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RESEARCH PAPER

The β-catenin pathway contributes to the effects of leptin on SREBP-1c expression in rat hepatic stellate cells and liver fibrosis

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BACKGROUND AND PURPOSE

Liver fibrosis is commonly associated with obesity and most obese patients develop hyperleptinaemia. The adipocytokine leptin has a unique role in the development of liver fibrosis. Activation of hepatic stellate cells (HSCs) is a key step in hepatic fibrogenesis and sterol regulatory element-binding protein-1c (SREBP-1c) can inhibit HSC activation. We have shown that leptin strongly inhibits SREBP-1c expression in rat HSCs. Hence, we aimed to clarify whether the β-catenin pathway, the crucial negative regulator of adipocyte differentiation, mediates the effects of leptin on SREBP-1c expression in HSCs and in mouse liver fibrosis.

EXPERIMENTAL APPROACH

HSCs were prepared from rats and mice. Gene expressions were analysed by real-time PCR, Western blot analysis, immunostaining and transient transfection assays.

KEY RESULTS

Leptin increased β-catenin protein but not mRNA levels in cultured HSCs. Leptin induced phosphorylation of glycogen synthase kinase-3β at Ser^{9} and subsequent stabilization of β-catenin protein was mediated, at least in part, by ERK and p38 MAPK pathways. The leptin-induced β-catenin pathway reduced SREBP-1c expression and activity but did not affect protein levels of key regulators controlling SREBP-1c activity, and was not involved in leptin inhibition of liver X receptor α. In a mouse model of liver injury, the β-catenin pathway was shown to be involved in leptin-induced liver fibrosis.

CONCLUSIONS AND IMPLICATIONS

The β-catenin pathway contributes to leptin regulation of SREBP-1c expression in HSCs and leptin-induced liver fibrosis in mice. These results have potential implications for clarifying the mechanisms of liver fibrogenesis associated with elevated leptin levels.

Abbreviations

α-SMA, alpha-smooth muscle actin; ER, endoplasmic reticulum; FFA, free fatty acid; GSK-3β, glycogen synthase kinase-3β; HSC, hepatic stellate cell; Insig, insulin-induced proteins; LXR, liver X receptor; SCAP, SREBP cleavage-activating protein; SREBP-1c, sterol regulatory element-binding protein-1c

Introduction

Hyperleptinaemia and insulin resistance are common in obese patients and, in turn, are associated with the development of type 2 diabetes mellitus (Stefanovic *et al.*, 2008; Yokaichiya *et al.*, 2008). Obesity and type 2 diabetes mellitus are important causes of non-alcoholic fatty liver disease (Watanabe *et al.*, 2008). Furthermore, liver fibrosis has been shown to be six times more prevalent in obese individuals than in the general population (McCullough and Falck-Ytter, 1999; Ratziu *et al.*, 2000).

The adipocytokine leptin is mainly produced by adipocytes and is an important mediator in the negative feedback loop regulating energy homeostasis. It inhibits free fatty acid (FFA) uptake and *de novo* fatty acid synthesis, stimulates the mobilization and oxidation of FFA and reverses the differentiation of adipocytes (Zhou *et al.*, 1999). In addition, in recent years, *in vitro* and *in vivo* studies have provided convincing evidence that leptin plays a unique role in the development of liver fibrosis (Aleffi *et al.*, 2005; Cao *et al.*, 2006; Elinav *et al.*, 2009). Liver fibrosis is the result of chronic liver injury from multiple causes and is associated with the accumulation of excess, abnormal extracellular matrix (ECM). Hepatic stellate cells (HSCs) are the major mesenchymal cell type in the liver. In normal liver, HSCs are in a quiescent state and reside in the perisinusoidal space of Disse, which contains an abundance of neutral lipids (Tsukamoto, 2005). Following chronic injury, quiescent HSCs transdifferentiate into activated myofibroblast-like cells (activated HSCs) and secret large amounts of ECM. The process of HSC activation is thought to be a key event in the development of liver fibrosis because, upon injury, HSCs in the liver become the primary source of ECM, such as collagen (Hernandez-Gea and Friedman, 2011).

The activation of HSCs requires global reprogramming of HSC gene expression, which must be orchestrated by changes in the expression and/or activity of key transcription regulators. Quiescent HSCs, once called fat-storing cells, are much like adipocytes and HSC transdifferentiation from quiescent to myofibroblastic cells appears to be analogous to adipocyte-to-pre-adipocyte (fibroblast) transdifferentiation (Tsukamoto, 2005). The key transcription factors for adipocyte differentiation include the sterol regulatory element-binding protein (SREBP)-1, PPAR-γ and CCAAT/enhanced binding protein family (C/EBP; Rangwala and Lazar, 2000). SREBP-1 exists as two isoforms, SREBP-1a and SREBP-1c. The mRNA ratio of SREBP-1a to SREBP-1c is 1:9 in liver (Shimomura *et al.*, 1997) and 1:4 in HSCs (Supporting Information Figure S1), suggesting that SREBP-1c is the predominant isoform of SREBP-1 in liver and in HSCs. Interestingly, it has been demonstrated that SREBP-1c plays a crucial role in the inhibition of HSC activation (Tsukamoto, 2005). Ectopic transduction of SREBP-1c causes a morphological and biochemical reversal of activated HSCs to quiescent cells (She *et al.*, 2005). It appears that leptin can directly target HSCs via activation of its receptor and stimulate HSC activation and fibrogenesis (Saxena *et al.*, 2002; Bertolani and Marra, 2010). This HSC activation is coupled to the sequential up-regulation of leptin and down-regulation of SREBP-1c (Potter *et al.*, 1998; She *et al.*, 2005). Based on these observations, it seemed of interest to examine the effect of leptin on SREBP-1c expression and activity in HSCs, as this might shed light on the mechanisms underlying the role of leptin in HSC activation and liver fibrosis. In previous studies, we demonstrated that leptin strongly inhibits SREBP-1c expression in HSCs *in vivo* and *in vitro* through down-regulation of liver X receptor α (LXRα) via p38 MAPK, which led to a decrease in collagen expression in HSCs (Yan *et al.*, 2012). However, the molecular mechanisms underlying this leptin-induced inhibition of SREBP-1c expression in HSCs are incompletely understood.

The canonical Wnt/β-catenin signalling pathway controls many cellular processes including proliferation and differentiation (MacDonald *et al.*, 2009). It has been shown that the Wnt/β-catenin pathway is a crucial negative regulator of adipogenesis and adipocyte differentiation (Arango *et al.*, 2005; Christodoulides *et al.*, 2009). β-Catenin is a key effector of the Wnt signalling pathway (Valenta *et al.*, 2012). In the absence of Wnt, the phosphorylation of the β-catenin protein by glycogen synthase kinase-3β (GSK-3β) is facilitated leading to the ubiquitination and degradation of β-catenin (a primary substrate of GSK-3β) (Christodoulides *et al.*, 2009). In the presence of Wnt, as Wnts are involved in the formation of a complex that induces the phosphorylation and inactivation of GSK-3β, the β-catenin protein is stabilized (MacDonald *et al.*, 2009). As a result, β-catenin is translocated into the nucleus, where it binds to T-cell factor (Tcf) binding site to regulate the expressions of specific target genes (MacDonald *et al.*, 2009). Of particular interest, the existence of functional β-catenin-dependent Wnt signalling has been demonstrated in HSC and, more importantly, the Wnt/β-catenin pathway has been shown to mediate bile duct ligation (BDL)-induced liver fibrosis (Cheng *et al.*, 2008). Therefore, in view of the roles of leptin and SREBP-1c in adipocyte differentiation and liver fibrosis, it is possible that leptin is associated with β-catenin signalling pathway in HSCs and thereby affects SREBP-1c expression in HSCs. Thus, the aim of this study is to investigate this possibility *in vitro* and *in vivo* by using HSC cultures and leptin-deficient mice (ob/ob mice).

Activation of SREBP-1c involves the proteolytic cleavage of a SREBP-1c precursor (125 kDa) embedded in the endoplasmic reticulum (ER). Within the ER, the inactive SREBP-1c protein is associated with two integral proteins, SREBP cleavage-activating protein (SCAP) and insulin-induced genes (insig-1 and insig-2), which play key roles in controlling this SREBP-1c cleavage process (Goldstein *et al.*, 2006; Sato, 2010). Insigs function as retention proteins for the SCAP/SREBP complex in the ER (Goldstein *et al.*, 2006). In the presence of specific signals, SCAP dissociates from insigs and escorts SREBP from the ER to the Golgi apparatus, where SREBPs undergo proteolytically processing to yield a transcriptionally active form (nuclear SREBP-1c, 68 kDa), leading to the translocation of SREBP-1c to the nucleus to activate transcription. Therefore, as leptin was found to influence β-catenin signalling and consequently affect SREBP-1c activity, we further investigated whether the leptin-induced β-catenin signalling pathway is associated with changes in the levels of SCAP as well as insigs in the HSCs.

Methods

Materials

Leptin was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel) and used to treat HSCs at 100 ng·mL^{-1} (Yan *et al.*, 2012). XAV939 (a potent inhibitor of the β-catenin pathway) and cycloheximide (an inhibitor of protein biosynthesis) were purchased from Santa Cruz (Santa Cruz, CA, USA). Thioacetamide (TAA), LiCl, IBMX, dexamethasone and insulin were purchased from Sigma (St. Louis, MO, USA). PD98059 (a MEK inhibitor) and SB203580 (a p38 MAPK inhibitor) were purchased from CalBiochem (La Jolla, CA, USA).

Treatment of animals

Male C57BL/6J ob/ob mice (obese mouse, leptin deficient), 5 weeks old, were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). After being housed for an additional week, the mice were used in the experiments. All the mice were given free access to water and standard chow diet and received humane care. Experimental protocols were conducted according Jiangsu and Nantong University guidelines. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Mice were randomly separated into four groups (six mice per group). All groups of mice were injected i.p. with TAA (200 μg·g^{-1} body weight, twice a week) for 4 weeks (Yan *et al.*, 2012). Throughout the 4-week period of TAA treatment, group 1–4 were administered, respectively, Ad.Fc (adenovirus encoding IgG2α Fc fragment), leptin plus Ad.Fc, leptin plus Ad.Dkk-1 [adenovirus encoding mouse Dickkopf-1 (Brugmann *et al.*, 2007)] or Ad.Dkk-1. Ad.Dkk-1 (2×10^7 pfu·g^{-1} body weight, once every two weeks) or Ad.Fc was injected into the tail vein of mice. Ad.Fc was used as a control virus. Mice received leptin (1 μg·g^{-1} body weight, i.p., once a day) (Yan *et al.*, 2012). Adenoviruses were amplified in HEK293 cells and purified on a caesium chloride gradient.

HSC isolation and culture

HSCs were isolated from Sprague-Dawley rats or ob/ob mice as described previously (Zhou *et al.*, 2010). The purity of the HSCs was assessed by phase contrast microscopy and a UV excited fluorescence microscope and was found to exceed 95%. Semiconfluent rat HSCs, after 4–8 passages, were used for the experiments. After being deprived of serum for 24 h in DMEM with 0.4% FBS, cells were treated with leptin in DMEM containing 0.4% FBS. HSCs isolated from ob/ob mice were used directly for Western blot analysis.

Western blot analysis

Western blot analysis was performed as described previously (Zhou *et al.*, 2010). The target protein was detected by primary antibodies against β-catenin (diluted 1:400), SREBP1 (diluted 1:500), GSK-3β (diluted 1:1000), phosphorylated GSK-3β at serine 9 (diluted 1:500), SCAP (diluted 1:1000), insig-1 (diluted 1:1000), insig-2 (diluted 1:1000), LXRα (diluted 1:500), α-smooth muscle actin (α-SMA) (1:2000), α1(I) collagen (1:1000), cyclin D1 (1:1000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, diluted 1:2000) or β-actin (diluted 1:2000) and subsequently by horseradish peroxidase-conjugated secondary antibodies (diluted 1:4000). β-actin or GAPDH was used as an internal control. LXRα, SREBP1 and α-SMA were purchased from Abcam (Cambrige, MA, USA) and other antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA).

mRNA isolation and real-time PCR

Total mRNA was extracted by using TRI-Reagent (Sigma) following the manufacturer's instructions. Real-time PCR was carried out as described previously (Zhou *et al.*, 2009). Fold changes in mRNA levels of target genes relative to the endogenous cyclophilin control were calculated as described by Schmittgen *et al.* (2000). The primers used in real-time PCR were as follows:

Rat β-catenin:
(forward) 5′-CCACGACTAGTTCAGCTGCTTGTAC-3′;
(reverse) 5′-ACTGCACAAACAGTGGAATGGTATT-3′.
Rat SREBP-1c:
(forward) 5′-AGCACAGCAACCAGAAACTCAA -3′;
(reverse) 5′-AGGTCTTTCAGTGATTTGCTTTTGT-3′.
Rat α-SMA:
(forward) 5′-ACAACGTGCCTATCTATGAGGGCT-3′;
(reverse) 5′-AGCGACATAGCACAGCTTCTCCTT-3′.
Rat α1(I) collagen:
(forward) 5′-TGGTCCCAAAGGTTCTCCTGGT-3′;
(reverse) 5′-TTAGGTCCAGGGAATCCCATCACA-3′.
Rat cyclophilin:
(forward) 5′-TGGATGGCAAGCATGTGGTCTTTG-3′;
(reverse) 5′-CTTCTTGCTGGTCTTGCCATTCCT-3′.

Plasmids and transient transfection assay

The Photinus luciferase-reporter plasmid pSREBP1c-Luc contains the 5′-flanking region (–1516 bp) of rat SREBP-1c gene promoter (Deng *et al.*, 2002). Plasmid pdncatenin and plasmid pwtcatenin encodes dominant-negative β-catenin and wild-type β-catenin respectively. Plasmid pMKK6E encodes MKK6E, an active mutant of the p38 MAPK upstream activator (MAPK kinase-6). Plasmid pcaMEK-1 encodes the constitutively active form of MEK-1, which stimulates ERK activation. Photinus luciferase-reporter plasmid pLXRE-Luc contains three tandem LXR-binding sites. Photinus luciferase-reporter plasmid pSRE-Luc contains three consensus SREBP-1c-binding sites. Plasmid ptwist2 encodes twist2, the repressor for SREBP-1c activity (Lee *et al.*, 2003). Photinus luciferase-reporter plasmid pGL3-OT (Addgene, Cambrige, MA, USA) contains three tandem wild-type Tcf-4 binding sites for evaluating β-catenin activity and pGL3-OF contains three tandem mutant Tcf-4 binding sites (Addgene).

HSCs were cultured in 12-well plastic plates and were transiently transfected with a reporter plasmid expressing Photinus luciferase (0.8 μg DNA per well) plus 30 ng of control vector expressing Renilla luciferase (pRL-TK; Promega, Madison, WI, USA) by using LipofectAMINE reagent (Life Technologies, New York, NY, USA) according to manufacturer's instructions. 0–0.8 μg (each well) and 2 μg or 8 μg (each 25-cm^2 flask) of other plasmids or the respective empty vector were used. The empty vectors were used to ensure an equal amount of total DNA in transfection assay. Luciferase activity was quantified fluorimetrically by using the

Dual-Luciferase Reporter Assay System (Promega). Data are expressed as the ratios of Photinus to Renilla luciferase activity for normalization of Photinus luciferase activity.

Immunostaining of β-catenin, SREBP-1c, synaptophysin and α-SMA and histological analysis of liver fibrosis

Single immunostaining of cultured HSCs and double immunostaining of liver sections were performed as described previously (Zhou *et al.*, 2010). Briefly, for single fluorescent staining of β-catenin on cultured HSCs, paraformaldehyde-fixed cells were blocked with normal serum and then incubated with primary antibody against β-catenin (diluted 1:100, Abcam) and a DyLight488-conjugated secondary antibody (diluted 1:500, ImmunoReagents, Inc. Raleigh, NC, USA). The nuclei were counterstained with Hoechst 33342 (Sigma). For single fluorescent staining of α-SMA in liver, paraformaldehyde-fixed liver sections were blocked with normal serum and incubated with primary antibody against α-SMA (diluted 1:100, Abcam) and DyLight488-conjugated secondary antibody (diluted 1:500). For single staining of SREBP1c in liver the avidin biotin complex (ABC) method was used; after being blocked with peroxidase blocking solution and normal serum, paraformaldehyde-fixed liver sections were incubated with primary antibody against SREBP1c (diluted 1:100, Abcam) and biotinylated secondary antibody (diluted 1:500, ImmunoReagents, Inc.). Thereafter, liver sections were incubated with ABC reagent (Vector Laboratories, Inc., Burlingame, CA, USA) and DAB reagent (Amresco, Solon, OH, USA), followed by counterstaining with the nuclear dye haematoxylin (Sigma). Images were captured with a light microscope. For examination of the expression of SREBP1c in HSCs in the liver, a double fluorescent staining was performed. Paraformaldehyde-fixed liver sections were blocked with normal serum and incubated with primary antibody against SREBP1c (diluted 1:100, Abcam) and primary antibody against synaptophysin (SYP, diluted 1:10, Abcam), a marker for quiescent and activated HSCs (Cassiman *et al.*, 1999). After being washed, liver sections were incubated with DyLight594-conjugated secondary antibody (diluted 1:500, ImmunoReagents, Inc.) and DyLight488-conjugated secondary antibody (diluted 1:500). The nuclei were counterstained with Hoechst 33342 (Sigma). Images were captured with the fluorescence microscope. For quantification of the SREBP-1c-positive HSCs as shown by the double fluorescence staining method, the SREBP-1c-positive HSCs were counted in six randomly chosen high-power fields at 200-fold magnification.

For histological analysis of liver fibrosis (sirius red staining of collagen), paraformaldehyde-fixed liver sections were stained with picric acid-fast green (Amresco) at room temperature for 10 min and then incubated with picric acid–sirius red (Amresco) for 1 h. Images were captured with a light microscope.

Statistical analysis

Each result was obtained from at least three independent experiments. The results are expressed as mean values ± SD. Differences between means were evaluated using Student's unpaired two-sided *t*-test. Where appropriate, comparisons of multiple treatment conditions with controls were analysed by ANOVA with Dunnett's test used for *post hoc* analysis. $P < 0.05$ is considered as significant.

Results

Leptin treatment increases β-catenin protein levels in cultured HSCs

To investigate whether leptin is associated with the β-catenin signalling pathway, the cultured HSCs were incubated with 100 ng·mL^{-1} of leptin (Yan *et al.*, 2012) or 10 mM of LiCl (Rodionova *et al.*, 2007) for different time periods (Figure 1A) or with different concentrations of leptin for 24 h (Figure 1B). As LiCl can inhibit GSK-3β activity, leading to stabilization of the β-catenin protein, which mimics the effects of activation of the Wnt signalling pathway (Hedgepeth *et al.*, 1997), LiCl treatment served as a control. Figure 1A and B indicate that leptin markedly increased β-catenin protein levels in a time-dependent and dose-dependent manner respectively. At the 6 h time point, the protein level of β-catenin had clearly been up-regulated by leptin. LiCl treatment also increased the β-catenin protein level. This effect of leptin on β-catenin was further confirmed by immunofluorescent staining of β-catenin protein (Figure 1C). In addition, nuclear accumulation of β-catenin protein was observed after leptin treatment (Figure 1C), which indicates activation of β-catenin (MacDonald *et al.*, 2009). Based on these results, the effect of leptin on β-catenin trans-activation activity was examined by transfection of HSCs with β-catenin activity reporter plasmid pGL3-OT. As expected, leptin increased β-catenin trans-activation activity (Figure 1D). Next HSCs were incubated with or without 100 ng·mL^{-1} of leptin for different time periods and the effect of leptin on β-catenin mRNA levels were further analysed by real-time PCR. Unexpectedly, leptin had no effect on β-catenin mRNA levels during the period of 24 h (Figure 1E).

Collectively, the results shown in Figure 1 indicate that leptin stimulates β-catenin signalling in HSCs by up-regulating the β-catenin protein level and activity but does not affect β-catenin mRNA level for the period of 24 h.

β-Catenin is required for leptin inhibition of SREBP-1c gene expression in cultured HSCs

We have shown that leptin strongly inhibits SREBP-1c expression in HSCs *in vitro* and *in vivo* (Yan *et al.*, 2012). To elucidate whether β-catenin mediated this leptin-induced inhibition of SREBP-1c expression in HSCs, XAV939, a potent inhibitor of the β-catenin pathway [promotes degradation of the β-catenin protein (Huang *et al.*, 2009)], was used. HSCs were pretreated with different concentrations of XAV939 before treatment with leptin for an additional 24 h. Western blot analysis showed that 5 μM XAV939 led to a marked reduction in β-catenin protein level (Figure 2A). Based on this result, HSCs were treated with or without 5 μM XAV939 or leptin for 24 h and the protein and mRNA levels of SREBP-1c were evaluated by Western blot analysis and real-time PCR respectively (Figure 2B, C). Figure 2B and C demonstrate that XAV939 attenuated the inhibitory effect of leptin on SREBP-1c expression at both the protein and mRNA levels,

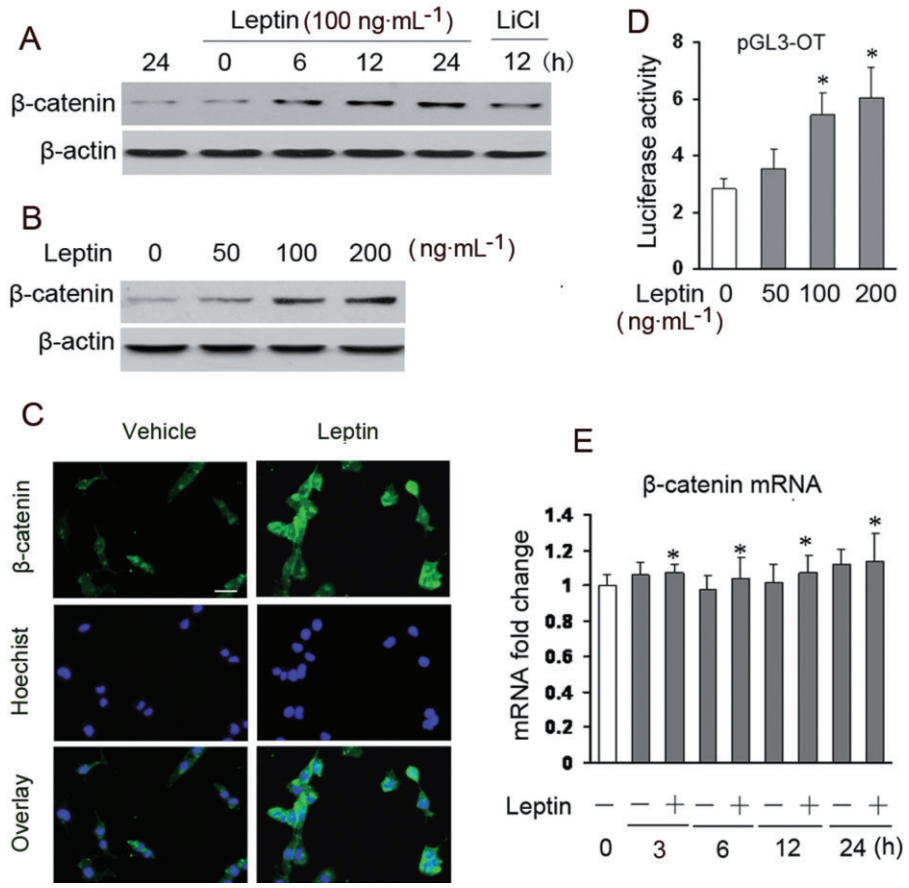

Figure 1

Leptin treatment increases β-catenin protein levels and activity but not β-catenin mRNA levels in cultured HSCs. (A, B) Western blot analysis of β-catenin levels. HSCs were incubated with 100 ng·mL^{-1} of leptin or 10 mM of LiCl for different time periods (A) or with different concentrations of leptin for 24 h (B). Representatives from three independent experiments are shown. (C) Immunofluorescent staining of β-catenin protein. After HSCs were treated with or without 100 ng·mL^{-1} of leptin for 24 h, immunofluorescent staining of β-catenin protein was performed by using primary antibody against β-catenin and DyLight488-conjugated secondary antibody (green fluorescence). The nuclei were counterstained with Hoechst 33342 (blue fluorescence). Representative images are from three independent experiments. Scale bar 25 μm. (D) Transfection assay for analysis of β-catenin trans-activation activity ($n = 6$). HSCs were transfected with plasmid pGL3-OT or pGL3-OF plus pRL-TK and then treated with different concentrations of leptin for 24 h. Luciferase activities denoted the ratio of signals detected with pGL3-OT and pGL3-OF after normalizing to the internal control pRL-TK activity. All values are expressed as means ± SD. *$P < 0.05$ versus the control without leptin. (E) Real-time PCR analysis of β-catenin mRNA levels ($n = 3$). HSCs were incubated with or without 100 ng·mL^{-1} of leptin for different time periods. All values are expressed as means ± SD. *$P > 0.05$ versus the respective control without leptin at the same time point.

suggesting that the β-catenin pathway was required for leptin-induced inhibition of SREBP-1c gene expression in cultured HSCs. To further confirm these results, the dominant-negative β-catenin expression plasmid pdncatenin (inhibits β-catenin activity, Supporting Information Figure S3) or the wild-type β-catenin expression plasmid pwtcatenin (increases β-catenin activity, Supporting Information Figure S3) were co-transfected into HSCs with plasmid pSREBP1c-Luc and the cells were stimulated with or without leptin. As shown in Figure 2D by luciferase assay, interruption of the leptin-induced β-catenin pathway by pdncatenin up-regulated the luciferase activity in a dose-dependent manner. Compared with the control without treatment (the first column on the right), leptin alone (the first column on the left) reduced luciferase activity, which was enhanced by pwtcatenin transfection (the second column on the right). These results suggest that the leptin-induced β-catenin pathway down-regulates SREBP-1c promoter activity, in line with the results presented in Figure 2B and C. Leptin did not appear to influence SREBP-1a expression (Supporting Information Figure S4).

Since LiCl increased the β-catenin protein level at the12 h time point (Figure 1A), HSCs were treated with different concentrations of LiCl for 12 h (Figure 2E) or HSCs were transfected with pSREBP1c-Luc and then incubated with or without LiCl for 12 h (Figure 2F). Western blot analysis and luciferase assay, respectively, indicated that LiCl reduced SREBP-1c protein levels (Figure 2E) and SREBP-1c promoter activity (Figure 2F), supporting the results in Figure 2B–D.

Together, these results strongly suggest that the β-catenin pathway mediates the leptin-induced decrease in SREBP-1c expression in HSCs *in vitro*.

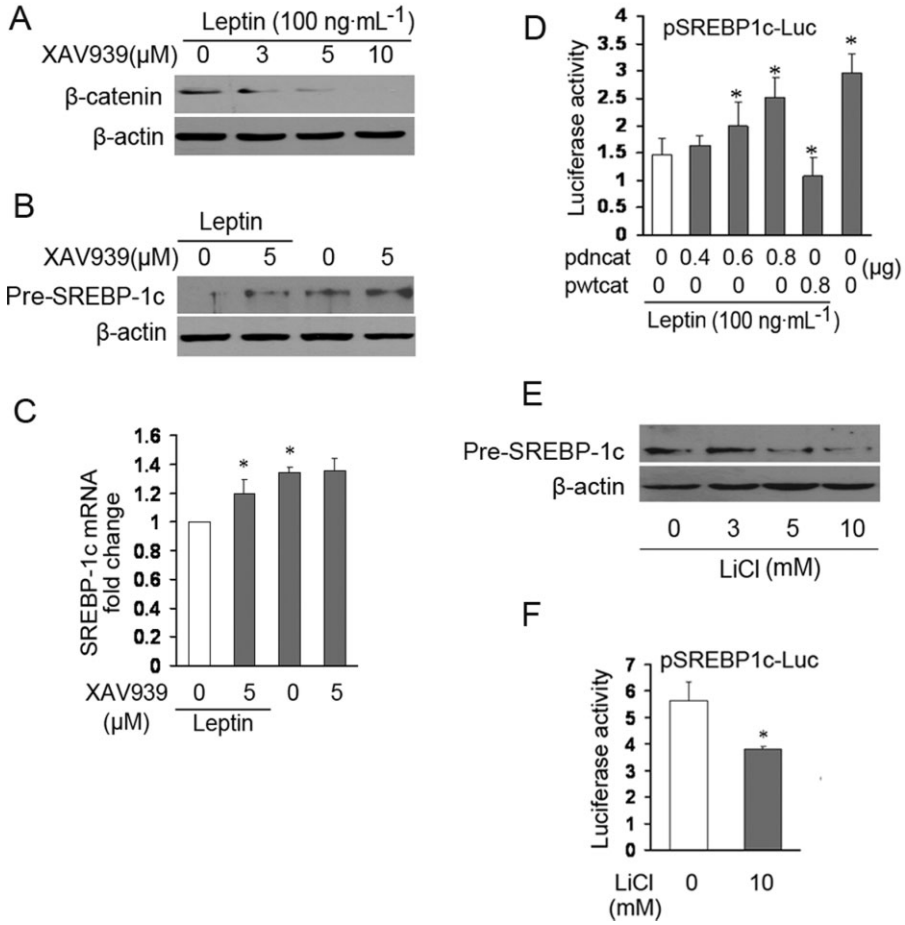

Figure 2

β-Catenin is required for leptin inhibition of SREBP-1c [precursor SREBP-1c (pre-SREBP-1c), 125 kDa] gene expression in cultured HSCs. (A, B) Western blot analysis of the protein levels of β-catenin (A) and precursor form of SREBP-1c (pre-SREBP-1c) (B). HSCs were pre-incubated with different concentrations of XAV939 before treatment with leptin (100 ng·mL^{-1}) for an additional 24 h. The results are representative of three independent experiments. (C) Real-time PCR analysis of SREBP-1c mRNA levels ($n = 3$). HSCs were pre-incubated with or without XAV939 before treatment with or without leptin (100 ng·mL^{-1}) for an additional 24 h. All values are expressed as means ± SD. *$P < 0.05$ versus cells with leptin alone (the first column on the left). (D) Transfection assay for analysis of SREBP-1c promoter activity ($n = 6$). HSCs were co-transfected with a fixed amount of a DNA mixture (including 0.8 μg of pSREBP1c-Luc, different doses of pwtcatenin (pwtcat) or pdncatenin (pdncat), 30 ng of pRL-TK and the empty vector) per well and stimulated with or without leptin for 24 h. The empty vector was used to ensure an equal amount of total DNA in the transfection assay. All values are expressed as means ± SD. *$P < 0.05$ versus cells with leptin alone (the first column on the left). (E) Western blot analysis of pre-SREBP-1c protein levels. HSCs were incubated with increasing doses of LiCl for 12 h. Results shown are representative of three independent experiments. (F) Transfection assay for analysis of SREBP-1c promoter activity ($n = 6$). HSCs were transfected with pSREBP1c-Luc plus pRL-TK and then incubated with or without LiCl for 12 h. All values are expressed as means ± SD. *$P < 0.05$ versus cells without LiCl.

The β-catenin pathway is involved in the leptin-induced reduction in SREBP-1c protein level in HSCs in the ob/ob mouse model of TAA-induced liver injury

To investigate whether the β-catenin pathway is involved in the leptin-induced reduction in SREBP-1c protein levels in HSCs *in vivo*, ob/ob mice (leptin deficient) and Ad.Dkk-1 were used. TAA administration can lead to liver fibrosis in normal mice and this is a well-established model of liver fibrosis (Honda *et al.*, 2002). In contrast, almost no liver fibrosis develops in ob/ob mice given TAA alone for 4 weeks whereas administration of TAA plus leptin to ob/ob mice for 4 weeks leads to prominent liver fibrosis (Honda *et al.*, 2002; Yan *et al.*, 2012). Ad.Dkk-1(encoding Dkk-1, an antagonist of the Wnt/β-catenin pathway) administration can interrupt the β-catenin pathway in the liver (Cheng *et al.*, 2008). Hence, throughout the 4-week period of TAA treatment, four groups of ob/ob mice were co-administered, with or without leptin, Ad.Dkk-1 or Ad.Fc as described in Figure 3A. Single staining of SREBP1c in liver sections by the ABC method was performed. HSCs are resident perisinusoidal cells with stellate projections. Figure 3A shows that in group 1 (TAA + Ad.Fc) SREBP-1c reactivity in perisinusoidal cells with stellate pro-

jections were clearly present, whereas SREBP-1c-positive perisinusoidal cells were barely detectable in group 2 (TAA + leptin + Ad.Fc). However, when mice in group 3 received TAA, leptin and Ad.Dkk-1, SREBP-1c reactivity in perisinusoidal cells with stellate projections appeared again. These results suggest that blockade of the β-catenin pathway by Dkk-1 attenuates the leptin-induced reduction in SREBP-1c protein levels in HSCs in the model of TAA-induced liver injury.

To verify this result *in vivo*, HSCs were isolated from each group and directly used for detecting β-catenin and SREBP-1c protein levels by Western blot analysis (Figure 3B). As expected, leptin clearly increased the β-catenin protein level but reduced the protein levels of the SREBP-1c precursor (125 kDa) and nuclear active form (68 kDa; the respective second band on the left) and these effects were partially reversed by Ad.Dkk-1treatment (the respective second band on the right). These results indicate that the β-catenin pathway mediates the leptin-induced reduction in SREBP-1c protein levels in HSCs in this mouse model.

To confirm the above results, double fluorescent staining of the same samples was also performed. SYP can be used as a marker for quiescent and activated HSCs (Cassiman *et al.*, 1999). Figure 3C shows representative photomicrographs of immunofluorescent analysis for SREBP-1c (red colour) and SYN (green colour) in the liver sections from each group. Double fluorescent staining and the SREBP-1c-positive HSC count demonstrated that, compared with group 1 (TAA + Ad.Fc), leptin reduced SREBP-1c-positive HSCs (group 2: TAA + leptin + Ad.Fc), which was partially counteracted by Ad.Dkk-1 treatment (group 3: TAA + leptin + Ad.Dkk-1). These double fluorescent staining results further support a role for the β-catenin pathway in the leptin-induced reduction in SREBP-1c protein level in HSCs.

Collectively, these data indicate that the β-catenin pathway mediates the effect of leptin on SREBP-1c protein level in HSCs *in vivo*.

Both ERK and p38 MAPK are associated with leptin-induced stabilization of the β-catenin protein in cultured HSCs

As leptin was shown to increase β-catenin protein levels but not β-catenin mRNA levels in HSCs (Figure 1), we speculated that leptin might inhibit the degradation of β-catenin protein. To examine this possibility, HSCs were stimulated with leptin for 24 h (to increase β-catenin protein level) and then treated with 5 μM of cycloheximide (Reynaert *et al.*, 2005) in the presence or absence of leptin for an additional 24 h. Western blot analysis indicated that leptin retarded the decline in the protein content of β-catenin (Figure 4A), suggesting an inhibitory effect of leptin on β-catenin protein degradation in cultured HSCs.

β-Catenin protein is the primary substrate of GSK-3β and GSK-3β can be negatively regulated by phosphorylation of an N-terminal serine (Ser^9), leading to the stabilization of β-catenin protein (Rodionova *et al.*, 2007; Caraci *et al.*, 2008). Since leptin has been shown to stimulate the activation of ERK and p38 MAPK in HSCs (Zhou *et al.*, 2009; Yan *et al.*, 2012) and it appears that ERK can phosphorylate (at Ser^9) and subsequently inactivate GSK-3β in human lung fibroblasts (Caraci *et al.*, 2008), we examined whether leptin-induced activation of ERK and p38 MAPK causes the phosphorylation of GSK-3β at Ser^9 and subsequent accumulation of β-catenin protein in HSCs. HSCs were firstly stimulated with leptin for different periods of time and the phosphorylation of GSK-3β (Ser^9) was analysed by Western blot. Figure 4B shows that leptin treatment increased the level of Ser^9-phosphorylated GSK-3β. At the 30 min time point, leptin significantly increased the Ser^9-phosphorylated GSK-3β level. Next, HSCs were pretreated with PD98059 (a MEK inhibitor) or SB203580 (a p38 MAPK inhibitor) before stimulation with leptin for an additional 30 min (for analysis of Ser^9-phosphorylated GSK-3β level) or 24 h (for analysis of β-catenin protein level). Western blot analysis (Figure 4C) showed that leptin alone increased the levels of Ser^9-phosphorylated GSK-3β and β-catenin protein and these effects were partially counteracted by pretreatment with PD98059 or SB203580. These results indicate that leptin-induced phosphorylation of GSK-3β at Ser^9 and subsequently stabilization of β-catenin protein were mediated, at least in part, by ERK and p38 MAPK pathways in HSCs.

We also evaluated the role of the leptin-stimulated ERK and p38 MAPK pathways in β-catenin trans-activation activity. Cultured HSCs were transfected with pGL3-OT or pGL3-OF and then treated with or without PD98059, SB203580 or leptin as shown in Figure 4D. These results demonstrate that leptin up-regulated β-catenin trans-activation activity (the respective second empty or solid column on the left) and this was, respectively, attenuated by PD98059 or SB203580. These results are consistent with the involvement of the leptin-stimulated ERK and p38 MAPK pathways in the stabilization of β-catenin protein.

Leptin-induced stabilization of the β-catenin protein through ERK and p38 MAPK leads to a reduction in SREBP-1c expression in cultured HSCs

As we had shown that β-catenin is involved in the leptin-induced inhibition of SREBP-1c expression in cultured HSCs (Figure 2), it was reasonable to hypothesize that the stabilization of β-catenin protein by activation of ERK and p38 MAPK might contribute to the reduction in SREBP-1c expression in HSCs. To verify this, HSCs were firstly transfected with or without a plasmid pMKK6E (stimulates p38 MAPK activation, Figure 5A) or a plasmid pcaMEK-1 (stimulates ERK activation, Figure 5B) and treated with or without XAV939 for 24 h. Western blot analysis showed that transfection with pMKK6E or pcaMEK-1 led to the accumulation of β-catenin protein (the second band on the left in Figure 5A and B respectively) and this was eliminated by treatment with XAV939 (the first band on the right in Figure 5A and B respectively). Based on these results, HSCs were co-transfected with pSREBP1c-Luc with or without pMKK6E (Figure 5C) or pcaMEK-1 (Figure 5D) and treated with or without 5 μM of XAV939 for 24 h. As expected, compared with the respective control (with vehicle), the inhibitory effect of pMKK6E (Figure 5C) or pcaMEK-1 (Figure 5D) on the luciferase activity was partially counteracted by XAV939 treatment. These results indicate that p38 MAPK- and ERK-dependent accumulation of β-catenin protein contributes to the leptin-induced inhibition of SREBP-1c promoter activity in cultured HSCs.

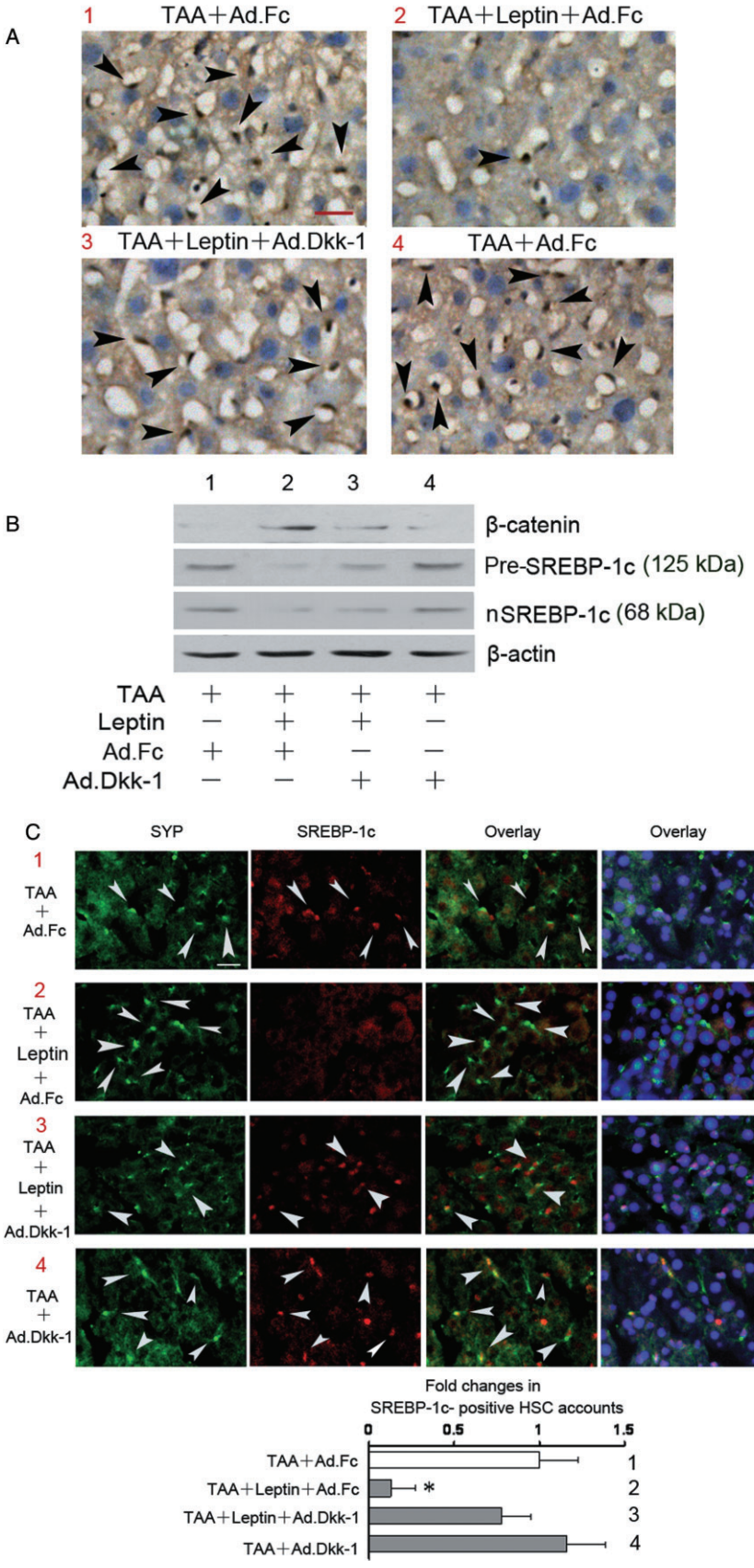
A
1 TAA+Ad.Fc
2 TAA+Leptin+Ad.Fc
3 TAA+Leptin+Ad.Dkk-1
4 TAA+Ad.Fc
B
1 2 3 4
β-catenin
Pre-SREBP-1c (125 kDa)
nSREBP-1c (68 kDa)
β-actin
TAA + + + +
Leptin − + + −
Ad.Fc + + − −
Ad.Dkk-1 − − + +
C
SYP
SREBP-1c
Overlay
Overlay
1 TAA + Ad.Fc
2 TAA + Leptin + Ad.Fc
3 TAA + Leptin + Ad.Dkk-1
4 TAA + Ad.Dkk-1
Fold changes in
SREBP-1c- positive HSC accounts
0 0.5 1 1.5
TAA+Ad.Fc 1
TAA+Leptin+Ad.Fc * 2
TAA+Leptin+Ad.Dkk-1 3
TAA+Ad.Dkk-1 4

Figure 3

β-Catenin is required for leptin inhibition of SREBP-1c gene expression in HSCs in an ob/ob mouse model of TAA-induced liver injury. Ob/ob mice were randomly separated into four groups: group 1 (with TAA + Ad.Fc); group 2 (with TAA + leptin + Ad.Fc); group 3 (with TAA + leptin + Ad.Dkk-1); group 4 (with TAA + Ad.Dkk-1). Mice were injected with TAA (200 $\mu g \cdot g^{-1}$ body weight, i.p., twice a week) or leptin (1 $\mu g \cdot g^{-1}$ body weight, once per day) for 4 weeks. Ad.Dkk-1 or Ad. Fc (2×10^7 $pfu \cdot g^{-1}$ body weight, once every 2 weeks) was injected into the tail vein. Ad. Fc was used as a control virus. After 4 weeks of treatment, the protein of SREBP1c or β-catenin in HSCs was examined by the following methods: (A) Single staining of SREBP1c on liver sections by ABC method. Representative images from each group ($n = 6$) showed the SREBP-1c-positive perisinusoidal cells with stellate projections (indicated by arrowhead). Scale bar 25 μm. (B) Western blot analysis of the protein level of SREBP1c [pre-SREBP1c, 125 kDa; nuclear active form (nSREBP1c), 68 kDa] or β-catenin in HSCs. HSCs were isolated from the four groups and directly used for analysis of the protein level of SREBP1c or β-catenin by Western blot. Representative images from three independent experiments are shown. (C) Double fluorescent staining for detecting and quantifying SREBP-1c-positive HSCs. SREBP-1c-positive HSCs were detected by double fluorescent staining of liver sections with primary antibody against SREBP1c plus primary antibody against synaptophysin (SYP, a marker for quiescent and activated HSCs) and subsequently with DyLight594-conjugated secondary antibody (red fluorescence) and DyLight488-conjugated secondary antibody (green fluorescence). The nuclei were counterstained with Hoechst 33342 (blue fluorescence). Representative images from each group ($n = 6$) are presented. Scale bar 25 μm. Arrowheads indicated examples of positively stained cells. The SREBP-1c-positive HSCs were counted in six randomly chosen high-power fields at 200-fold magnification and the counts of SREBP-1c-positive HSCs are shown in the histogram. All values are expressed as means ± SD. $^*P < 0.05$ versus group 1 (TAA + Ad.Fc) or group 3 (TAA + leptin + Ad.Dkk-1).

Leptin-induced effect on β-catenin pathway reduces the active form of SREBP-1c and its trans-activation activity but has no effect on SCAP, insigs and LXRα in cultured HSCs

The nuclear active form of SREBP-1c (68 kDa) is generated by the cleavage of the SREBP-1c precursor (125 kDa). Therefore, the effects of the leptin-stimulated β-catenin pathway on the protein level of the active form of SREBP-1c and on SREBP-1c activity were examined in cultured HSCs. HSCs were pretreated with or without XAV939 and subsequently incubated with or without leptin for 24 h (Figure 6A), or HSCs were co-transfected with increasing doses of the plasmid pdncatenin plus pSRE-Luc (contains SREBP-1c-binding sites) and incubated with or without leptin for 24 h (Figure 6B). The results show that leptin reduced the level of the active form of SREBP-1c (Figure 6A) and its trans-activation activity (Figure 6B), effects which were reversed, respectively, by XAV939 (Figure 6A) and pdncatenin (Figure 6B).

SCAP, insig-1 and insig-2 play key roles in controlling the cleavage of the SREBP-1c precursor to generate its nuclear active form (Goldstein *et al.*, 2006; Sato, 2010). Hence, we examined the effect of the leptin-stimulated β-catenin pathway on the expression of SCAP, insig-1 and insig-2. HSCs were treated with or without leptin for different periods of time and Western blot analyses indicated that leptin increased insig-1protein level in a period of 12–24 h but had no effect on the protein levels of SCAP and insig-2 (Figure 6C). Next, HSCs were pretreated with or without XAV939 before treatment with leptin for an additional 24 h (Figure 6D). Western blot analysis showed that XAV939 failed to affect the effect of leptin on insig-1 protein level, suggesting that the β-catenin pathway is not involved in the effects of leptin on insig-1 expression in HSCs.

Our previous studies indicated that leptin inhibits SREBP-1c expression by reducing the expression and activity of LXRα (Yan *et al.*, 2012). Hence, we investigated the effect of the leptin-stimulated β-catenin pathway on LXRα expression and activity. HSCs were pretreated with or without XAV939 before treatment with or without leptin for an additional 24 h (Yan *et al.*, 2012; Figure 6E), or HSCs were co-transfected with increasing doses of pdncatenin plus the plasmid pLXRE-Luc (containing LXRα-binding sites) before stimulation with or without leptin for 24 h (Yan *et al.*, 2012; Figure 6F). Unexpectedly, Western blot analysis and luciferase assay indicated that blockade of the β-catenin pathway had no effect on LXRα protein level (Figure 6E) and trans-activation activity (Figure 6F).

The negative effect of the leptin-upregulated β-catenin pathway on SREBP1c expression influences mRNA levels of α-SMA and α1(I) collagen in cultured HSCs

SREBP-1c inhibits HSC activation (She *et al.*, 2005) and reduces the expression of α1(I) collagen, the major component of ECM, in activated HSCs (She *et al.*, 2005). Hence, we examined whether the negative effect of the leptin-induced β-catenin pathway on SREBP1c expression can affect the transcriptions of α-SMA (a well-established marker for HSC activation) and α1(I) collagen. HSCs were transfected with or without plasmid ptwist2 [encoding twist2, a repressor for SREBP-1c activity (Lee *et al.*, 2003)], and then maintained in DMEM containing the adipogenic differentiation cocktail (MDI: 0.5 mM IBMX, 1 μM dexamethasone and 1 μM insulin) for 24 h. MDI was used to reduce the expressions of α-SMA and α1(I) collagen and increase SREBP-1c expression in HSCs (She *et al.*, 2005). After incubation with MDI, cells were switched to the medium with or without XAV939 and pretreated for 30 min before stimulation with or without leptin for an additional 24 h. Real-time PCR analysis revealed that leptin alone increased the mRNA levels of α-SMA and α1(I) collagen as compared with the control (the corresponding first column on the left; Figure 7A). Pretreatment with XAV939 attenuated the leptin-induced increases in the mRNA levels of α-SMA and α1(I) collagen (the corresponding third column on the left); these effects were partially eliminated by transfection with ptwist2 (the corresponding third column on the right).

These results suggest that the inhibitory effect of leptin on SREBP1c expression mediated through the β-catenin pathway leads to increases in the transcriptions of α-SMA and α1(I) collagen in cultured HSCs.

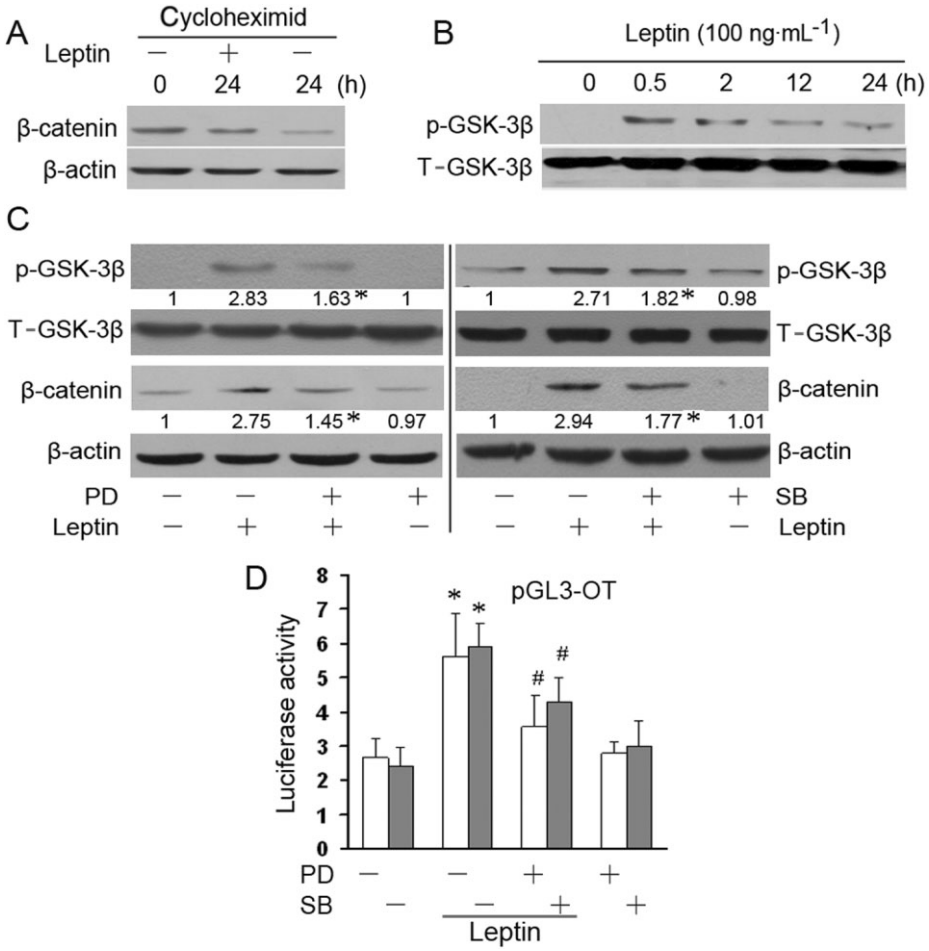

Figure 4

Both ERK and p38 MAPK mediate leptin-induced stabilization of β-catenin protein in cultured HSCs. (A) Western blot analysis of β-catenin protein levels. HSCs were stimulated with leptin for 24 h and then treated with 5 μM of cycloheximide in the presence or absence of leptin for an additional 24 h. The results are representatives of three independent experiments. (B) Western blot analysis of the protein level of Ser^9-phosphorylated GSK-3β (p-GSK-3β). HSCs were stimulated with leptin (100 ng·mL^{-1}) for different time periods. Total GSK-3β (T-GSK-3β) was used as an internal control. The results are representatives of three independent experiments. (C) Western blot analysis of the protein levels of Ser^9-phosphorylated GSK-3β (p-GSK-3β) and β-catenin. HSCs were pretreated with or without PD98059 (PD, a MEK inhibitor, 10 μM) or SB203580 (SB, a p38 MAPK inhibitor, 10 μM) before stimulation with or without leptin (100 ng·mL^{-1}) for an additional 30 min (for analysis of p-GSK-3β level) or 24 h (for analysis of β-catenin level). Total GSK-3β (T-GSK-3β) and β-actin were used as the respective internal controls. Results are representatives of three independent experiments. The intensities of protein bands were determined densitometrically and the numbers beneath the blots indicated the fold changes in the band densities relative to the respective control (the first band on the left) after normalization to the internal control. *$P < 0.05$ versus the respective sample with leptin alone. (D) Transfection assay for analysis of β-catenin activity ($n = 6$). HSCs were transfected with β-catenin activity reporter plasmid pGL3-OT or pGL3-OF plus pRL-TK and then treated with or without PD98059 (10 μM), SB203580 (10 μM), or leptin (100 ng·mL^{-1}) for 24 h. Luciferase activities denote the ratio of signals detected with pGL3-OT and pGL3-OF after normalizing to the internal control pRL-TK activity. All values are expressed as means ± SD. *$P < 0.05$ versus the respective control without treatment (the respective first column on the left). $^{\#}P < 0.05$ versus the respective sample with leptin alone (the respective second column on the left).

The β-catenin pathway is required for leptin-induced liver fibrosis in the ob/ob mouse model of TAA-induced liver injury

As we had shown that the β-catenin pathway is required for leptin-induced inhibition of SREBP1c expression and activity in HSCs, the role of this pathway in leptin-induced liver fibrosis in mouse model of TAA-induced liver injury was further investigated. An identical experimental design to that shown in Figure 3 was conducted. Briefly, ob/ob mice were randomly separated into four groups: group 1 (TAA + Ad.Fc), group 2 (TAA + leptin + Ad.Fc), group 3 (TAA + leptin + Ad.Dkk-1) and group 4 (TAA + Ad.Dkk-1). After all the mice had received the respective reagent for 4 weeks, liver fibrosis was demonstrated by sirius red staining of collagen and HSC activation was evaluated by fluorescent staining of α-SMA. As shown in Figure 7B, group 1 (TAA + Ad.Fc) did not develop signs of liver fibrosis or HSC activation, whereas when mice were co-administered TAA, Ad.Fc and leptin, liver fibrosis and HSC activation clearly appeared (group 2). These effects were

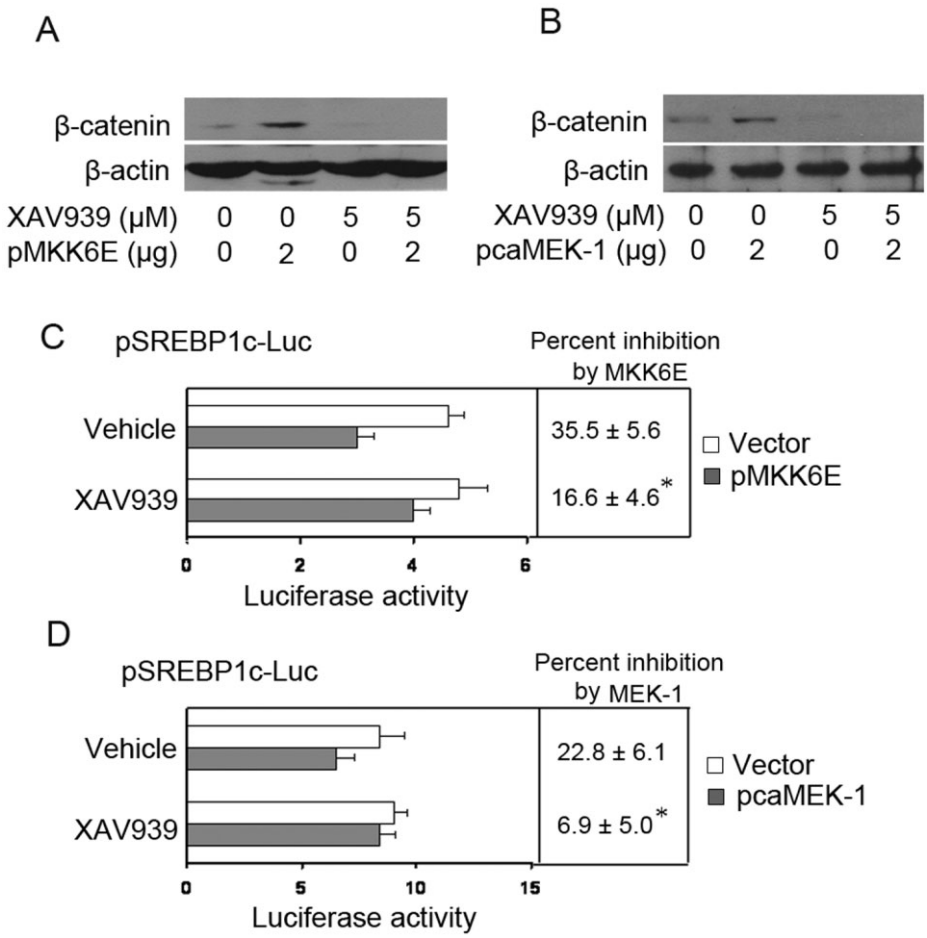

Figure 5

Blockade of the β-catenin signalling pathway attenuates the inhibitory effects of both ERK and p38 MAPK on SREBP-1c promoter activity in cultured HSCs. (A, B) Western blot analysis of β-catenin. HSCs were transfected with pMKK6E (A), pcaMEK-1(B) or the empty vector per 25-cm^2 flask and treated with XAV939 or vehicle for 24 h. Representatives were shown from three independent experiments. (C, D) Transfection assay for analysis of SREBP-1c promoter activity ($n = 6$). HSCs were co-transfected with a fixed amount of a DNA mixture (including 0.8 μg of pSREBP1c-Luc, 0.3 μg of pMKK6E or 0.3 of μg pcaMEK-1, 30 ng of pRL-TK and the empty vector) per well and treated with 5 μM of XAV939 or vehicle for 24 h. Percentage inhibition of SREBP-1c promoter activity by pMKK6E (C) or pcaMEK-1 (D) was calculated for cells treated with XAV939 or vehicle. All values are expressed as means ± SD. *$P < 0.05$ versus cells with vehicle alone. The empty vector was used to ensure an equal amount of total DNA in the transfection assay.

partially suppressed by treatment with Ad.Dkk-1 (group 3). Activated HSCs are the main ECM-producing cells in the injured liver and demonstrate increased proliferation. The D-type cyclins, such as cyclin D1, play a critical role in the proliferation of activated HSCs (Kawada *et al.*, 1999). Therefore, isolated HSCs from groups 1–4 were directly used to examine the protein levels of α1(I) collagen, α-SMA and cyclin D1 by Western blot analysis (Figure 7C). The results revealed that, compared with group 1, leptin treatment increased the expressions of α1(I) collagen, α-SMA and cyclin D1 (group 2) and these effects were attenuated by co-treatment with Ad.Dkk-1 (group 3). Hence, these data from Western blot analysis are in line with those obtained from histological analysis.

Collectively, these results suggest that the β-catenin pathway is involved in leptin-induced liver fibrosis in the mouse model of TAA-induced liver injury.

Discussion and conclusions

The results from this study demonstrate that leptin treatment increases the level of β-catenin protein in HSCs through stabilization and this is associated with leptin-induced activation of p38 MAPK or ERK and subsequent inactivation of GSK-3β. The effect of leptin on β-catenin protein could cause the reduction in SREBP-1c expression and activity in HSCs *in vivo* and *in vitro*, which might lead to the increases in α1(I) collagen and the marker of HSC activation in HSCs and contribute to liver fibrogenesis in the ob/ob mouse model of TAA-induced liver injury. Insigs and SCAP are the key regulators controlling SREBP-1c activation (Goldstein *et al.*, 2006; Sato, 2010). Our studies revealed that leptin treatment up-regulated the protein level of insig-1, but not those of SCAP or insig-2 in cultured HSCs. The β-catenin pathway did not appear to be involved in the effects of leptin on insig-1

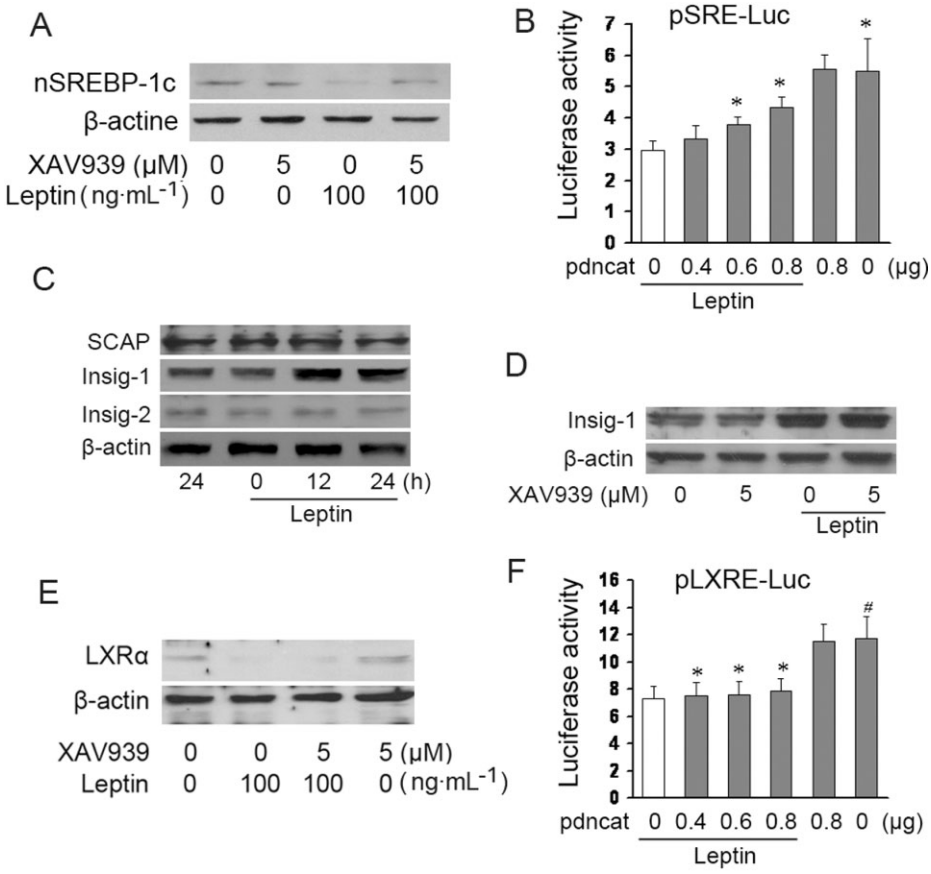

Figure 6

Leptin-induced β-catenin signalling pathway inhibits SREBP-1c activation, but has no effect on insigs, SCAP and LXRα in cultured HSCs. (A) Western blot analysis of the protein levels of the nuclear active form of SREBP-1c (nSREBP-1c, 68 kDa). HSCs were pretreated with or without XAV939 before treatment with or without leptin for an additional 24 h. Representative results shown are from three independent experiments. (B) Transfection assay for analysis of the trans-activation activity of SREBP-1c ($n = 6$). HSCs were co-transfected with a fixed amount of a DNA mixture [including 0.8 μg of pSRE-Luc, different doses of pdncatenin (pdncat), 30 ng of pRL-TK and the empty vector] per well and treated with or without leptin (ng·mL^{-1}) for 24 h. Values are expressed as means ± SD. *$P < 0.05$ versus the control with leptin alone (the first column on the left). (C, D, E) Western blot analysis of the protein levels of SCAP, insig-1, insig-2 and LXRα. HSCs were treated with or without leptin (100 ng·mL^{-1}) for different time periods (C) or pretreated with or without XAV939 before treatment with or without leptin (100 ng·mL^{-1}) for an additional 24 h (D, E). Representative results shown are from three independent experiments. (F) Transfection assay for analysis of LXRα trans-activation activity ($n = 6$). HSCs were co-transfected with a fixed amount of a DNA mixture [including 0.8 μg of pLXRE-Luc, different doses of pdncatenin (pdncat), 30 ng of pRL-TK and the empty vector] per well and treated with or without leptin (100 ng·mL^{-1}) for 24 h. Values are expressed as means ± SD. *$P > 0.05$, $^{\#}P < 0.05$ versus the control with leptin alone (the first column on the left). The empty vector was used to ensure an equal amount of total DNA in the transfection assay.

or on LXRα protein levels and trans-activation activity. However, our results, obtained from a variety of experiments, demonstrate that there is a relationship between the leptin-upregulated β-catenin pathway and SREBP-1c expression in HSCs and that this pathway is involved in leptin-induced liver fibrosis. There is an abundance of evidence indicating a unique role for leptin in the development of liver fibrosis (Aleffi *et al.*, 2005; Cao *et al.*, 2006; Elinav *et al.*, 2009) and our results provide new insights into the mechanisms underlying leptin-induced inhibition of SREBP-1c expression (a key factor for inhibiting HSC activation) in HSCs and liver fibrosis.

The influence of inappropriate β-catenin pathway signalling on fibrogenesis is just beginning to be understood (Lam and Gottardi, 2011). The Wnt/β-catenin pathway has been shown to mediate BDL-induced liver fibrosis in mice (Cheng *et al.*, 2008). In addition, activation of the Wnt/β-catenin pathway has also been observed in pulmonary fibrosis in mice and in humans (Königshoff and Eickelberg, 2010). While we were preparing this article, Akhmetshina and his colleagues found that β-catenin activity was correlated with transforming growth factor-β1(TGF-β1)-induced dermal fibrosis (Akhmetshina *et al.*, 2012). Combined with our results showing that the β-catenin pathway is involved in leptin-induced liver fibrosis in mice, these observations suggest that the β-catenin pathway functions as an important mediator in the development of tissue fibrosis. Less clear is the precise mechanism by which this occurs. It is worth noting that the Wnt/β-catenin pathway is a crucial negative regulator of adipogenesis and adipocyte differentiation (Arango *et al.*,

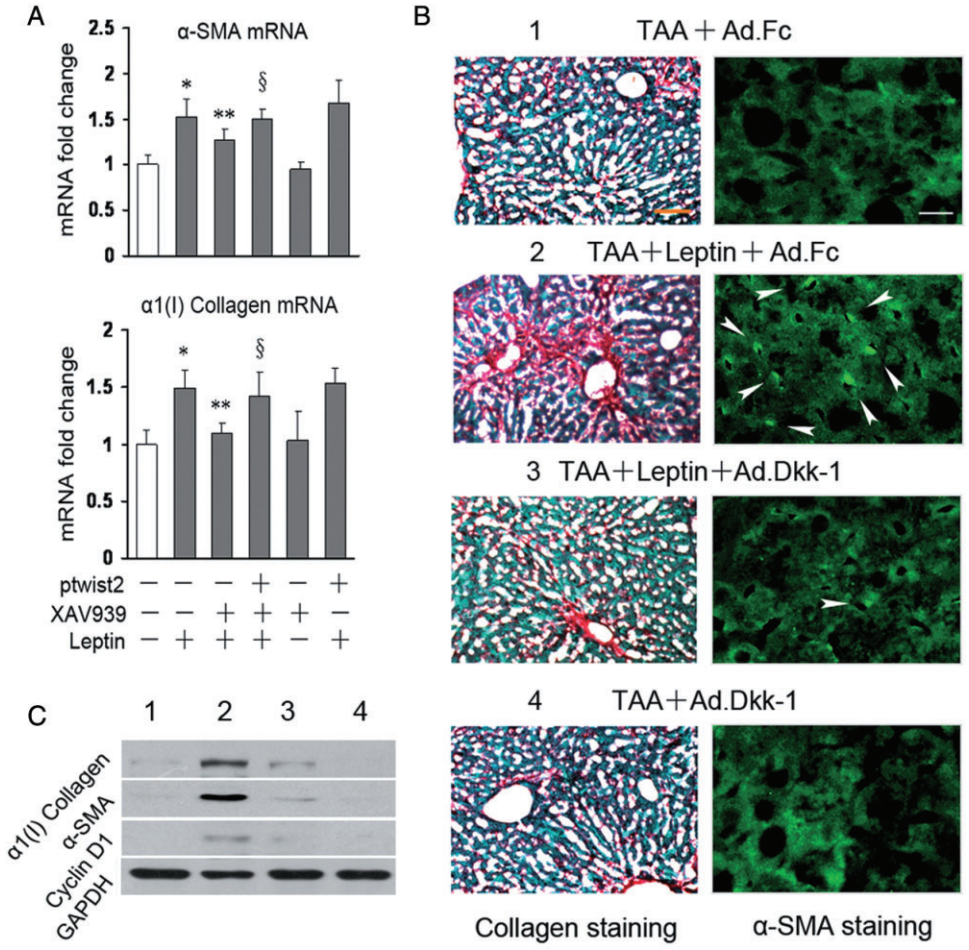

Figure 7

Leptin inhibition of SREBP1c expression by β-catenin pathway causes a decline in the mRNA levels of α-SMA and α1(I) collagen in cultured HSCs and the β-catenin pathway is required for leptin-induced liver fibrosis in ob/ob mouse model of TAA-induced liver injury. (A) Real-time PCR analysis mRNA levels of α-SMA and α1(I) collagen. HSCs in 25 cm^2 flask were transfected with or without 8 μg of plasmid ptwist2 (inhibiting SREBP1c activity) or empty vector. After being maintained in DMEM containing the adipogenic differentiation cocktail (MDI: 0.5 mM IBMX, 1 μM dexamethasone and 1 μM insulin) with 0.4% FBS for 24 h, HSCs were switched to the medium with or without XAV939 (5 μM) and pretreated before incubation with or without leptin (100 $ng \cdot mL^{-1}$) for an additional 24 h. The mRNA levels of α-SMA and α1(I) collagen were examined by real-time PCR analysis ($n = 3$). Values are expressed as means ± SD. *$P < 0.05$ versus cells with no treatment (the corresponding first column on the left). **$P < 0.05$ versus cells with leptin alone (the corresponding second column on the left). §$P < 0.05$ versus cells with XAV939 plus leptin (the corresponding third column on the left). (B, C) Evaluation of liver fibrosis and HSC activation. An identical experimental design to Figure 3 was used. Ob/ob mice were randomly divided into four groups: group 1 (with TAA + Ad.Fc), group 2 (with TAA + leptin + Ad.Fc), group 3 (with TAA + leptin + Ad.Dkk-1) and group 4 (with TAA + Ad.Dkk-1). After mice received the respective reagent for 4 weeks, liver fibrosis was examined by sirius red staining of collagen in the liver section and HSC activation was detected by single fluorescent staining of α-SMA (indicated by arrowhead) with primary antibody against α-SMA and the DyLight488-conjugated secondary antibody (B) or HSCs were isolated from each group and directly used for examining protein levels of α1(I) collagen, α-SMA and cyclin D1 by Western blot analysis (C). Representative images from each group ($n = 6$) are presented. Scale bar (on the left) 80 μm. Scale bar (on the right) 25 μm.

2005; Christodoulides *et al.*, 2009), whereas SREBP-1c (also called adipocyte determination and differentiation-dependent factor 1) is a key transcription factor for promoting adipocyte differentiation (Rangwala and Lazar, 2000). Furthermore, HSC activation is analogous to reverse adipocyte-to-pre-adipocyte (fibroblast) transdifferentiation (Tsukamoto, 2005) and SREBP-1c plays a pivotal role in inhibition of HSC activation (Tsukamoto, 2005). Our results demonstrated a negative relationship between the leptin-upregulated β-catenin pathway and SREBP-1c expression in HSCs *in vivo* and *in vitro* and indicate that this pathway has a role in leptin-induced liver fibrosis. Thus, we hypothesize that SREBP-1c might be a pivotal target of the β-catenin pathway in promoting HSC activation and liver fibrogenesis. Abiola *et al.* found that activation of the Wnt/β-catenin signalling in skeletal muscle cells improved insulin sensitivity by decreasing intramyocellular lipid deposition through the down-regulation of SREBP-1c (Abiola *et al.*, 2009), suggesting that SREBP-1c is also the molecular target of the β-catenin pathway in skeletal muscle cells.

A leptin-induced increase in β-catenin protein was shown to be associated with leptin inhibition of β-catenin protein degradation in HSCs. This result was based on the following observations: firstly, leptin treatment increased the levels of β-catenin protein, but not mRNA. Secondly, when protein biosynthesis was inhibited, leptin retarded the decline in the protein content of β-catenin. Thirdly, leptin induced the phosphorylation of GSK-3β at Ser^9 (inactive form), a key regulator of the stability of β-catenin protein (Rodionova *et al.*, 2007; Caraci *et al.*, 2008), leading to the accumulation of β-catenin protein. In accord with our results, leptin has also been shown to increase Ser^9-phosphorylated GSK-3β and subsequent β-catenin protein levels in neural stem/progenitor cells (Garza *et al.*, 2012). Of note, Endo *et al.* (2011) revealed that the activation of β-catenin pathway could increase leptin receptor expression in colorectal cells, which, combined with the positive effect of leptin on the stability of β-catenin protein in HSCs and neural stem cells, suggests a reciprocal relationship between the β-catenin signalling pathway and leptin signalling pathway.

GSK-3β is negatively regulated by phosphorylation of Ser^9, leading to the stabilization of β-catenin protein (Rodionova *et al.*, 2007; Caraci *et al.*, 2008) and MAPK appears to be linked to the Wnt/β-catenin pathway in some cell types (Bikkavilli and Malbon, 2009). Our results indicate that leptin-induced activation of p38 MAPK and ERK caused the phosphorylation of GSK-3β at Ser^9 and stabilization of the β-catenin protein in HSCs. Wnt3a was shown to inactivate GSK3β through p38 MAPK, leading to the accumulation of β-catenin in F9 teratocarcinoma cells (Bikkavilli *et al.*, 2008). Interestingly, Wnt3a-induced p38 MAPK activation increases β-catenin trans-activation activity but has no effect on Wnt3a-induced changes in β-catenin protein levels in C3H10T1/2 mesenchymal cells (Caverzasio and Manen, 2007), whereas Wnt3a-induced ERK does not affect either β-catenin activity or β-catenin protein levels in the same cell type (Caverzasio and Manen, 2007). In contrast, ERK activation induced by TGF-β1 phosphorylates (at Ser^9) and inactivates GSK-3β in human lung fibroblasts (Caraci *et al.*, 2008). These observations suggest that the influence of p38 MAPK and ERK on β-catenin pathway is dependent on cell type. Our results do not exclude the involvement of other signalling pathways in leptin regulation of β-catenin protein levels in HSCs. Recently, it was found that leptin could induce frizzled expression through activation of Janus kinase 2, resulting in activation of the β-catenin pathway in chondrocytes (Ohba *et al.*, 2010). Our data suggest the existence of crosstalk between the p38 MAPK and ERK pathways and that of β-catenin and that, in HSCs, this crosstalk is mediated by phosphorylation of GSK-3β at Ser^9 induced by p38 MAPK and ERK.

LXR plays a central role in insulin-mediated activation of SREBP-1c transcription in liver (Chen *et al.*, 2004). Our previous study revealed that leptin-induced p38 MAPK resulted in a decrease in the expression and activity of LXRα, thereby causing a decline in SREBP-1c expression in HSCs (Yan *et al.*, 2012). Although the present study demonstrated that leptin-induced increase in p38 MAPK leads to the accumulation of β-catenin protein and the subsequent reduction in SREBP-1c expression in HSCs, our results also indicated that the β-catenin pathway has no effect on LXRα protein levels and activity, implying that the β-catenin pathway is not associated with the effect of p38 MAPK on LXRα in HSCs. However, in contrast to our results, β-catenin was shown to enhance LXRα activity in human embryonic kidney cells (Uno *et al.*, 2009). From our results we cannot explain this discrepancy.

Activation of SREBP-1c is regulated by its expression and the proteolytic cleavage of its precursor. The latter process is controlled by several key factors such as SCAP and insigs (Goldstein *et al.*, 2006; Sato, 2010). In our study, leptin failed to affect SCAP and insig-2 protein levels suggesting that the β-catenin pathway does not mediate the effect of leptin on insig-1 protein levels in HSCs. Therefore, leptin inhibition of SREBP-1c activity in HSCs is probably mainly due to leptin-induced decrease in SREBP-1c expression mediated by the β-catenin pathway.

In summary, by using *in vitro* and *in vivo* approaches, we have obtained evidence linking the β-catenin pathway to leptin-induced decline in SREBP-1c expression and activity in HSCs and demonstrated the role of the β-catenin pathway in leptin-induced HSC activation and liver fibrosis. Our results do not exclude the possibility that leptin might also influence other cell types (that express leptin receptors), which could also mediate the role of leptin in liver fibrosis. Given that most obese patients develop hyperleptinaemia and their serum leptin concentrations can reach as high as 169 $ng \cdot mL^{-1}$ (Liuzzi *et al.*, 1999), the results in the present study might have potential implications for clarifying the mechanisms of liver fibrogenesis associated with these elevated leptin levels.

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Real-time PCR analysis of SREBP-1a and SREBP-1c mRNA levels in HSCs. HSCs were cultured in Dullbecco's modified Eagle's medium with 10% of fetal bovine serum. Total mRNA was extracted and real-time PCR was carried out. Fold changes in mRNA levels of target gene relative to the endogenous cyclophilin control were calculated as suggested by Schmittgen et al. (Schmittgen TD, *et al*. Anal Biochem 2000, 285:194–204).

Figure S2 Real-time PCR analysis of SREBP-1a and SREBP-1c mRNA levels in rat liver (n = 3). Total mRNA was extracted from rat liver by using TRI-Reagent (Sigma, St. Louis, USA) following the manufacturer's instructions. The mRNA levels of SREBP-1a and SREBP-1c were analysed by real-time PCR as described in Supporting Information Figure S1.

Figure S3 Transfection assay for analysis of β-catenin activity (n = 6). HSCs were cotransfected with a fixed amount of a DNA mixture (including 0.8 μg of plasmid pGL3-OT or pGL3-OF, 0.8 μg of pwtcatenin (pwtcat) or pdncatenin (pdncat), 30 ng of pRL-TK, and the empty vector) per well and stimulated with or without leptin (100 ng·mL^{-1}) for 24 h. The empty vector was used to ensure the equal amount of total DNA in transfection assay. Luciferase activities denoted the ratio of signals detected with pGL3-OT and pGL3-OF after normalizing by the internal control pRL-TK activity. All values are expressed as means ± SD. $^{*}P < 0.05$ versus cells without leptin (the first column on the left). $^{**}P < 0.05$, $^{\#}P < 0.05$ versus cells with leptin alone (the second column on the left).

Figure S4 Real-time PCR analysis of SREBP-1a mRNA levels (n = 3). HSCs were preincubated with or without XAV939 for 30 min before treatment with or without leptin (100 ng·mL^{-1}) for an additional 24 h. The mRNA levels of SREBP-1a was analysed by real-time PCR as described in Supporting Information Figure S1.