5 μl of CCl_4/g b.wt) by intraperitoneal injection twice a week for 6 weeks. Control animals received vehicle alone (n=5).

Viral Infection

Cells were treated with short-hairpin lentiviral particles against HuR [CCGGCCCACAAATGTTAGACCAATTCTCGAGAATTGGTCTAACATTTGTGGGTTTTTG], or against LKB1 [CCGGCATCTACACTCAGGACTTCACCTCGAGGTGAAGTCCTGAGTGT-AGATGTTTTT] in the presence of hexadimethrine bromide (8 μg/ml). For control cells, HSC were infected with pLKO.1 lentiviral vector (SIGMA). After 24h transduction, the cells were selected using puromycin (1.25 μg/ml).

Migration assay

Migration using the "scratch-assay" was performed in *LKB1*- and *HuR*-silenced cells seeded onto PDL-coated dishes, as described (13).

RNA isolation and real-time PCR (qPCR)

PCR was performed with primers described in Supplementary Table I.

RNA immunoprecipitation-qPCR (RIP-qPCR)

Immunoprecipitation (IP) of endogenous RNA–protein complexes were performed as described (8,10).

Western blot analysis

Total proteins were extracted in RIPA buffer. Cytoplasmic and nuclear lysates were prepared with the subcellular proteome extraction kit (Calbiochem). Immunoblotting analysis was performed with specific antibodies (Supplementary Table II).

Immunohistochemistry

Detailed immunohistochemistry protocol of paraffin-embedded sections is provided in Supplemental Material and Methods.

RESULTS

HuR expression in HSC from human chronic liver diseases

We found that activated HSC (α-SMA^+ cells) strongly expressed HuR in surgically resected liver samples from patients with alcoholic (Figure 1A) and hepatitis C (VHC) cirrhosis (Figure 1B). Similarly, activated HSC expressed HuR in the nucleus of liver sections from two animal models of induced fibrosis, bile duct ligated (BDL) mice (Figure 1C) and rats treated with CCl_4 (5) (Figure 1D), suggesting that HuR could play a role during HSC activation.

HuR silencing attenuated hepatic fibrosis in BDL-mice

To confirm the role of HuR in liver fibrosis, we silenced HuR *in vivo* in BDL mice. Thus, mice were injected in the tail vein with a HuR-specific or control Sh RNA at time 0h, days 3 and 6 after BDL, and then sacrificed 9 days after BDL. *HuR* silencing was confirmed by RT-PCR and Western Blot in whole liver extracts (Supplementary Figures 1A and 1B) and specifically in HSC by immunohistochemistry (Supplementary Figures 1C). HuR silencing

resulted in reduced histological liver damage, as seen by hematoxilin/eosin staining (Figure 2A) and decreased ALT and bilirubin serum levels (Supplementary Figure 1D and 1E). Notably, fibrosis development in these mice was significantly attenuated as shown by reduced collagen deposition (Figure 2B), α-SMA expression (Figure 2C), and *col1a1, α-SMA* and *TGF-β* mRNA levels (Figure 2D).

HuR silencing also led to reduced protein oxidation (Figure 3A and Supplementary Figure 1F and 1G), proliferation (Figure 3B), macrophage infiltration (Figure 3C), and lower expression of genes involved in inflammation (*iNOS*, *IL-1α*, *TNF-α*) and infiltration (*MCP-1*, *F4/80*, *ICAM-1*, *MMP9* and *Actin*) (Figure 3D). Altogether, our results suggest that HuR plays a crucial role in the pathogenesis of cholestatic liver injury.

HuR expression during HSC activation

The data above suggest that HuR could be regulating HSC activation and fibrosis development either directly, and/or indirectly by a decrease in liver damage and inflammation. To characterize the effect of HuR in HSC activation only, we examined its expression in primary HSC isolated from sham and BDL mice, nine days after surgery. *HuR* mRNA levels increased in HSC isolated from BDL mice, correlating with HSC activation, as seen by induction of *α-SMA* mRNA expression (Figure 4A). Total, cytoplasmic and nuclear HuR protein levels were also upregulated (Figure 4B).

Similarly, during *in vitro* activation of primary HSCs on plastic surface (14), *HuR* mRNA levels also increased after 3, 5 and 7 days in culture compared to quiescent HSC (day 1), correlating with HSC activation, as seen by induction of *α-SMA, cyclin D1* and *TGF-β*, and down-regulation of *GFAP* (Glial fibrillary acidic protein) expression (Figure 4C). This up-regulation of HuR was confirmed by Western blotting in 5-days cultured HSC compared to quiescent HSC (Figure 4D). *HuR* silencing in primary HSC, as confirmed by immunocytochemsitry (Figure 4E), induced morphological changes (F-actin immunostaining) (Figure 4E), significantly reduced levels of activation (*α-SMA, col1a1* and *TGF-β*) and proliferation markers (*cyclin D1*), and markedly increased expression of the quiescent marker *GFAP* (15) (Figure 4F). Taken together, our data show that HuR could play a role during HSC activation.

Role of HuR in PDGF-induced migration and proliferation

We next examined if HuR activity controlled the functions of two principal mediators of HSC activation, PDGF and TGF-β. PDGF potently promotes HSC migration and proliferation during fibrosis (16). *HuR* silencing in primary HSC isolated from BDL mice (Supplementary Figure 2A) significantly reduced their migratory rate both basally (Supplementary Figure 2C) and after PDGF treatment (Supplementary Figure 2D), and decreased BrDU incorporation after PDGF stimulation (Supplementary Figure 2E).

HuR silencing in a cell line of activated HSC, CFSC-8B cells (12) (Supplementary Figure 2B) also blocked PDGF-induced migration and proliferation (Figure 5A and 5B). In CFSC-8B cells, *HuR* silencing prevented PDGF-induced increase in mRNA levels of genes regulating proliferation (*cyclin D1* and *B1*), migration [*MMP9* and *Actin* (17)], and infiltration [*MCP-1* (18)] (Figure 5D) as well as cyclin D1 protein (Supplementary Figure 2B). RIP-qPCR analyses revealed a significantly increased binding of HuR to these mRNAs after PDGF stimuli (Figure 5E).

These data demonstrate the importance of HuR in PDGF-mediated HSC proliferation and migration.

Role of PI3K and ERK in PDGF-induced HuR

The abundance and subcellular localisation of HuR are important determinants of its activity (19,20). PDGF treatment increased the expression of HuR mRNA (Figure 6A) and protein (Figure 6B and 6C) levels in CFSC-8B cells as well as its cytoplasmic localization (Figure 6D). Inhibition of both ERK and PI3K blocked PDGF-induced up-regulation of HuR mRNA and protein (Figure 6A, 6B and 6C), thus controlling HuR abundance. Recently, it was reported that *HuR* transcription is controlled by NFκB/p65 (21). We found that both ERK and PI3K induced nuclear translocation of the NFκB subunit p65 in response to PDGF (Supplementary Figure 3A and 3C), and inhibition of this translocation by BAY117802 treatment prevented PDGF-mediated up-regulation of HuR protein expression (Supplementary Figure 3B and 3D).

Conversely, we found that cytoplasmic localisation of HuR was mediated by the ERK pathway only, and not by the PI3K pathway, unlike above (Figure 6D and 6E, Supplementary Figure 3E). Post-translational modifications of HuR such as phosphorylation play an important role in its subcellular localisation (19,20). We performed mutagenesis of six serine (S) and two threonines (T) residues to the non-phosphorylable residue alanine (A) of HuR protein. Mutation of serine residue 100 and threonine residues 293 or 295 prevented the translocation to the cytosol of the mutant protein after PDGF treatment (Figure 6F and Supplementary Figure 3F), without affecting the nuclear levels (data non shown) suggesting that these phosphorylation sites are important for PDGF-induced HuR nucleo-cytoplasmic shuttling.

Role of p-LKB1 in PDGF induced HuR

Recent studies have shown that PDGF induces LKB1 (Ser428) phosphorylation, via ERK-induced activation, in a cell type dependent manner (22). Here, using the CFSC-8B cell line, we found that PDGF-induced LKB1 phosphorylation was blocked by the MEK inhibitor U0126 (Figure 6D and Supplementary Figure 3E). No regulation by the PI3-Kinase inhibitor LY294002 was observed (Figure 6E and Supplementary Figure 3E). *LKB1* silencing did not affect PDGF-induced ERK and AKT phosphorylation (Supplementary Figure 4A), showing that LKB1 is a downstream kinase of ERK. Importantly, *LKB1* knockdown (Supplementary Figure 4A) prevented HuR cytoplasmic localization (Figure 7A and Supplementary Figure 4B), and blocked PDGF-induced cyclin D1 protein expression (Supplementary Figure 4C and 4D), and *MMP9*, *actin*, *MCP-1*, *cyclin D1* and *cyclin B1* mRNA expression (Figure 7B). Finally, basal and PDGF-induced HSC migration (Figure 7C) and PDGF-induced proliferation (Figure 7D) were both reduced after *LKB1* silencing.

It is known that LKB1 phosphorylates and regulates AMPK, and recent studies have shown that activation of AMPK in HSC leads to reduction of induced proliferation and migration of HSC (23,24). Here, however, we show that in activated HSC (CFSC-8B), PDGF induced pLKB1 without affecting pAMPK levels (Supplementary Figure 5A), and that AMPK silencing did not affect PDGF-induced HuR cytosolic translocation (Supplementary Figure 5B). Altogether our results suggest that in activated HSC, AMPK does not mediate LKB1-induced HuR translocation in response to PDGF.

In primary HSC isolated from BDL mice, PDGF-induced HuR cytosolic localization was also accompanied by LKB1 phosphorylation (Supplementary Figure 3G) and LKB1 silencing (Supplementary Figure 6A) also reduced migration both basally and after PDGF treatment (Supplementary Figure 6B and 6C) and inhibited PDGF-induced proliferation (Supplementary Figure 6D). Finally, we found strong LKB1 phosphorylation in activated HSC (α-SMA$^+$ cells) from BDL mice and CCl_4-treated rats (Supplementary Figure 6E), and more importantly in human cirrhotic samples (Figure 7E). In summary, our data suggest that

LKB1 activation by ERK could be mediating PDGF-induced proliferation and migration in HSC by regulating, at least partly, cytoplasmic localisation of HuR and expression of its critical target genes.

Role of HuR in TGF-β induced profibrogenic response

TGF-β is another major mediator of liver fibrogenesis (25). *HuR* silencing in the CFSC-8B cell line markedly reduced up-regulation of *col1a1, α-SMA and TGF-β* mRNA after TGF-β treatment (Figure 8A). RIP-qPCR analysis showed that *α-SMA and TGF-β*, but not *col1a1*, were bound to HuR in TGF-β–stimulated cells (Figure 8A).

In HSC, TGF-β also plays a major role in inhibiting proliferation in HSC (26). TGF-β treatment decreased levels of the cell cycle activators *cyclin D1* and *B1*, whilst increasing levels of the cell cycle inhibitor *p21* (Supplementary Figure 7A and 7B). *HuR* knockdown abrogated the anti-proliferative effects of TGF-β in primary HSC from BDL mice (Supplementary Figure 7C) and in the CFSC-8B cell line (Figure 8B). This anti-proliferative effect of TGF-β was likely due to reduced *p21* levels (Figure 8C). RIP-qPCR showed that TGF-β treatment induced an increased binding of HuR to *p21*, whilst reducing the interaction of *cyclin D1* and *B1* mRNA with HuR (Figure 8C).

TGF-β treatment did not regulate HuR at mRNA and protein levels, unlike PDGF (Supplementary Figure 7D and 7E). However, TGF-β induced increased cytoplasmic localisation of HuR both in primary HSC (Supplementary Figure 3G) and in the CFSC-8B cell line (Figure 8D and Supplementary Figure 7F). This translocation is unlikely to be mediated by ERK, AKT or LKB1 since TGF-β did not activate any of these kinases (Figure 8E). However, TGF-β activated p38 MAPK (Figure 8E) and inhibition of this pathway prevented TGF-β-induced HuR translocation (Figure 8F). TGF-β did not affect phosphorylation at any of the 8 residues that we previously tested for PDGF-induced translocation (data not shown) suggesting that TGF-β and PDGF mediates HuR translocation by different post-translational modifications.

In summary, we found that the profibrogenic and anti-proliferative actions of TGF-β could be controlled by HuR-mediated regulation of critical genes.

DISCUSION

Liver fibrosis and cirrhosis result from the majority of chronic liver insults and represent a difficult clinical challenge. Recent studies have shown that HuR regulates Angiotensin II-induced kidney fibrosis (27) and ventricular remodeling after myocardial infarction (28). However, HuR functions during liver fibrosis development are unknown. Several studies have shown that HuR regulates expression of several mRNAs encoding pro-inflammatory cytokines (TNF-α, IL-6, TGF-β and INF-γ), proinflammatory mediators (iNOS) and chemoattractant factors (MCP-1) (29). Most of these factors are involved in the pathogenesis of liver fibrosis (4). Here, we show that *HuR* silencing in a cholestactic liver injury model (BDL) reduces expression of several of these genes leading to decreased liver damage, oxidative stress, inflammation, macrophage infiltration and liver fibrosis development. This suggests that HuR silencing would have a beneficial effect after cholestactic liver injury.

More importantly, our study also shows that HuR regulates HSC activation, which likely results in the reduced fibrosis observed *in vivo* after HuR silencing. HSC activation is highly regulated with hundreds of genes up- and down-regulated (5). Modulation of mRNA stability and translation rates plays an important role in regulation of gene expression during liver fibrosis development and hepatic stellate activation (1). Here, we show that HSC activation *in vitro* and *in vivo* after BDL is accompanied by an increase in HuR. *HuR*

silencing significantly reduces expression of HSC activation markers. Importantly, we observed that HuR mediates the response of two of the principal mediators of HSC activation, PDGF and TGF-β (30,31). These data, together with the finding that HSC from human samples of hepatic cirrhosis expressed HuR, suggest that HuR has a significant role in fibrosis development after liver injury by controlling HSC activation itself in addition to liver damage and inflammation.

HuR regulates PDGF-induced proliferation and migration, controlling the expression of several genes involved in these processes. PDGF binding to its receptor leads to the sequential activation of Raf-1, MEK and ERK-1 and -2. ERK signalling is involved in PDGF-stimulated mitogenesis, migration and chemotaxis. PI3K also mediates PDGF-induced proliferation, migration and chemotaxis, at least in part via ERK-independent pathways (30). Here, we demonstrated that ERK1/2, but not PI3K, regulates cytoplasmic translocation of HuR. PDGF also induces LKB1 (Ser428) phosphorylation via ERK activation (22). LKB1 has been classically described as a tumor suppressor (32) but seems to have the opposite role in liver, controlling HuR nucleo-cytoplasmic shuttling and proliferation in HGF-stimulated hepatocytes and during apoptosis in hepatoma cell lines (8,9). Here, we also identified LKB1 as a downstream target of ERK1/2 in PDGF-stimulated HSC, and silencing *LKB1* significantly reduced PDGF-induced migration and proliferation. These functions of LKB1 are possibly mediated by HuR activity, since LKB1 regulates nucleo-cytoplasmic shuttling of HuR and both regulate expression of a common set of mRNAs. It is known that LKB1 phosphorylates and regulates AMPK, however we observed that PDGF-induced HuR cytosolic localization was independent of AMPK activity. This observation is in agreement with previous work describing that AMPK exerts anti-proliferative properties in HSC (23,24), and with studies in melanoma cells, which show that LKB1 can be active without affecting AMPK activity (22). Previous studies have shown that PI3K and ERK are activated in HSC *in vivo* following liver injury (33,34). Here we found that similarly, LKB1 (Ser428) phosphorylation, is also expressed *in vivo* in activated HSC in two animal models of hepatic fibrosis (BDL and CCl_4) and importantly in human cirrhotic patients.

TGF-β1 is another major mediator of liver fibrogenesis (35). We found that TGF-β1 treatment increased binding of HuR to several target mRNAs such as *α-SMA* and *TGF-β*, and *HuR* silencing significantly reduced their expression. Increasing evidence supports a mechanism by which autocrine production of TGF-β is required to maintain the pathogenic myofibroblast phenotype in several cell types (36). We found that *col1a1* was significantly reduced after *HuR* silencing likely due to reduced TGF-β autocrine secretion, rather than by regulation of its stability and translation since we did not find increased binding of *col1a1* to HuR. TGF-β1 is also an important negative regulator of proliferation in activated HSC (25). Our results showed that TGF-β increased the stabilization or translation of *p21* mRNA increasing its binding to HuR. Conversely, we observed a markedly reduced association between HuR and *cyclin D1* and *cyclin B1* mRNAs in response to TGF-β. The TGF-β-induced decrease in proliferation was abrogated by *HuR* silencing, suggesting that HuR is an important mediator of the anti-proliferative effects of TGF-β.

This role of HuR in TGF-β-treated cells is in sharp contrast to its effects in PDGF-treated cells, where we showed that HuR positively regulated HSC proliferation. Whilst PDGF activates the ERK-LKB1 signalling pathway to promote HuR translocation, TGF-β induced HuR translocation via p38 MAPK activation. In addition, TGF-β does not phosphorylate the same residues of HuR protein that control its cytoplasmic translocation, induced by PDGF. Thus, it is possible that the specific post-translational modification of HuR induced by the two signals could determine its binding to different mRNA targets. Similarly, PDGF and TGF-β have contrasting roles in regulating the levels of HuR. PDGF, via ERK- and PI3K-

mediated activation of NFκB, is sufficient to increase *HuR* transcription. This is in agreement with other studies, which show that NFκB activity is regulated by cytokines in activated HSC (11), and that p65 binds to the *HuR* promoter in gastric tumour cells (21).

HuR has been implicated in several biological events such as carcinogenesis, cell proliferation, differentiation, and inflammation (29). However both low and high levels of HuR have been correlated with good prognosis in cancer, making careful designs of interventions to modulate HuR functions necessary. These generate the need to study the advantages or disadvantages of *HuR* silencing in different pathologies, as well as the identification of its specific mediators (29). Here, we have demonstrated that *HuR* silencing has pleiotropic and beneficial functions during cholestactic liver injury and HSC activation. Importantly, we find that HuR levels in human cirrhotic samples strongly correlate with the degree of HSC activation, suggesting that it could be a valuable therapeutic target for treatment of liver fibrosis, and possibly its progression to hepatocellular carcinoma in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AREs	AU-rich elements
CCl_4	carbon tetrachloride
col1a1	collagen type I α1
ECM	extracellular matrix proteins
ERK	Extracellular signal-regulated kinases
GFAP	Glial fibrillary acidic protein
HSC	hepatic stellate cells
HuR	human antigen R
MEK	MAPK/ERK kinases (MAP2Ks)
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NAFLD	non-alcoholic fatty liver disease
p38 MAPK	p38 mitogen-activated protein kinases
PI 3-kinases or PI3Ks	Phosphatidylinositol 3-kinases
PDGF	platelet-derived growth factor
Raf-1	RAF proto-oncogene serine/threonine-protein kinase

qPCR	real-time PCR
RIP-qPCR	RNA immunoprecipitation of ribonucleotide complexes coupled to qPCR
RBPs	RNA-binding proteins
STK11	Serine/threonine kinase 11
LKB1	or liver kinase B1
TGF-β	Transforming growth factor beta
HCV	viral hepatitis
α-SMA	α-smooth muscle actin

References

1. Friedman SL. Transcriptional regulation of stellate cell activation. J Gastroenterol Hepatol. 2006; 21 (Suppl 3):S79–83. [PubMed: 16958680]
2. Milani S, Herbst H, Schuppan D, Hahn EG, Stein H. In situ hybridization for procollagen types I, III and IV mRNA in normal and fibrotic rat liver: evidence for predominant expression in nonparenchymal liver cells. Hepatology. 1989; 10:84–92. [PubMed: 2737606]
3. Hendriks HF, Verhoofstad WA, Brouwer A, de Leeuw AM, Knook DL. Perisinusoidal fat-storing cells are the main vitamin A storage sites in rat liver. Exp Cell Res. 1985; 160:138–49. [PubMed: 4043241]
4. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005; 115:209–18. [PubMed: 15690074]
5. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, Schwabe RF. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. Gastroenterology. 2007; 132:1937–46. [PubMed: 17484886]
6. Hinman MN, Lou H. Diverse molecular functions of Hu proteins. Cell Mol Life Sci. 2008; 65:3168–81. [PubMed: 18581050]
7. Martinez-Chantar ML, Vazquez-Chantada M, Garnacho M, Latasa MU, Varela-Rey M, Dotor J, et al. S-adenosylmethionine regulates cytoplasmic HuR via AMP-activated kinase. Gastroenterology. 2006; 131:223–32. [PubMed: 16831604]
8. Vazquez-Chantada M, Fernandez-Ramos D, Embade N, Martinez-Lopez N, Varela-Rey M, Woodhoo A, et al. HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis. Gastroenterology. 2010 May; 138(5):1943–53. [PubMed: 20102719]
9. Martinez-Lopez N, Varela-Rey M, Fernandez-Ramos D, Woodhoo A, Vazquez-Chantada M, Embade N, et al. Activation of LKB1-Akt pathway independent of phosphoinositide 3-kinase plays a critical role in the proliferation of hepatocellular carcinoma from nonalcoholic steatohepatitis. Hepatology. 2010; 52:1621–31. [PubMed: 20815019]
10. Embade N, Fernandez-Ramos D, Varela-Rey M, Beraza N, Sini M, de Juan VG, et al. Mdm2 regulates HuR stability in human liver and colon cancer through neddylation. Hepatology. 2011
11. Hellerbrand C, Jobin C, Iimuro Y, Licato L, Sartor RB, Brenner DA. Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. Hepatology. 1998; 27:1285–95. [PubMed: 9581682]
12. Greenwel P, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl4-cirrhotic livers. Differences in the production of interleukin-6. Lab Invest. 1991; 65:644–53. [PubMed: 1753710]
13. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc. 2007; 2:329–33. [PubMed: 17406593]

14. Herrmann J, Gressner AM, Weiskirchen R. Immortal hepatic stellate cell lines: useful tools to study hepatic stellate cell biology and function? J Cell Mol Med. 2007; 11:704–22. [PubMed: 17760834]
15. Carotti S, Morini S, Corradini SG, Burza MA, Molinaro A, Carpino G, et al. Glial fibrillary acidic protein as an early marker of hepatic stellate cell activation in chronic and posttransplant recurrent hepatitis C. Liver Transpl. 2008; 14:806–14. [PubMed: 18508359]
16. Pinzani M, Gesualdo L, Sabbah GM, Abboud HE. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. J Clin Invest. 1989; 84:1786–93. [PubMed: 2592560]
17. Kenagy RD, Hart CE, Stetler-Stevenson WG, Clowes AW. Primate smooth muscle cell migration from aortic explants is mediated by endogenous platelet-derived growth factor and basic fibroblast growth factor acting through matrix metalloproteinases 2 and 9. Circulation. 1997; 96:3555–60. [PubMed: 9396455]
18. Kisseleva T, Brenner DA. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. J Gastroenterol Hepatol. 2007; 22 (Suppl 1):S73–8. [PubMed: 17567473]
19. Doller A, Pfeilschifter J, Eberhardt W. Signalling pathways regulating nucleo-cytoplasmic shuttling of the mRNA-binding protein HuR. Cell Signal. 2008; 20:2165–73. [PubMed: 18585896]
20. Doller A, Schlepckow K, Schwalbe H, Pfeilschifter J, Eberhardt W. Tandem phosphorylation of serines 221 and 318 by protein kinase Cdelta coordinates mRNA binding and nucleocytoplasmic shuttling of HuR. Mol Cell Biol. 2010; 30:1397–410. [PubMed: 20086103]
21. Kang MJ, Ryu BK, Lee MG, Han J, Lee JH, Ha TK, et al. NF-kappaB activates transcription of the RNA-binding factor HuR, via PI3K-AKT signaling, to promote gastric tumorigenesis. Gastroenterology. 2008; 135:2030–42. 2042, e1–3. [PubMed: 18824170]
22. Esteve-Puig R, Canals F, Colome N, Merlino G, Recio JA. Uncoupling of the LKB1- AMPKalpha energy sensor pathway by growth factors and oncogenic BRAF. PLoS One. 2009; 4:e4771. [PubMed: 19274086]
23. Caligiuri A, Bertolani C, Tosti C, et al. Adenosine Monophosphate–Activated Protein Kinase Modulates the Activated Phenotype of Hepatic Stellate Cells. Hepatol. 2008; 47:669–676.
24. Adachi M, Brenner DA. High Molecular Weight Adiponectin Inhibits Proliferation of Hepatic Stellate Cells via Activation of Adenosine Monophosphate–Activated Protein Kinasa. Hepatol. 2008; 47:677–685.
25. Breitkopf K, Haas S, Wiercinska E, Singer MV, Dooley S. Anti-TGF-beta strategies for the treatment of chronic liver disease. Alcohol Clin Exp Res. 2005; 29:121S–131S. [PubMed: 16344596]
26. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci. 2002; 7:d793–807. [PubMed: 11897555]
27. Doller A, Gauer S, Sobkowiak E, Geiger H, Pfeilschifter J, Eberhardt W. Angiotensin II induces renal plasminogen activator inhibitor-1 and cyclooxygenase-2 expression post-transcriptionally via activation of the mRNA-stabilizing factor human-antigen R. Am J Pathol. 2009 Apr; 174(4):1252–63. [PubMed: 19246637]
28. Krishnamurthy P, Lambers E, Verma S, Thorne T, Qin G, Losordo DW, Kishore R. Myocardial knockdown of mRNA-stabilizing protein HuR attenuates post-MI inflammatory response and left ventricular dysfunction in IL-10-null mice. FASEB J. 2010 Jul; 24(7):2484–94. [PubMed: 20219984]
29. Srikantan S, Gorospe M. HuR function in disease. Front Biosci. 2012 Jan 1.17:189–205. [PubMed: 22201738]
30. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. Cytokine Growth Factor Rev. 2004; 15:255–73. [PubMed: 15207816]
31. Borkham-Kamphorst E, Herrmann J, Stoll D, et al. Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. Lab Invest. 2004; 84:766–77. [PubMed: 15077122]

32. Bardeesy N, Sinha M, Hezel AF, Signoretti S, Hathaway NA, Sharpless NE, et al. Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. Nature. 2002; 419:162–7. [PubMed: 12226664]

33. Marra F, Arrighi MC, Fazi M, et al. Extracellular signal-regulated kinase activation differentially regulates platelet-derived growth factor's actions in hepatic stellate cells, and is induced by in vivo liver injury in the rat. Hepatology. 1999; 30:951–8. [PubMed: 10498647]

34. Marra F, Gentilini A, Pinzani M, Choudhury GG, Parola M, Herbst H, et al. Phosphatidylinositol 3-kinase is required for platelet-derived growth factor's actions on hepatic stellate cells. Gastroenterology. 1997; 112:1297–306. [PubMed: 9098016]

35. Yata Y, Gotwals P, Koteliansky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for antifibrotic therapy. Hepatology. 2002; 35:1022–30. [PubMed: 11981752]

36. Dabiri G, Tumbarello DA, Turner CE, Van de Water L. Hic-5 promotes the hypertrophic scar myofibroblast phenotype by regulating the TGF-beta1 autocrine loop. J Invest Dermatol. 2008 Oct; 128(10):2518–25. [PubMed: 18401422]

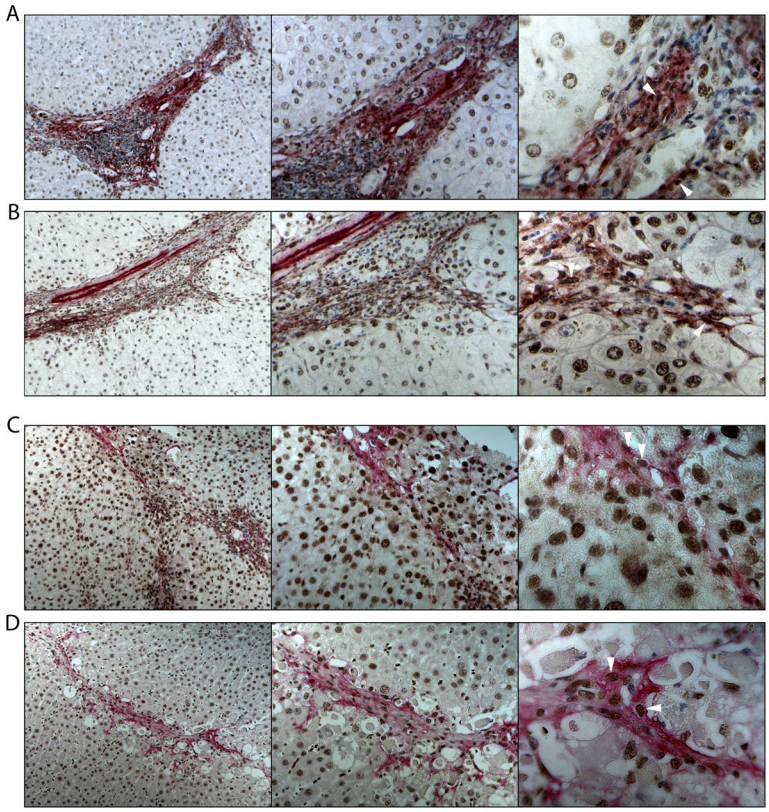

Figure 1. HuR in human cirrhosis, BDL-mice and CCl_4-treated rats

IHC showing expression of HuR (DAB^+ cells, brown, arrowheads) in activated HSC (α-SMA^+ cells, alkaline phosphatase, red) in liver sections from patients with (**A**) alcoholic and (**B**) VHC cirrhosis. Representative pictures of 9 alcoholic and 7 VHC samples are shown. (**C**), BDL-mice, 7 days after surgery (n=5) (**D**) and CCl_4-treated rats (n=5).

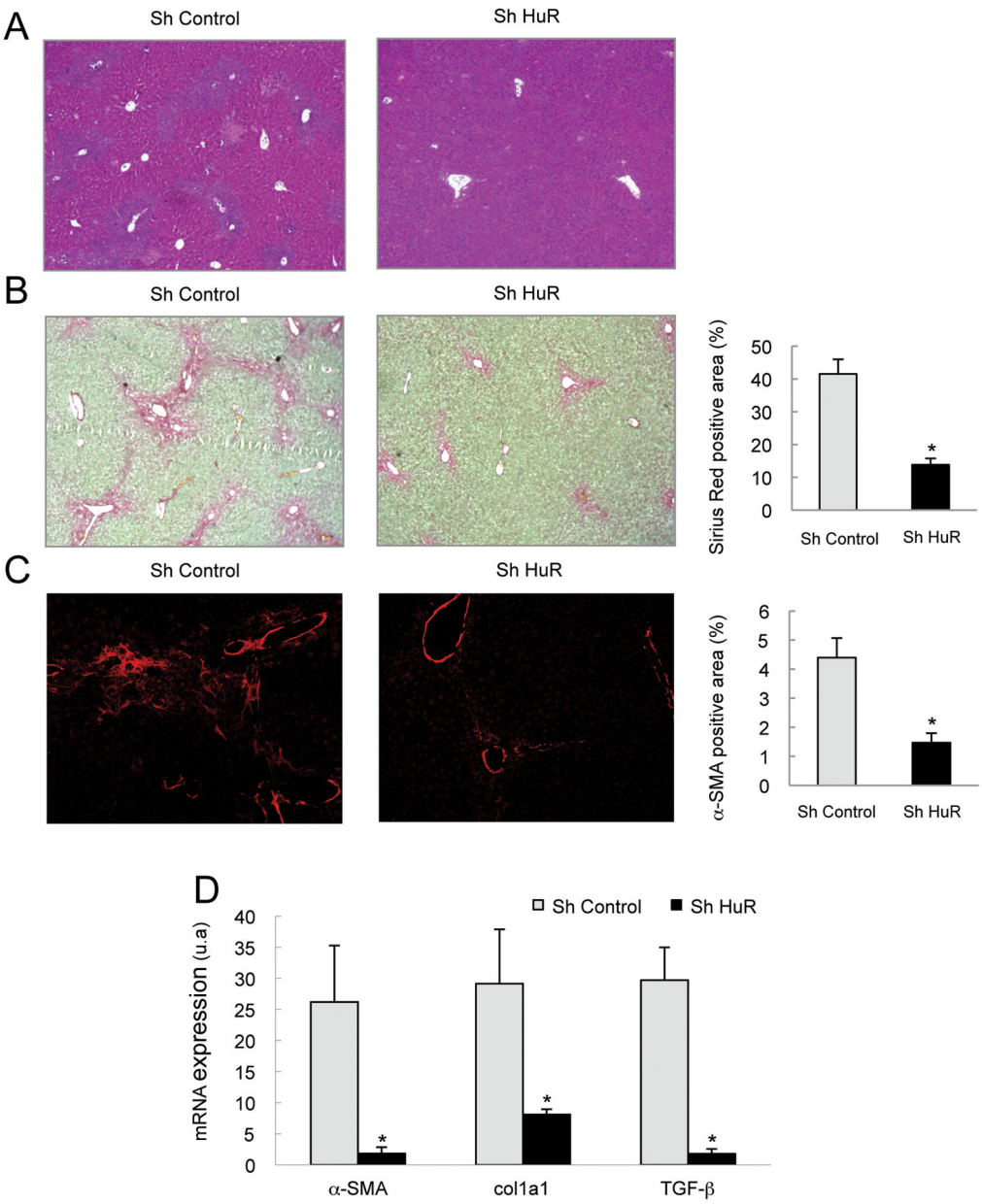

Figure 2. HuR silencing attenuated liver damage and hepatic fibrosis in BDL-mice

Livers from BDL mice, injected with Sh control or Sh HuR plasmids, were extracted and the following analyses performed: (**A**) Hematoxilin/eosin staining, (**B**) Sirius red staining and quantification of positive areas (**C**) α-SMA immunostaining and quantification of positive areas and (**D**) qPCR of fibrogenic genes. (*p<0.05).

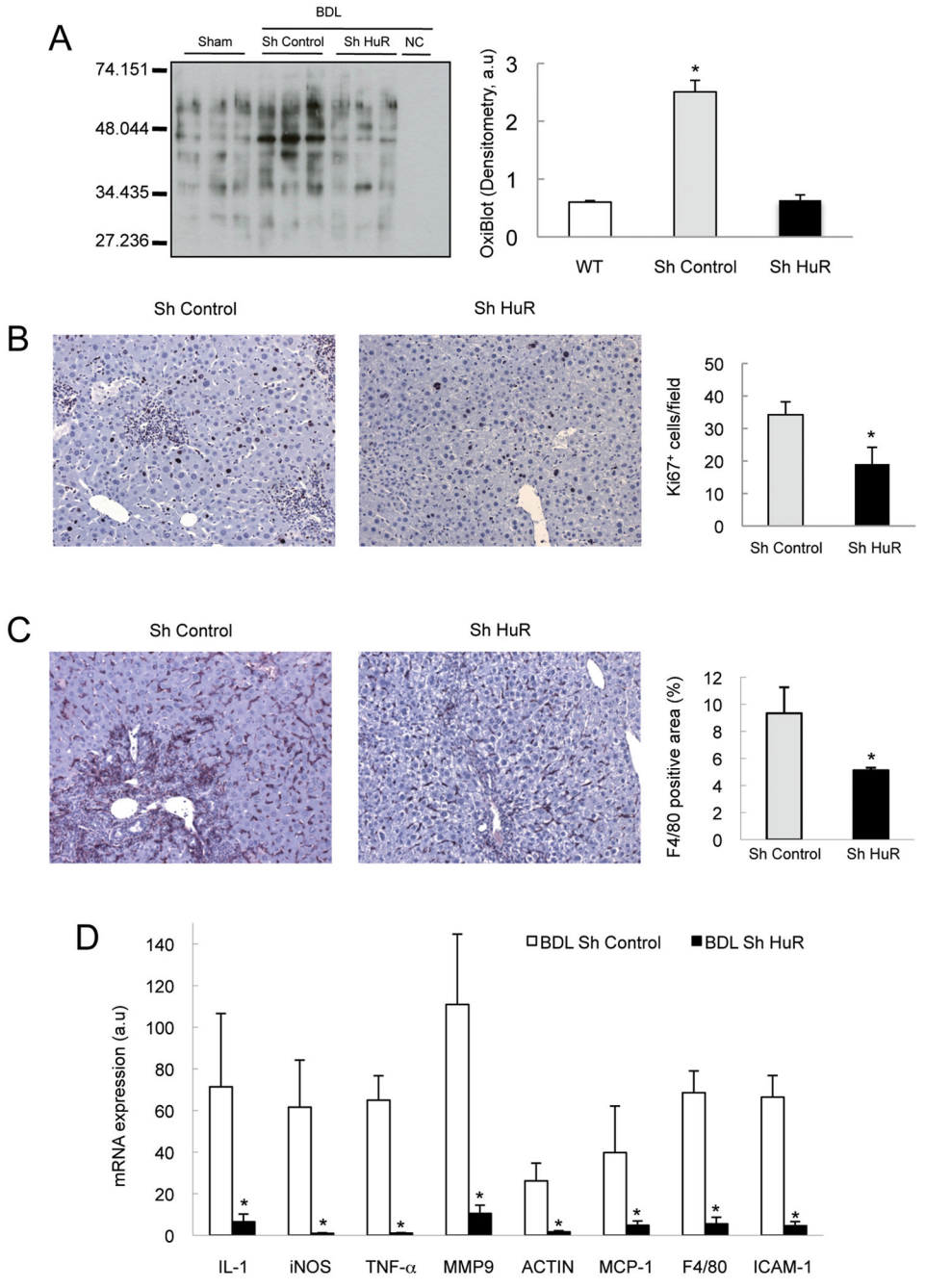

Figure 3. HuR silencing attenuated oxidative stress and hepatic inflammation in BDL- mice

(**A**) Quantification of protein oxidation by WB, (**B**) analysis of proliferation by Ki67 IHC and quantification of positive cells, (**C**) analysis of macrophage infiltration determined by F4/80 IHC and quantification of positive areas (**D**) qPCR of inflammatory genes. (*p<0.05).

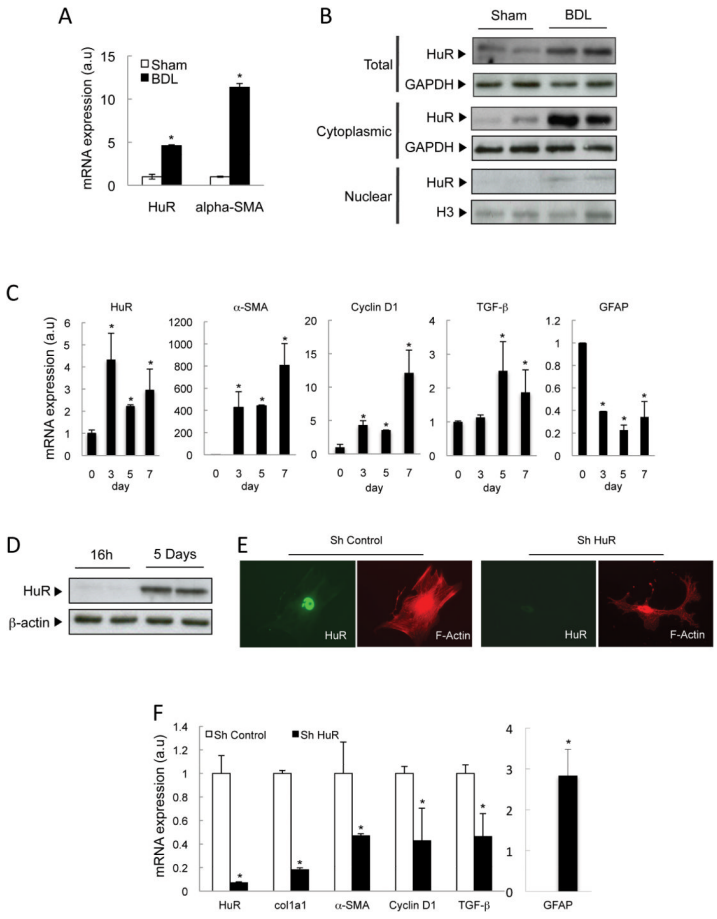

Figure 4. HuR is expressed in activated HSC

(**A**) qPCR and (**B**) WB analysis in HSC from sham and BDL mice 9 days after surgery. (**C**) qPCR and (**D**) WB analysis in culture-activated HSC. (**E**) HuR and F-actin staining and (**F**) qPCR analysis of selected mRNAs after *HuR* silencing in culture-activated HSC (7 days). *p<0.05

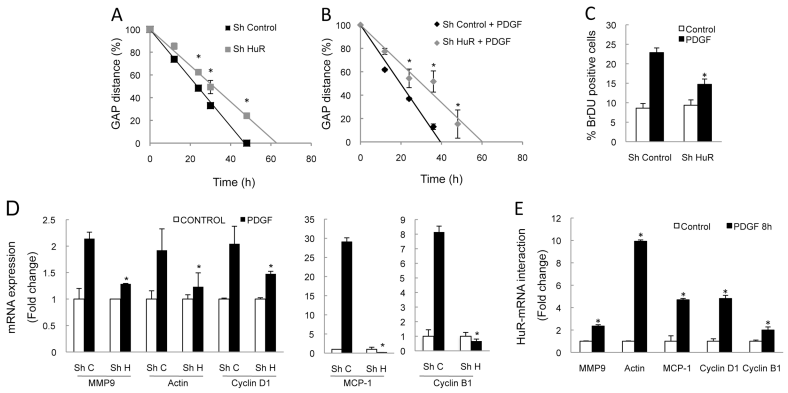

Figure 5. PDGF-induced responses are mediated by HuR

HuR silencing in CFSC-8B cells reduced PDGF-mediated (**A,B**) migration, as shown by slower rate of the gap closure at different time-points, (**C**) proliferation, as shown by percentage of $BrDU^+$ cells, 24h after treatment, (**D**) up-regulation of specific mRNAs (Sh C = Sh Control, Sh H = Sh HuR). (**E**) RIP-qPCR showing binding of selected genes with HuR after PDGF stimulation. *$p<0.05$.

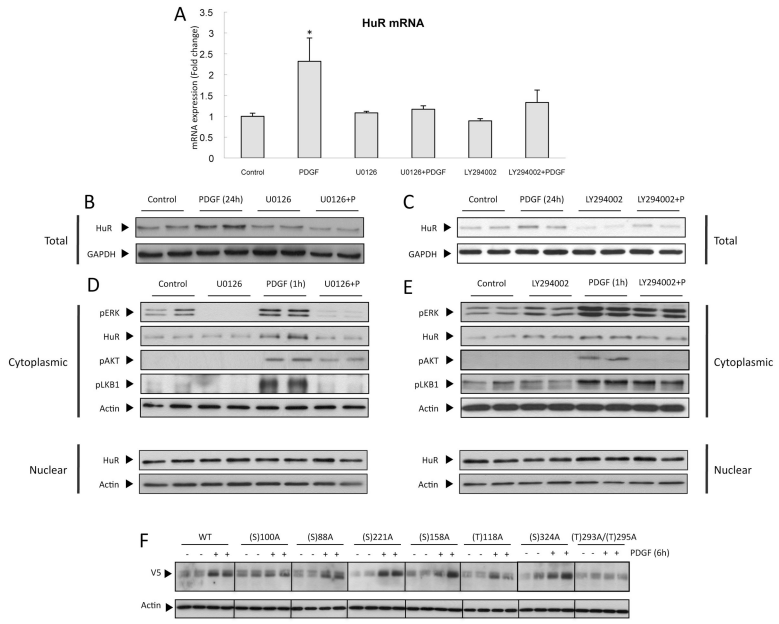

Figure 6. Role of PI3K and ERK in PDGF-induced HuR

(**A**) qPCR and (**B,C**) WB analysis of total protein extracts showing that ERK inhibition using UO126 and PI3K inhibition using LY-294002, prevents PDGF-induced increase in HuR expression. (**D,E**) WB showing that ERK inhibition, but not PI3K inhibition, prevents PDGF-induced HuR cytoplasmic translocation. (**F**) WB showing V5 expression in cytoplasmic extracts of PDGF-treated CFSC-8B cells, transfected with plasmids containing wild-type or mutant HuR. *p<0.05.

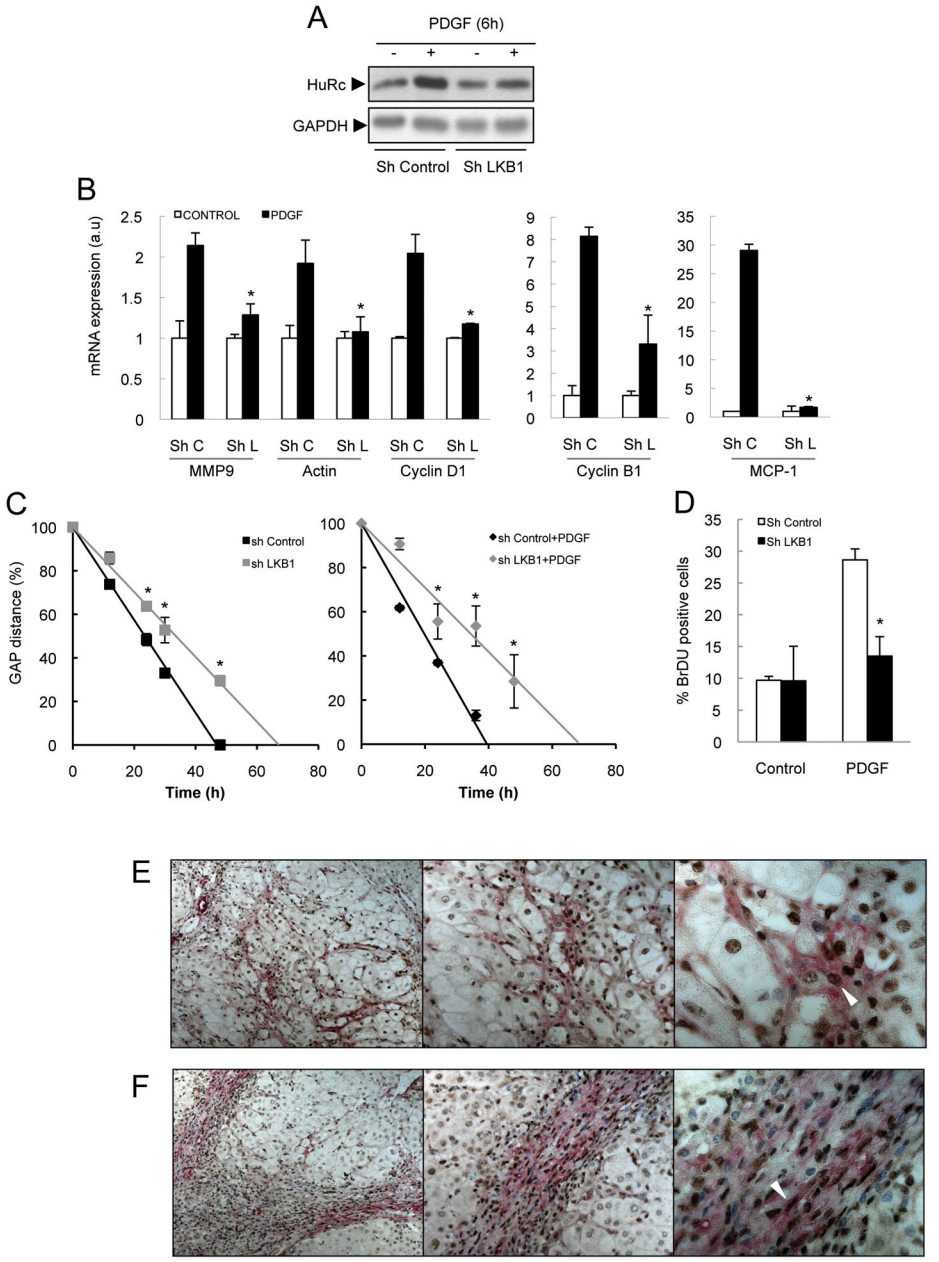

Figure 7. LKB1 is a downstream target of ERK in PDGF-treated cells

LKB1 silencing in CFSC-8B cells reduces PDGF-induced (**A**) HuR translocation, (**B**) up-regulation of specific mRNAs (**C**) migration using the scratch assay, and (**D**) proliferation, as measured by BrDU incorporation, IHC showing expression of p-LKB1 (DAB^+ cells, brown, arrowheads) in activated HSC (α-SMA^+ cells, alkaline phosphatase, red) in liver sections from patients with alcoholic (**E**) and VHC cirrhosis (**F**). Representative pictures of 9 alcoholic and 7 VHC samples are shown. *$p<0.05$.

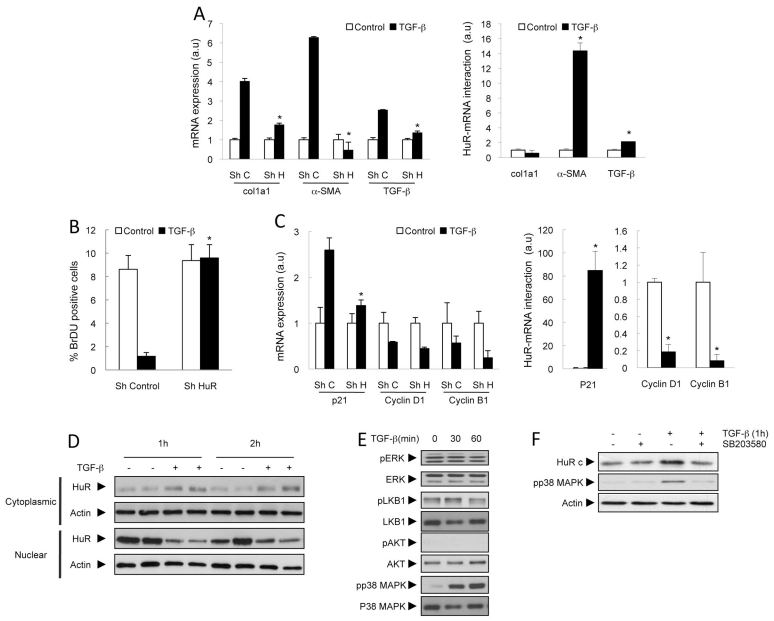

Figure 8. TGF-β-induced fibrogenesis is mediated by HuR

(**A**) qPCR showing reduced expression of profibrogenic mRNAs after *HuR* silencing and RIP-qPCR showing binding of selected genes with HuR after TGF-β stimulation in CFSC-8B cells. (**B**) *HuR* silencing prevents the anti-proliferative effects of TGF-β, as measured by BrDU incorporation. (**C**) qPCR showing reduced *p21* expression after *HuR* silencing, and RIP-qPCR showing its binding to HuR after TGF-β stimulation. WB showing TGF-β-induced (**D**) HuR translocation and (**E**) p38 MAPK activation. (**F**) WB showing that p38 MAPK inhibition prevents TGF-β-induced HuR translocation. *p<0.05.