Amphiregulin activates human hepatic stellate cells and is upregulated in non-alcoholic steatohepatitis

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Amphiregulin (AR) involvement in liver fibrogenesis and hepatic stellate cells (HSC) regulation is under study. Non-alcoholic fatty liver disease (NAFLD) and its more severe form non-alcoholic steatohepatitis (NASH) may progress to cirrhosis and hepatocellular cancer (HCC). Our aim was to investigate ex vivo the effect of AR on human primary HSC (hHSC) and verify in vivo the relevance of AR in NAFLD fibrogenesis. hHSC isolated from healthy liver segments were analyzed for expression of AR and its activator, TNF-α converting enzyme (TACE). AR induction of hHSC proliferation and matrix production was estimated in the presence of antagonists. AR involvement in fibrogenesis was also assessed in a mouse model of NASH and in humans with NASH. hHSC time dependently expressed AR and TACE. AR increased hHSC proliferation through several mitogenic signaling pathways such as EGFR, PI3K and p38. AR also induced marked upregulation of hHSC fibrogenic markers and reduced hHSC death. AR expression was enhanced in the HSC of a murine model of NASH and of severe human NASH. In conclusion, AR induces hHSC fibrogenic activity via multiple mitogenic signaling pathways, and is upregulated in murine and human NASH, suggesting that AR antagonists may be clinically useful anti-fibrotics in NAFLD.

Non-alcoholic fatty liver disease (NAFLD), the commonest cause of chronic liver disease in affluent countries, is a spectrum of liver diseases ranging from hepatic steatosis (simple intrahepatic accumulation of lipid droplets) through steatosis with inflammation and fibrosis (non-alcoholic steatohepatitis, NASH) to cirrhosis and hepatocellular cancer (HCC)¹,². Hepatic stellate cells (HSC), found in the space of Disse (perisinusoidal space between sinusoids and hepatocytes), are the predominant fibrogenic cells in the liver, are activated by liver injury to transdifferentiate from a quiescent state to proliferative matrix producing myofibroblasts³. Excessive matrix production may result in cirrhosis with the possibility of HCC onset, although HCC may also develop in a minority of cases in a background of NAFLD/NASH without cirrhosis⁴,⁵.

The epidermal growth factor receptor (EGFR) ligand amphiregulin (AR) plays a central role in branching morphogenesis in organs and is expressed both in healthy and in cancer tissues. It is an autocrine growth factor as well as a mitogen for fibroblasts and regulatory T-cells⁶. Various studies have highlighted the functional role of AR in multiple aspects of tumorigenesis, including transducing growth signals, modulating tissue invasion and metastasis, angiogenesis, and resistance to apoptosis⁷. AR participates in the modulation of the hepatic acute-phase reaction that occurs during inflammation and liver regeneration, and is important for allowing normal hepatocellular proliferation and the restoration of homeostasis⁸. AR has also been shown to be a trigger of liver regeneration after partial heptectomy⁹,¹⁰.

The association of AR expression with liver disease has been demonstrated in different animal models of liver damage and in human samples. AR is induced in the fibrotic liver of mice chronically treated with CCl4 and in models of acute liver damage induced by CCI4, activation of Fas and LPS administration. AR induction was also demonstrated in the liver of cirrhotic patients and rats, as well as in human HCC, suggesting that AR is also...
implicated in hepatic carcinogenesis\textsuperscript{9,11–14}. Moreover, it has been suggested to protect from immune mediated liver injury\textsuperscript{10}. Interestingly, AR has been suggested to be pro-fibrogenic since mice lacking AR develop less hepatic fibrosis after carbon tetrachloride challenge compared to wild type littermates\textsuperscript{15}. However, our understanding of AR function in NASH is far from being complete and, importantly, whether and how AR regulates human (h) HSCs, the major cellular determinant of hepatic fibrosis in NASH, and plays a role in demographically important specific human liver diseases such as NAFLD/NASH is under study.

In this study, our aims were primarily to study the \textit{ex vivo} role of AR signaling mechanisms in the fibrogenic physiology of primary hHSC, and secondarily to determine the \textit{in vivo} significance and disease relevance of these findings by assaying AR in the HSC compartment of a mouse model of NASH and of human livers with NAFLD/NASH at different stages of severity. We show for the first time that AR is expressed in hHSC; further and importantly, we describe AR up-regulation in the HSC of mice and patients with NASH.

**Results**

**AR expression in hHSC increases in early passages and parallels ASMA expression and TACE activation.** AR has been detected in the whole liver but has not previously been reported specifically in HSC. To determine if AR is expressed by freshly isolated (quiescent) HSC and activated HSC we analysed hHSC for AR mRNA and protein expression, as shown in Figure 1: on days 0 and 2 when quiescent, and day 4 and 10 when activated. hHSC expressed AR mRNA and protein, with expression levels at day 4 greater than that at day 10, at which time AR expression became lower at the mRNA level and returned to basal as for protein levels (Figure 1A and 1C).

We then analysed the mRNA and protein expression of the AR regulating factor tumour necrosis factor (TNF)\textsuperscript{α} converting enzyme (TACE), which is known to solubilise AR and promote its interaction with its cognate receptors, including EGFR\textsuperscript{16}. We found that TACE was expressed by hHSC and its mRNA, protein and activity levels reach all peak values preceding the one of AR mRNA and protein, with expression levels at day 4 greater than that at day 10, at which time AR expression became lower at the mRNA level and returned to basal as for protein levels (Figure 1A and 1C). We then analysed the mRNA and protein expression of the AR regulating factor tumour necrosis factor (TNF)\textsuperscript{α} converting enzyme (TACE), which is known to solubilise AR and promote its interaction with its cognate receptors, including EGFR\textsuperscript{16}. We found that TACE was expressed by hHSC and its mRNA, protein and activity levels reach all peak values preceding the one of AR mRNA and protein (Figure 1B, 1C and 1D), suggesting that in hHSC TACE is likely to be required for activation of AR. These observations were corroborated by immunocytochemistry data showing that AR expression was confirmed at peak levels at day 2–4, with expression at day 10 lower than that at day 4 (Figure 2). Parallel analysis of the expression of \textit{α}-smooth muscle actin (ASMA), a marker of HSC activation\textsuperscript{17}, showed a progressive increase from day 0, clearly indicating that AR expression

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**Figure 1 | AR mRNA expression in hHSC.** (A) Cultures of hHSC show significantly higher expression (* \textit{p} < 0.05) of AR by day 4 compared to day 0. Expression is effectively reduced by day 10 (** \textit{p} < 0.01). (B) AR mRNA expression is preceded by the up-regulation of the TACE gene, required for AR activity. TACE expression is highest at day 2 (* \textit{p} < 0.05) and is reduced by day 4 (** \textit{p} < 0.01). (C) Western blot analysis for AR and TACE protein levels in hHSC at day 0, 2, 4 and 10 (left panel); images were obtained from separate gels, run under the same experimental conditions, blotted on separate PVDF membranes and processed for detection with the respective antibodies. Densitometric quantification normalized to β-actin levels (right panel). Images are representative of three independent experiments (* \textit{p} < 0.05; ** \textit{p} < 0.01). (D) TACE activities in hHSC at day 0, 2, 4 and 10 were measured using SensoLyte 520 TACE Activity Assay kit. N = 3, \textasteriskcentered \textit{p} < 0.01.
preceded that of ASMA which increased progressively from day 0, indicating that AR expression preceded that of ASMA (Figure 2). Our findings also clearly show that hHSC were AR producing cells because of the observed co-localisation of ASMA and AR (Figure 2).

**Exogenous AR stimulates proliferation of hHSC.** AR has been suggested to be pro-fibrogenic because mice lacking AR develop less hepatic fibrosis after carbon tetrachloride challenge compared to controls15, but whether this effect of AR is directly dependent on hepatic fibrosis after carbon tetrachloride challenge compared to controls15, but whether this effect of AR is directly dependent on HSC is not known. Here, we found that AR significantly enhanced hHSC proliferation with a maximal effect at 100 pg/ml (Figure 3A). To further explore these proliferation effects, equal numbers of hHSC were seeded into 96-well plates and grown under serum free (SF) conditions, 10% fetal bovine serum (FBS) or with AR (10 pg/mL) plus 10% FBS. As expected, an increased number of cell nuclei was visible between FBS and FBS plus AR (10 pg/mL) conditions compared to SF (Figure 3B). In addition, annexin-PI staining by flow cytometry demonstrated a significantly higher number of live cells in the AR + FBS condition compared to FBS alone (Figure 3C).

**AR-induced hHSC proliferation requires EGFR and the PI3K, MEK, p38 MAPK, PKC survival pathways.** AR is thought to act through the EGFR in non-liver cells19, but whether its action in hHSC is similarly mediated via EGFR is not known. We show here that treatment with EGFR antagonist PD153035 markedly inhibits AR-induced hHSC proliferation (Figure 4A), pointing for the first time to a role for EGFR in AR-induced hHSC proliferation. Previous studies have shown that AR influenced the phosphorylation of one or more growth signals and MAP kinases involved in the proliferation of vascular smooth muscle cells20. Consistently, here we find that culturing hHSC with AR (10 pg/mL) triggers activation of EGFR, AKT, ERK1/2 and p38 MAP kinases, as evidenced by increased phosphorylation levels on key residues [pEGFR(Y1068), pAKT(Ser473), pERK1/2(Thr202/Tyr204) and p-p38(Thr180/Tyr182)] (Figure 4B). To confirm that several mitogenic pathways, other than EGFR signalling, are involved in AR-induced hHSC proliferation, we cultured hHSC with AR (10 pg/mL) in the presence of various specific inhibitors20: pertussis toxin (G-protein inhibitor, 100 nM), wortmannin (PI3K inhibitor, 100 nM), PD98059 (MEK inhibitor, 100 nM), SB202190 (p38 MAPK inhibitor, 10 μM), and Ro-32-0432 (PKC inhibitor, 1 μM). Our data show that blockage of intra-cellular mitogenic kinases invariably dampen AR-induced hHSC proliferation (Figure 4C). The most potent reductions were observed after treatment with wortmannin and SB202190, indicating that the PI3K and p38 MAPK pathways are of particular importance in AR-induced hHSC proliferation (Figure 4C).

**AR induces fibrogenic markers in hHSC and is upregulated in a murine model of NASH.** Besides proliferation, a characteristic of activated hHSC is the production of matrix proteins such as collagen, and over-exuberant matrix over-production results in fibrosis and cirrhosis21. The effect of AR on the induction of collagen in hHSC is not known. We analysed the induction of hHSC collagen 1a2 gene expression in the presence of AR. As shown in Figure 5A, AR increased dose-dependently hHSC collagen 1a2 gene expression of 2 to 3 fold in primary hHSC. Similar findings were obtained for ASMA mRNA expression (data not shown). The effect is comparable to the effect of the pro-fibrogenic cytokine TGF-β used here as a positive control22 (Figure 5A).

To confirm the in vivo relevance of these cellular studies, C57/B6 mice were fed for 4 weeks either normal chow or a methionine-cysteine-deficient (MCD) diet, a well-known regimen inducing NASH23,24. Mice fed a MCD diet had a more abundant hepatic mRNA expression of AR of about 5 times compared to controls (Figure 5B). To discern if the increase in hepatic AR was due to an increase of its expression in hepatocytes (the predominant hepatic cell type) or in HSC, we isolated hepatocytes and HSC (mouse HSC, mHSC) from the livers of control C57/B6 mice and mice fed a MCD diet, and measured AR protein expression by immunoblotting (Figure 5C): while AR protein expression was detectable in hepatocytes at similar levels in control and MCD fed mice, it was significantly higher in mHSC and enhanced in mHSC isolated from the NASH model (Figure 5C), confirming mRNA data and highlighting a predominant role of mHSC in NASH-dependent increase in AR expression.

**AR is increased in human patients scored for NASH.** To study in humans the in vivo relevance of these ex vivo and animal studies, we analyzed specimens obtained from healthy liver segments of patients undergoing resection of metastases and compared them to samples from patients with histologically proven NASH scored for fibrosis according to both Brunt and NAFLD Activity Score (NAS) of fibrosis severity25,26. Semi-quantitative RT-PCR analysis of liver RNA from...
controls and from patients with NASH induced cirrhosis demonstrated a marked upregulation of AR in human livers with NASH compared with controls, where it was only minimally expressed (Figure 6A). Quantification of AR expression in these samples confirmed that AR expression was approximately 6-fold greater in NASH livers compared to controls (Figure 6B).

These results were corroborated by immunohistochemical analyses of liver biopsies from patients with increased fibrosis scores (F1, F2, F3 and F4), as visualized by Masson trichrome stainings (Figure 7A). Immunopositivity for AR and for fibrogenic marker ASMA in hHSC was present and abundant only in F3 and F4, whereas virtually no positive hHSC were detected in F1 and F2 (Figure 7B and 7C).

**Discussion**

HSCs have previously been identified as the primary subtype responsible for remodeling liver tissue following injury. However, the factors that stimulate their proliferation are not fully understood. AR, a protein member of the EGFR-ligand family, is shown here to act both as an hHSC mitogen in primary culture and also to influence the production of hHSC matrix proteins (Figure 8). AR was also shown to be up-regulated in a murine model of NASH and in patients with liver scored for advanced levels of fibrosis and cirrhosis, suggesting that AR may be an important factor in a spectrum of liver diseases ranging from NAFLD through NASH to cirrhosis. This is fully consistent with the association of AR expression and liver disease that has been previously shown in different animal models of acute liver damage and in human samples with cirrhosis and HCC. AR has been widely described as a protein capable to induce mitosis. A link to human diseases was initially reported when AR was found to be increased in patients with lung fibrosis. The role of AR in the liver is under investigation but has been reported to assist in the recovery of hepatic tissues following injury. This observation is interesting in respect to the liver, which defaults to hepatic fibrogenesis as a means of...
regenerating injured tissue. Therefore, the question is whether AR is expressed in the restorative HSCs and AR level change upon HSCs activation is relevant.

AR is expressed as a 252 amino acid glycoprotein that exists stably in an uncleaved pro-form. AR activation has been shown to require cleavage via the matrix-metalloproteinase TACE (also known as ADAM17), allowing “shedding” of the ligand from its plasma membrane anchoring and subsequent interaction with cognate receptors. TACE mRNA levels were observed to increase early in primary hHSC (day 2) and precede the expression of AR (day 4), suggesting its activation from the pro-form. The rise in AR expression mirrors an increase in ASMA during this period, indicating an association between hHSC activation and AR expression. The activation of AR might be expected to induce proliferation in HSC. AR is most commonly identified as a growth factor and acts as a mitogen in a varied range of tissues, including keratinocytes, renal cells, and mammary epithelial cells. Consistently, HSC of AR knock-out mice show an inability to proliferate at the same level as the ones of wild type mice. As expected, the exogenous addition of AR in the picogram range was sufficient to stimulate proliferation of hHSC. Furthermore, in addition to greater hHSC growth, live cell populations were increased while apoptosis levels did not change significantly. AR can act as a survival factor when expressed in liver. The survival pathway by which AR operates has only been partially described. AR has been previously shown to interact with EGFR in various cell types and EGFR expression may in fact constitute a requirement for liver recovery. Data are

![Graph showing proliferative response](imageURL)

**Figure 4** | Inhibition of AR-induced proliferative pathways in hHSC. (A) Proliferation was assessed via ELISA with and without EGFR receptor blocker PD153035 (1 μmol/L). AR induced hHSC proliferation (*p < 0.001) that was prevented by blocking the EGF receptor (# p < 0.05). (B) Western blot analysis for pEGFR(Y1068), EGFR, pAKT(Ser473), AKT, pERK1/2(Thr202/Tyr204), ERK1/2, p-p38(Thr180/Tyr182), p38 and β-actin was assayed in primary hHSC cultured with AR (10 pg/ml). Images are representative of three independent experiments. Images were obtained from separate gels, run under the same experimental conditions, blotted on separate PVDF membranes and processed for detection with the respective antibodies. Asterisks indicate increased phosphorylation levels in presence of AR. (C) Involvement of mitogenic intracellular pathways in AR-induced hHSC proliferation was assayed in primary hHSC cultured with AR and in the presence of specific inhibitors: pertussis toxin (G-protein inhibitor, 100 nM), wortmannin (PI3K inhibitor, 100 nM), PD98059 (MEK inhibitor, 100 nM), SB202190 (p38 MAPK inhibitor, 10 μM), and Ro-32-0432 (PKC inhibitor, 1 μM) (*p < 0.001).
emerging that AR is expressed at the onset of hepatocyte injury within the liver either by partial hepatectomy or exposure to carbon tetrachloride\(^{10}\). The protein is assumed to act through AKT and potentially through one or more mitogen-activated protein (MAP) kinase signaling pathways\(^{19}\). However, previous research has limited investigations to only a few potential pathways. In this study, numerous inhibitors have been employed to investigate a broad spectrum of survival pathways including \(\gamma\)-coupled proteins, MAP kinases, and mitogens. Each inhibitor was shown to decrease the ability of AR to induce a proliferative response compared to the addition of AR alone. The results agree with previous reports that AR-EGFR complexes are mediated through MAP kinase pathways, and our investigations reveal that specifically the p38 and PI3 kinase pathways were the most affected by inhibitor treatment.

Our work and others have suggested that AR is capable of inducing proliferation in multiple cell types. Additionally, our investigations revealed that collagen 1\(\alpha\), ASMA and positive control TGF-\(\beta\) mRNA are increased upon stimulation of AR in hHSCs (Figure 4 and data not shown). Collagen 1\(\alpha\) is important for recovery of damaged liver tissues, but its over-production is a clinical hallmark of fibrosis\(^{36,37}\). One question our investigation sought to answer was whether AR was expressed in a model of liver injury. To understand if AR expression is also associated with liver disease, its expression was investigated initially in mice fed a MCD diet, known to induce fat accumulation in the liver. The model, within its limitations, is able to provoke NASH. Mice fed a MCD diet in fact showed AR up-regulation associated with the presence of NASH specifically in HSC rather than in hepatocytes. These data were corroborated by the evaluation of biopsy specimens obtained from resected human livers scored for increased levels of fibrosis and associated cirrhosis according to both Brunt and NAFLD Activity Score (NAS) of fibrosis severity (scored from 1 to 4; 1 = centrilobular/perisinusoidal; 2 =

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Figure 5 | Effect of AR on collagen 1\(\alpha\)2 production. (A) AR is capable of inducing collagen production at both picogram and nanogram concentrations (*\(p < 0.05\)). The effect is similar to that of TGF-\(\beta\), inductor of collagen production, a major step in the pathogenesis of fibrosis. (B) Mice were fed either a normal chow diet or a methionine-choline deficient (MCD) diet modelling NASH. Mice on MCD diet expressed higher hepatic AR mRNA levels than mice fed a normal chow diet (*\(p < 0.001\)). (C) Western blot analysis for AR protein levels in hepatocytes and mHSC isolated from MCD mice (left panel). Images were obtained from separate gels, run under the same experimental conditions, blotted on separate PVDF membranes and processed for detection with the respective antibodies. Densitometric quantification normalized to \(\beta\)-actin levels (right panel). Images are representative of three independent experiments (* \(p < 0.05\)).
The mRNA expression of AR in F4 NASH patients was found to be at a level seven times higher respect to that of liver obtained from patients not exhibiting NASH. Consistently, the number of HSC immunopositive for AR in NASH patient liver histological sections was abundant and only detectable in NASH stages F3 and F4. It is known that AR expression is minimal in hepatocytes and other cell types under normal conditions. In agreement with this 1) AR mRNA was detectable but at very low levels in human liver without fibrosis while in NASH F4 livers AR gene expression was much higher; 2) at the protein levels, in NASH F1 and F2, HSC cells positive for AR where very rare or absent, as determined by immunohistochemistry. As livers with grade 3 and 4 scores are very fibrotic, the expression profiles make a strong case that AR is up-regulated in response to damage to the organ and may be important for wound healing. However, an over-expression may contribute to development of fibrosis and onset of cirrhosis.

Fibrosis and progression to cirrhosis have been identified as positive factors in the development of cancer, specifically HCC. Currently, why this relation exists is not known. However, proteins promoting HSC activation and proliferation may act as a contributor to the process. HSCs were recently shown to express WNT and Hedgehog proteins, both of which have been linked to signaling pathways involved in carcinogenesis. Furthermore, human AR is believed to be a target of WNT as it possesses a binding site for the protein in its promoter. Thus, mitogens expressed in hHSC such as AR may aid in cancer progression. In support of this, AR knock-out animals or knock-downs cell lines have shown a lesser incidence of HCC characteristics. A future approach would therefore be to determine whether manipulation of AR would be useful as an HCC preventative agent in the treatment of patients with cirrhosis.

In conclusion, AR is implicated in liver regeneration, NAFLD and its fibrogenic form NASH are HCC risk factors, and hepatic fibrogenesis involves activation of HSC. We show here that AR activates HSC function in mouse and human NASH. With relatively few investigations relating to AR in liver function, the reported results may be of value in therapeutic applications. We present for the first time that AR is expressed in hHSC. Our results have also shown that AR may activate and induce proliferation in hHSC and enhance live cell populations in primary culture. In keeping with what is known about stellate cells in liver, their increased proliferation is thought to be aided by AR during liver wound healing, likely producing a...
protective layer of extra-cellular matrix (ECM) that supports repopulating hepatocytes. The data compiled here supports the hypothesis, as collagen and ASMA production is concurrent with AR-induced proliferation of hHSC. The observation is complemented in both a mouse model of NASH and patient biopsies with NASH. The human data lends added importance to this study as we suggest that AR expression may be a component of the liver’s survival response to NAFLD. However, overproduction of AR may lead to increased

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**Figure 7** | AR and ASMA expression in liver sections in fibrotic patients with increasing NASH severity (F1 to F4), according to both Brunt and NAS Scores; (n = 10). (A) Representative pictures of Masson trichrome staining for collagen (green/blue) in samples with fibrosis F1 to F4. Bar: 100 μm. Magnification 400×. (B) Representative pictures of AR and ASMA immunostainings. Bar: 100 μm. Arrows indicate positive cells. Magnification 400×; highlighted insets were obtained with a magnification 1000×. (C) The quantification values of AR and ASMA immunopositive cells refer to the average number of positive cells for High Power Fields (HFP, 400×). Three independent observers examined the specimens. The means of triplicate counts were used for statistical analyses.

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**Figure 8** | Scheme illustrating the signaling events induced by amphiregulin and influencing hepatic stellate cell dynamics and fate. Continuous lines ending with arrows render positive interactions, while continuous line ending with sphere renders negative interaction. TACE = tumour necrosis factor (TNF) α converting enzyme; AR = amphiregulin.
levels of fibrosis and lead to cirrhosis and potentially HCC. Thus, regulation of AR may be used to control the severity of the disease, using a regime that will promote liver recovery but control over-stimulation and the potential formation of fibrosis.

**Methods**

**Animals.** Adult C57BL6 mice (Charles River Laboratories, UK) were used. All animal studies were approved by the University Hospitals of Leuven, Belgium, and all the procedures described have been carried out in accordance with EU Directive 2010/63/EU for animal experiments. During study murine models of NAFLD, the administration of murine NASH was confirmed by histological examination of liver specimens. HSCs were isolated from murine livers and further cultured using a regime that will promote liver recovery but control over-stimulation and the potential formation of fibrosis.

**Human Biopsies and Histological Evaluations.** Liver specimens were obtained from patients undergoing clinically indicated hepatic biopsies, with the approval of the University of Palermo (Department of Pathology), patient consent and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Liver biopsy specimens were scored for the presence of NASH according to the Brunt and NAFLD Activity Score (NAS) of MCD model is an established dietary model of murine NASH23. After 4 weeks of feeding, the liver specimens were flash frozen in liquid nitrogen and stored at minus 80°C. Sections of human liver biopsies with a thickness of 4–5 mm were obtained using a microtome. Immunohistochemistry and immunofluorescence studies were performed using a regime that will promote liver recovery but control over-stimulation and the potential formation of fibrosis.

**Immunohistochemistry.** Immunostainings were performed by iVIEW DAB Detection Kit for Ventana BenchMark XT automated slide stainer on human biopsies43. Primary antibodies for AR (H-155, Santa Cruz Biotechnology) and ASMA (ACTA-2, SIGMA) were diluted 1:1000. Positive and negative controls were run concurrently. Cell identity was confirmed by autofluorescence, and expression of smooth muscle actin (ASMA), an accepted HSC marker. As stated in the Materials and Methods section, sections of human liver biopsies were consistently found to be suitable for immunohistochemical analysis. The amount of transcript was calculated and expressed as the difference relative to the control gene GADPH (2^-ΔΔCT, where ΔCT represents the difference in threshold cycles between the target and control genes).

**Western Blotting.** Total caspase-3 was evaluated by western blot analysis, as previously described13. Briefly, samples were homogenized with RIPA buffer (NaCl, Tris-HCl, 1% sodium deoxycholate, 0.1% SDS, and 1% NP-40, Sigma) plus protease cocktail (Roche). Samples (≥20 μg protein) were then loaded onto a 10% bis-tris polyacrylamide gel gel (Invitrogen, NP30). Western blots were performed using primary antibodies for caspase-8 (Cell Signaling #9746), β-actin (Santa Cruz Biotechnology), and ASMA (ACTA-2, SIGMA) as controls. Signal detection was achieved using the Western Lightning Chemiluminescence kit (Perkin Elmer). Optical densities were read from 96-well plates with an optical density of at least 3 separate experiments. Statistical analyses were performed using the Mann-Whitney test. A p value was considered statistically significant if p < 0.05.

**Visualization of caspase-3 activity** was achieved using in cultured hHSC using the commercial fluorimetric SensoLyte® 520 TACE (z-Secretase) Activity Assay Kit (Anaspec, CA, USA), according to manufacturer’s instructions. Statistical Analysis. All data are expressed as mean (±SEM) of at least 3 separate experiments. Statistical analyses were performed using the Student’s t-test. A p value was considered statistically significant if p < 0.05.

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Author contributions
Study concept, design and supervision: I.A.O.; Acquisition of data: C.M., B.S., J.S., A.M., M.M., F.R. and V.P.; Analysis and interpretation of data: F.C., V.P., C.M., B.S., J.S., A.M., M.M. and I.A.O.; Drafting of the manuscript: G.M., M.V. and I.A.O.; Statistical analysis: G.M.; Obtained funding: M.V. and I.A.O.; Technical support: D.C., C.S. and T.R.

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