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Curcumin eliminates oxidized LDL roles in activating hepatic stellate cells by suppressing gene expression of lectin-like oxidized LDL receptor-1

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Abstract

Type II diabetes mellitus (T2DM) is often accompanied by non-alcoholic steatohepatitis (NASH) and associated with hypercholesterolemia, *i.e.* increased levels of plasma low-density lipoprotein (LDL) and oxidized LDL (ox-LDL). Approximately one third of NASH develops hepatic fibrosis. The hypercholesterolemia role in T2DM & NASH-associated hepatic fibrogenesis remains obscure. We previously reported that the phytochemical curcumin inhibited the activation of hepatic stellate cells (HSCs), the major effector cells during hepatic fibrogenesis, and protected the liver from fibrogenesis *in vitro* and *in vivo*. The aims of this study are to evaluate ox-LDL roles in activation of HSCs, to assess curcumin effects on eliminating the ox-LDL roles, and to further explore the underlying mechanisms. In this report, we observe that ox-LDL alters expression of genes closely relevant to HSC activation, which is eliminated by curcumin. Curcumin suppresses gene expression of lectin-like oxidized LDL receptor-1 (LOX-1), leading to the blockade of the transport of extracellular ox-LDL into cells. This suppressive effect of curcumin results from the interruption of Wnt signaling and the activation of peroxisome proliferator-activated receptor-gamma (PPAR γ). In conclusion, these results support our initial hypothesis and demonstrate that ox-LDL stimulates HSC activation, which is eliminated by curcumin by suppressing *lox-1* expression via interrupting Wnt signaling and stimulating PPAR γ activity. These results provide novel insights into roles of ox-LDL in T2DM & NASH-associated hepatic fibrogenesis and mechanisms by which curcumin suppresses ox-LDL-induced HSC activation, as well as the implication of curcumin in treatment of T2DM & NASH-associated hepatic fibrosis.

Keywords

Hepatic fibrosis; hepatic stellate cell; hypercholesterolemia; diabetes; phytochemical

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Introduction

Almost 6% of the world's adult population now lives with diabetes mellitus. Type II diabetes mellitus (T2DM), *i.e.* non-insulin-dependent diabetes mellitus, represents over 80% of all diabetics and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index 1. T2DM are often associated with non-alcoholic steatohepatitis (NASH) 2, 3. NASH is characterized by fat accumulation and inflammation in the liver. Approximate one third of NASH patients develop hepatic fibrosis and even cirrhosis 4. Both T2DM and NASH are most commonly present in obese patients with hypercholesterolemia, *i.e.* increased levels of plasma low-density lipoprotein (LDL) and oxidized LDL (ox-LDL) 4. However, the role of hypercholesterolemia in hepatic fibrogenesis remains obscure.

LDL becomes the highly reactive form of ox-LDL after lipid peroxidative modification. High concentrations of circulating ox-LDL are associated with high incidences of metabolic syndromes, such as type II diabetes and coronary heart disease 5. Cellular uptake of ox-LDL is mediated by binding to its scavenger receptors, such as lectin-like oxidized LDL receptor-1 (LOX-1), leading to the elevation of intracellular levels of ox-LDL and reactive oxygen species (ROS), as well as to the activation of intracellular signaling 6, 7. LOX-1 was originally identified as a major scavenger receptor for ox-LDL in endothelial cells, and was subsequently detected in many other cell types 7, 8. LOX-1 gene expression is induced by ox-LDL 9 and is up-regulated in obese patients with hyperlipidemia 6.

Hepatic fibrosis is a progressive disorder characterized by accumulation of extracellular matrix (ECM) components 10, 11. Hepatic stellate cells (HSCs) are the major effector cells during hepatic fibrogenesis and are the primary source of ECM production in the liver 10, 11. During liver injury, quiescent HSCs undergo dramatic phenotypic changes from vitamin A, fat-storing cells to proliferative myofibroblast-like cells with acquisition of fibrogenic properties 10, 11. This process is coupled with activation of signaling pathways for pro-fibrogenic transforming growth factor-beta (TGF β) 12, pro-mitogenic platelet-derived growth factor-beta (PDGF- β) 13 and Wnt signaling 14-16, as well as the depletion of peroxisome proliferator activated receptor-gamma (PPAR γ) 17-19. It is important to note that culturing quiescent HSCs on plastic plates causes spontaneous activation, mimicking the process seen *in vivo*, which provides a good model for elucidating underlying mechanisms of HSC activation and for studying therapeutic intervention of the process 10, 11.

Curcumin (diferuloylmethane), the yellow pigment in curry from turmeric, is one of the best-studied natural compounds. Although the underlying mechanisms remain elusive, curcumin has shown diverse and versatile beneficial effects, including anti-inflammatory, anti-oxidative stress, anti-viral, anti-hypercholesterolemic, anti-infective and anti-carcinogenic effects 20. Curcumin has recently received attention as a promising dietary supplement for liver protection 21. We recently reported that curcumin inhibited HSC activation by inhibiting cell proliferation, inducing apoptosis and attenuating oxidative stress *in vitro* and *in vivo* 22-27. In addition, we demonstrated that curcumin dramatically induced gene expression of endogenous PPAR γ and stimulated its activity in activated HSCs *in vitro*

and *in vivo*, which is required for curcumin to inhibit HSC activation 22-24. Furthermore, curcumin suppressed gene expression of LDL receptor in activated HSCs *in vitro* by activating PPAR γ and regulating gene expression of the transcription factors sterol regulatory element binding proteins (SREBPs), leading to the reduction in the level of intracellular cholesterol and to the attenuation of the stimulatory effects of LDL on HSC activation (Kang and Chen, manuscript accepted by British Journal of Pharmacology, in press).

The aims of this study are to evaluate the role of ox-LDL in activating HSCs, to assess the effects of curcumin on eliminating the stimulatory role, and to further explore the underlying mechanisms. Results in the current report supported our initial hypothesis that ox-LDL might stimulate HSC activation, which could be eliminated by curcumin by suppressing gene expression of LOX-1.

Materials and Methods

Isolation and culture of rat HSCs and chemicals

Primary HSCs were isolated from male Sprague-Dawley rats (200-250g) as we previously described 23. Passaged HSCs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Cultured HSCs were used at Passage #4-9. Human oxidized LDL was purchased from Intracel Company (Frederick, MD) and used before expiration. The presence of endotoxin in ox-LDL was < 0.1 units/ml, as measured by *Limulus* Amebocyte lysate assay kit (Whittaker M.A. Bioproducts, Walkersville, MD). Wnt3a was purchased from R & D Systems (Minneapolis, MN). PPAR γ antagonist PD68235 was kindly provided by Pfizer (Ann Arbor, MI). Curcumin (purity > 94%) and κ -carrageenan were purchased from Sigma (St. Louis, MO). 15-deoxy-^{12,14}-prostaglandin J₂ (PGJ₂), a natural PPAR γ agonist, was a product of Cayman Chemical Company (Ann Arbor, MI).

Western blotting analyses

Protein samples were prepared from whole cell extracts, separated by 10% SDS-PAGE, and electro-blotted onto PVDF membrane. Target proteins were visualized by using the ECL method (Amersham, Piscataway, NJ). Mouse anti-LOX-1 monoclonal antibody was kindly provided by Dr. Tatsuya Sawamura (National Cardiovascular Center Research Institute, Japan). Other primary and secondary antibodies were presented in Table 1. Densities of bands in Western blotting analyses were normalized with the internal invariable control. Levels of target protein bands were densitometrically determined by using Quantity One[®] 4.4.1 (Bio-Rad, Hercules, CA). Variations in the density were expressed as fold changes compared to the control in the blot.

Immuno-staining

Serum-starved HSCs were treated with or without human ox-LDL (10 μ g/ml) in the presence or absence of curcumin (20 μ M) for 24 hr before the fixation with 4% paraformaldehyde for 15 min at room temperature (RT). After blocking with 5% BSA for 1 hr, fixed cells were incubated with anti-human ox-LDL antibody (1: 100) (Cat# AB3230,

Chemicon Company, Billerica, MA) overnight at 4°C. After three washes with phosphate-buffered saline (PBS), sections on slides were incubated with goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (1:500) (Invitrogen, Carlsbad, CA) at RT for 1 hr. Sections incubated with secondary antibodies alone were used as negative controls (data not shown). DAPI (100ng/ml) (Molecular Probes Company, Eugene, Oregon) was added in the mounting solution for nuclear staining. Sections were viewed under Leica DM 400 B microscope (North Central Instruments, Maryland Heights, MO).

Preparation of nuclear protein extracts

Nuclear extracts were prepared as we previously described 28. In brief, after washing twice with PBS, cells were evenly re-suspended in Buffer A (10 mM HEPES-KOH pH7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM PMSF). After incubation on ice for 10 min, cells were mixed by vortex for 30 seconds and centrifuged at 8000g for 10 seconds. Pellets were re-suspended in Buffer C (20 mM HEPES-KOH pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 15 min before vortex. The lysates were centrifuged at 8000g at 4°C for 2 min, and the resulting supernatants were taken as nuclear protein extracts, and stored at -80°C until use.

Plasmids and transient transfection assays

The *lox-1* promoter luciferase reporter plasmid (p-2336/+36-Luc), containing a 2336 bp fragment of the 5'-flanking promoter region of the LOX-1 gene, and some of the reporter plasmids with various lengths of the *lox-1* promoter were kindly provided by Dr. Jawahar L. Mehta (Department of Internal Medicine, University of Arkansas for Medical Sciences) 29. Additional *lox-1* promoter luciferase reporter constructs with shorter promoter regions were created by PCR and enzyme ligation using p-2336/+36-Luc as a template (see the following for details). The PDGF-βR promoter luciferase reporter plasmid pPDGF-βR-Luc (pβ12) was a gift from Dr. Keiko Funa (Ludwig Institute for Cancer Research, Uppsala, Sweden) 30. The type I TGF-β receptor promoter luciferase reporter plasmid pTβ-RI-Luc (pES1.0) was kindly provided by Dr Michael Centrella (Yale University, New Haven, CT, USA) 31. The PPARγ cDNA expression plasmid pPPARγ, containing a full size of PPARγ cDNA, was a gift from Dr. Reed Graves (Department of Medicine, University of Chicago). The dominant negative PPARγ expression construct pdn-PPARγ was a gift from Dr. Krishna V. Chatterjee 32. The Wnt signaling luciferase reporter plasmids TOPflash and its mutant FOPflash were kindly provided by Dr. Randall T. Moon (Department of Pharmacology, School of Medicine, University of Washington) 33. TOPflash contained 8 copies of TCF/LEF binding sites. FOPflash was a counterpart control for TOPflash with site-directed mutations in the TCF/LEF binding sites 33.

Transient transfection of semi-confluent HSCs in 6-well plates was performed using the LipofectAMINE[®] reagent (Invitrogen Corp.), as we previously described 23. Each treatment had triplicates in every experiment. Each experiment was repeated, at least, three times. Luciferase activity assays were conducted as we previously described 23. Transfection efficiency was determined by co-transfection of a β-galactosidase reporter, pSV-β-gal (Promega, Madison, WI). β-galactosidase assays were performed using an assay kit from Promega Corp, according to the manufacturer's instruction. Luciferase activities were

expressed as relative unit after normalization with β -galactosidase activities. Results were combined from, at least, three independent experiments.

Generation of a LOX-1 cDNA expression plasmid

Total RNA was extracted from HSCs using Trizol reagent and reversely transcribed into cDNA, which was used as a template for PCR amplification of LOX-1 cDNA. The PCR primers were designed according to GenBank accession number: NM_138648, and use the following primers: (F) 5'-CCC AAG CTT ATG ACT TTT GAT GAC AAG ATG AAG C-3'; (R) 5'-GG GGT ACC CC CTA AAT TTG CAA ATG ATTT GTC TTC-3'. The primers were tailed with *HindIII* site (the forward primer) and *KpnI* site (the reverse primer). The PCR product was subcloned into pCDNA3.1/Zeo+ expression vector (Invitrogen Corp. Carlsbad, CA) at *HindIII/KpnI* sites. The subcloned LOX-1 cDNA was verified by DNA sequencing.

Promoter deletion and site-directed mutagenesis

The *lox-1* promoter luciferase reporter plasmid p-2336/+36-Luc, also termed pLOX-1-Luc, was used as a PCR template to generate constructs with various lengths of the gene promoter. Primers in Table 2 were used for generating the plasmids by PCR. The primers were tailed with *KpnI* site (forward primers) or *BglII* site (reverse primer), and the PCR products were subcloned into pGL3-Basic (Promega) at *KpnI/BglII* sites. The site-directed mutagenesis was carried out using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen), according to the manufacture's instruction. The sequence of 5'-GGC ACA TTT TTT ACA AAT GTA GTG TGA CTT ACT CTC TTT GAA TTT CAG TTT C-3' (-310 to -258) containing the putative TCF/LEF-1 binding site in the plasmid p-2336/+36-Luc, was mutated to 5'-GGC ACA TTT TTT ACA AAT GTA GTG TGA CTT ACT CTC ATA GAA TTT CAG TTT C-3' in p-2336/+36-Mut-Luc. The primers used for mutagenesis were in Table 3. The mutations were verified by DNA sequencing.

Real-time polymerase chain reactions (PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen) following the manufacturer's instruction. Real-time PCR was carried out using SYBR green, as previously described 34. Total RNA was treated with DNase I prior to the synthesis of the first strand of cDNA. First-strand cDNA was synthesized using total RNA as templates and oligo-dT as primers. Samples were run on a Bio-Rad MyiQ™ real-time PCR machine (Bio-Rad). mRNA levels were expressed as fold changes after normalization with endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as suggested by Schmittgen et. al. 35. The primers for PCR were presented in Table 3.

Electrophoretic mobility shift assays (EMSA)

Synthesized single-stranded oligonucleotide probes were biotin-labeled using the Biotin 3' End DNA Labeling Kit (Pierce Company, Rockford, IL), and annealed to form oligonucleotide duplex after labeling. EMSA were conducted using LightShift Chemiluminescent EMSA kit (Pierce Company, Rockford, IL), following the protocol provided by the manufacturer. Briefly, the annealed, biotin-labeled double-stranded

oligonucleotide probes were incubated with 3 μ g of nuclear extracts at room temperature for 30 min. The mixtures were subjected to 6% non-denatured polyacrylamide gel electrophoresis, and electroblotted onto nylon membrane. Proteins bound to the biotin end-labeled oligonucleotide probes were detected using the streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate. The following probes were used. P(TCFwt) contained a putative TCF-1 binding site: 5'-GTG TGA CTT ACT CTC TTT GAA TTT CAG TTT CTG TC-3' (-289/-259). P(TCFmut) contained a mutated TCF-1 binding site: 5'-GTG TGA CTT ACT CTC ATA GAA TTT CAG TTT CTG TC-3'.

Statistical analyses

Differences between means were evaluated by an unpaired two-sided Student's *t*-test ($P < 0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with controls were analyzed by ANOVA with the Dunnett's test for post hoc analysis.

Results

Ox-LDL induces expression of genes relevant to the activation of HSCs, which is abrogated by curcumin in cultured HSCs

It was previously demonstrated that ox-LDL prompted biosynthesis of ECM components in HSCs, implying ox-LDL being a pro-fibrogenic stimulator 36. We further examined the effect of ox-LDL on expression of genes closely relevant to HSC activation. Semi-confluent cultured HSCs were serum-starved for 24 hr before the stimulation with exogenous ox-LDL at various concentrations (0-15 μ g/ml) in serum-depleted media for additional 24 hr. Serum starvation rendered cells more sensitive to stimuli, including exogenous ox-LDL.

Subsequent culture in serum-depleted media excluded the interference from other factors in FBS 37, 38. Total RNA and whole cell protein extracts were prepared from the cells for real-time PCR and Western blotting analyses, respectively. As shown in Fig. 1A & B, ox-LDL increased gene expression of α (I) collagen and alpha-smooth muscle actin (α -SMA), the markers of activated HSCs, in a dose-dependent manner. In addition, ox-LDL dose-dependently stimulated expression of pro-fibrogenic genes, including connective tissue growth factor (CTGF) and type I and II TGF- β receptors (T β -RI & T β -RII), as well as pro-mitogenic genes, including receptors for PDGF- β (PDGF- β R) and EGF (EGFR), as well as cyclin D1, a key regulator in cell cycle progression. These data collectively indicated that exogenous ox-LDL markedly induced expression of genes closely relevant to HSC activation, suggesting the role of ox-LDL in the stimulation of HSC activation *in vitro*.

To evaluate the effect of curcumin on the elimination of the stimulatory role of ox-LDL in the activation of HSCs, serum-starved HSCs were stimulated with ox-LDL (10 μ g/ml) in the presence of curcumin at various concentrations (0-30 μ M) in serum-depleted media for 24 hr. Total RNA and whole cell extracts were prepared to analyze expression of genes related to HSC activation by real-time PCR and Western blotting analyses. As indicated in Fig. 1C & D, curcumin dose-dependently inhibited expression of the pro-fibrogenic and pro-mitogenic genes induced by ox-LDL in cultured HSCs. Taken together, these results

demonstrated that ox-LDL induced expression of genes closely relevant to the activation of HSCs, which was dose-dependently abrogated by curcumin.

LOX-1 plays a mediating role in ox-LDL-induced HSC activation and in the curcumin elimination of the stimulatory effect of ox-LDL

LOX-1 has been identified as a specific receptor for ox-LDL, responsible for transporting extracellular ox-LDL into cells 6, 7. LOX-1, as a scavenger receptor, facilitates the accumulation of ox-LDL and stimulates the transformation of smooth muscle cells and monocyte/macrophages into foam cells 39. LOX-1 gene expression is induced by ox-LDL 9. We assumed that LOX-1 mediated the stimulatory effects of ox-LDL on HSC activation and fibrogenesis. To elucidate the link role of LOX-1 in ox-LDL-induced HSC activation, serum-starved HSCs were pre-treated with or without κ -carrageenan (250 $\mu\text{g/ml}$), a LOX-1 antagonist 40, 41, for 1 hr prior to the stimulation with ox-LDL (10 $\mu\text{g/ml}$) for additional 24 hr. Whole cell extracts were prepared for Western blotting analyses of genes relevant to HSC activation. As shown in Fig. 2A, compared with the untreated control (the corresponding 1st wells), ox-LDL, as expected, significantly increased the abundance of proteins closely relevant to HSC activation, including pro-mitogenic PDGF- β R and EGFR and pro-fibrogenic CTGF, T β -RI and T β -RII (the corresponding 2nd wells). It was of interest to observe that the pretreatment with the LOX-1 antagonist κ -carrageenan dramatically eliminated the stimulatory effects of ox-LDL on the abundance of the proteins (the corresponding 4th wells). These results suggested a direct link of LOX-1 between ox-LDL and HSC activation and fibrogenesis.

To determine the role of LOX-1 in the curcumin elimination of the stimulatory effects of ox-LDL, HSCs were co-transfected with LOX-1 cDNA expression plasmid pLOX-1 at various doses plus the PDGF- β R gene promoter luciferase reporter plasmid pPDGF- β R-Luc, or the T β -RI gene promoter luciferase reporter plasmid pT β -RI-Luc. A total of 4.5 μg of plasmid DNA per well was used for co-transfection of HSCs in 6-well culture plates. It included 2 μg of pPDGF- β R-Luc or pT β -RI-Luc, 0.5 μg of pSV- β -gal, and 2.0 μg of pLOX-1 at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were stimulated with or without ox-LDL (10 $\mu\text{g/ml}$) in the presence or absence of curcumin (20 μM) in serum-depleted media for 24 hr. As shown in Fig. 2B by luciferase activity assays, compared with the untreated control (the corresponding 1st columns), ox-LDL, as expected, significantly increased luciferase activities in the cells (the corresponding 2nd columns). Curcumin significantly diminished the ox-LDL-elevated luciferase activities (the corresponding 3rd columns). Further experiments revealed that forced expression of LOX-1 cDNA dose-dependently eliminated the inhibitory role of curcumin and elevated luciferase activities (the last three corresponding columns). These results indicated that forced expression of LOX-1 cDNA eliminated the inhibitory effect of curcumin on the ox-LDL-induced promoter activities of pro-mitogenic PDGF- β R and pro-fibrogenic T β -RI genes. Taken together, our results demonstrated the mediating role of LOX-1 in ox-LDL-induced HSC activation and in the curcumin elimination of the stimulatory effect of ox-LDL.

Curcumin suppresses *lox-1* expression in cultured HSCs

Based on the above observations, we hypothesized that curcumin might eliminate the stimulatory effects of ox-LDL on the activation of HSCs by suppressing gene expression of LOX-1, leading to the reduction in the bioavailability of the receptor to transport extracellular ox-LDL into HSCs. To test the hypothesis, cultured HSCs were transiently transfected with the LOX-1 promoter luciferase reporter plasmid pLOX-1-Luc. This plasmid contained a 5'-flanking fragment (2336 bp) of the LOX-1 gene promoter, subcloned in a luciferase reporter plasmid 29. After overnight recovery, cells were treated with curcumin at various concentrations (0-30 μM) for 24 hr. As shown in Fig. 3A by luciferase activity assays, curcumin caused a dose-dependent reduction in luciferase activities, suggesting that curcumin reduced the promoter activity of LOX-1 gene in HSCs. To verify the observation, semi-confluent passaged HSCs were treated with curcumin at various concentrations (0-30 μM) for 24 hr. Total RNA and whole cell extracts were prepared for real-time PCR and Western blotting analyses. As shown in Fig. 3B & C, curcumin dose-dependently reduced the steady state mRNA levels and the protein abundance of LOX-1 in the cells.

To evaluate the impact of curcumin on the level of intracellular ox-LDL, serum-starved HSCs were treated with or without human ox-LDL (10 $\mu\text{g}/\text{ml}$) in the presence or absence of curcumin (20 μM) for 24 hr. Results from immuno-cytochemistry in Fig. 3D indicated that ox-LDL treatment significantly increased the abundance of intracellular ox-LDL, which was apparently ameliorated by curcumin. Taken together, these results collectively demonstrated that curcumin suppressed gene expression of LOX-1 in activated HSCs *in vitro*, which likely contributed to the reduction in the abundance of intracellular ox-LDL.

The activation of PPAR γ likely plays a critical role in the curcumin-caused inhibition of *lox-1* expression in activated HSCs

We have previously reported that curcumin induces expression of endogenous PPAR γ gene and stimulates its activity in activated HSCs, which is a prerequisite for curcumin to inhibit HSC activation *in vitro*^{23, 24}. To elucidate the mechanisms by which curcumin suppressed gene expression of LOX-1 in HSCs, we postulated that PPAR γ mediated the inhibitory effect of curcumin on *lox-1* expression. To test this postulation, HSCs were transiently transfected with the LOX-1 promoter luciferase reporter pLOX-1-Luc. After recovery, cells were pretreated with or without the PPAR γ antagonist PD68235 (20 μM) for 30 min prior to the addition of curcumin at 20 μM for additional 24 hr. As shown in Fig. 4A by luciferase activity assays, compared to the untreated control cells (the 1st column on the left), cells treated with curcumin showed a significant reduction in luciferase activity (the 2nd column), confirming the inhibitory impact of curcumin on the *lox-1* promoter activity in HSCs. In great contrast, the pretreatment of cells with the PPAR γ antagonist PD68235 abrogated this inhibitory impact of curcumin (the 4th column), suggesting the necessity of the activation of PPAR γ in the curcumin-caused inhibition of the *lox-1* promoter activity. To verify the observation, HSCs were pretreated with or without PD68235 (20 μM) for 30 min prior to the addition of curcumin (20 μM) for additional 24 hr. Whole cell extracts were prepared for Western blotting analyses. As demonstrated in Fig. 4B, compared to the untreated control (the 1st well), curcumin, as expected, significantly reduced the abundance of LOX-1 in the cells (the 2nd well). The inhibition of the PPAR γ activity by the pretreatment with PD68235

apparently eliminated the inhibitory effect of curcumin on the abundance of LOX-1 (the 4th well). These results collectively suggested that the activation of PPAR γ might play a critical role in the curcumin-caused inhibition of *lox-1* expression in activated HSCs *in vitro*.

The activation of PPAR γ results in the suppression of *lox-1* expression in activated HSCs *in vitro*

To verify the role of PPAR γ activation in the inhibition of *lox-1* expression, semi-confluent HSCs were co-transfected with pLOX-1-Luc and the plasmid pPPAR γ , or the plasmid pdn-PPAR γ , at indicated doses. The cDNA expression plasmid pPPAR γ contained a full size of wild-type PPAR γ cDNA 24. pdn-PPAR γ contained a full length of cDNA encoding dominant negative PPAR γ (dn-PPAR γ) 32. A total of 4.5 μ g of plasmid DNA per well was used for co-transfection of HSCs in 6-well culture plates. It included 2 μ g of pLOX-1-Luc, 0.5 μ g of pSV- β -gal, and 2.0 μ g of pPPAR γ , or pdn-PPAR γ , at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were treated with or without curcumin (20 μ M) in DMEM with FBS (10%) for 24 hr. Prior experiments have suggested that 10% of FBS in the medium contains enough agonists to activate PPAR γ in HSCs 19, 23, 24. Luciferase activity assays demonstrated that forced expression of wild-type PPAR γ cDNA dose-dependently reduced luciferase activities (Fig. 5A). In contrast, forced expression of dn-PPAR γ dose-dependently abrogated the inhibitory effect of curcumin on the *lox-1* promoter activity (Fig. 5B). These results collectively suggested the inhibitory role of PPAR γ activation and its requirement in the curcumin-caused inhibition of the *lox-1* promoter activity.

To further confirm the inhibitory role of PPAR γ activation, serum-starved HSCs were treated with the natural PPAR γ agonist 15-deoxy-^{12,14}-prostaglandin J₂ (PGJ₂) at 0-15 μ M for 24 hr. Total RNA and whole cell extracts were prepared for real-time PCR and Western blotting analyses. As showed in Fig. 5C & D, the activation of PPAR γ by PGJ₂ dose-dependently reduced the levels of the transcript and protein of LOX-1. Taken together, our data demonstrated that the activation of PPAR γ resulted in the suppression of *lox-1* expression in activated HSCs *in vitro*.

Identification of putative curcumin response element(s) in the *lox-1* promoter

Additional experiments were conducted to elucidate the molecular mechanisms by which curcumin inhibited the *lox-1* promoter activity in HSCs. To localize curcumin response element(s) in the *lox-1* promoter, a group of luciferase reporter plasmids with various lengths of the *lox-1* promoter region, including 2336 base pair (bp), 996 bp, 289 bp, 259 bp and 99 bp nucleotides, were used. Passaged HSCs were transfected with the group of luciferase reporter plasmids. After recovery, cells were treated with or without curcumin at 20 μ M for 24 hr. As shown in Fig. 6A, compared to the corresponding untreated control, curcumin significantly reduced luciferase activities by approximately 50% in cells transfected with a plasmid containing a 5'-flanking fragment longer than -289 bp nucleotides of the *lox-1* promoter. However, loss of the promoter region between -289 and -259 bp nucleotides in p259-Luc resulted in a marked reduction in luciferase activities and in response to curcumin. Although cells transfected with p259 or pp99 still showed the response to the inhibitory effect of curcumin, the difference in luciferase activities between

cells with or without curcumin treatment was dramatically reduced from ~50% to ~30%. We could not exclude the presence of additional curcumin response element(s) within the promoter region of -259 bp, even -99 bp. However, our results suggested that a major curcumin response element(s) might be located within the fragment between -289 and -259 bp, which also controlled the basal transcription activity of the promoter. We, therefore, paid our major attention to this region and studied the major curcumin response element(s) in the *lox-1* promoter.

Computer-aided analyses found a putative T cell factor/lymphoid enhancer factor (TCF/LEF) binding site, *i.e.* CTTTGA, at -275bp to -269bp within the promoter region. TCF/LEF binding sites are targets of canonical Wnt signaling and bound by a complex of β -catenin with TCF/LEF, mediating the regulation of target gene transcription 42, 43. The plasmid pLOX-1(mut)-Luc with site-directed mutation in the TCF/LEF-1 binding site was generated from the parental plasmid pLOX-1-Luc. Passaged HSCs were transfected with pLOX-1-Luc or pLOX-1(mut)-Luc and subsequently treated with or without curcumin (20 μ M) for 24 hr. Luciferase activity assays in Fig. 6B demonstrated that compared to the untreated control, curcumin significantly reduced, as expected, luciferase activities by 50.5% in cells transfected with wild-type pLOX-1-Luc. However, the site-directed mutation of the TCF/LEF-1 binding site in pLOX-1(mut)-Luc resulted in a significant reduction from ~50% to ~25% in the difference of luciferase activities between cells treated with or without curcumin, indicating a significant loss in response to curcumin. In addition, compared to pLOX-1-Luc, pLOX-1(mut)-Luc also led to an apparent reduction in luciferase activities in cells without curcumin treatment, suggesting an important role of the TCF/LEF-1 binding site in regulating the basal promoter activity of LOX-1 in HSCs. Taken together, these results *suggested* that the TCF/LEF-1 binding site might be responsible for *both* controlling the basal promoter activity, *as well as* responding to the curcumin treatment, *i.e.* a major curcumin response element, which played a critical role in the curcumin-caused inhibition of the *lox-1* promoter activity.

The TCF/LEF-1 binding site in the *lox-1* promoter mediates canonical Wnt signaling and PPAR γ in the regulation of the *lox-1* promoter activity in HSCs

Our earlier results demonstrated the roles of curcumin and PPAR γ activation in the inhibition of the *lox-1* promoter activity and gene expression (Fig. 3-5). Results in Fig. 6 suggested that the TCF/LEF-1 binding site was the curcumin response element, which might mediate the curcumin-caused inhibition of the *lox-1* promoter activity. To explore the underlying mechanisms, it is plausible to hypothesize that the curcumin-activated PPAR γ interrupted canonical Wnt signaling, leading to the reduction in the *lox-1* promoter activity and to the inhibition of *lox-1* expression in HSCs. To test the hypothesis, HSCs were co-transfected with the plasmid pLOX-1-Luc, or the mutant plasmid pLOX-1(mut)-Luc, and the PPAR γ cDNA expression plasmid pPPAR γ or the empty control vector pcDNA. After recovery, cells were cultured for 24 hr in DMEM with FBS (10%), which contained enough agonists to activate PPAR γ in HSCs 19, 23, 24. Luciferase activity assays in Fig. 7A demonstrated that forced expression of PPAR γ from pPPAR γ significantly reduced luciferase activity by 63.4% in cells co-transfected with the wild-type plasmid pLOX-1-Luc (the upper black column), compared to the empty control vector pcDNA (the upper white

column). In great contrast, forced expression of PPAR γ from pPPAR γ failed to reduce luciferase activity in cells co-transfected with the mutant plasmid pLOX-1(mut)-Luc (the lower black column), compared to the empty control vector pcDNA (the lower white column). Luciferase activity was much lower in cells transfected with pLOX-1(mut)-Luc (the lower white column), compared to that in cells transfected with the wild-type pLOX-1-Luc (the upper white column), confirming the role of the TCF/LEF-1 binding site in controlling the basal activity of the gene promoter observed in Fig. 6.

To further test our hypothesis, HSCs were transfected with pLOX-1-Luc, or the mutant pLOX-1(mut)-Luc. After over-night recovery, cells were serum-starved for 24 hr prior to the stimulation with or without exogenous Wnt3a (50 ng/ml), a stimulator of canonical Wnt signaling, in serum-free medium for another 24 hr. As shown in Fig. 7B by luciferase activity assays, exogenous Wnt3a significantly increased luciferase activity by 38.5% in cells transfected with the wild-type pLOX-1-Luc. However, Wnt3a showed no stimulatory effect on luciferase activity in cells transfected with the mutant pLOX-1(mut)-Luc, verifying the critical role of the TCF/LEF-1 binding site in regulating the *lox-1* promoter activity in HSCs. These results collectively indicated that the TCF/LEF-1 binding site in the *lox-1* promoter mediated both canonical Wnt signaling and PPAR γ in the regulation of the *lox-1* promoter activity in HSCs.

Activation of canonical Wnt signaling induces *lox-1* expression, which is dose-dependently eliminated by curcumin in activated HSCs

Additional experiments were conducted to clarify the impact of canonical Wnt signaling on regulating *lox-1* expression in activated HSCs. HSCs were transfected with the *lox-1* promoter luciferase reporter plasmid pLOX-1-Luc. After recovery, cells were serum-starved for 3 hr prior to the stimulation with Wnt3a at 0-100 ng/ml in serum-free medium for additional 24 hr. Luciferase activity assays demonstrated that the activation of canonical Wnt signaling by exogenous Wnt3a dose-dependently increased luciferase activities in the cells (Fig. 8A), suggesting the stimulatory effect of Wnt signaling on the *lox-1* promoter activity. This observation was confirmed by Western blotting analyses in serum-starved HSCs treated with Wnt3a at 0-100 ng/ml in serum-depleted media for 24 hr (Fig. 8B). Wnt3a dose-dependently increased the abundance of LOX-1 in the cells.

Further experiments examined whether curcumin could antagonize the stimulatory effect of Wnt3a on *lox-1* expression. Serum-starved HSCs were pretreated with curcumin at different concentrations for 30 min prior to the stimulation with exogenous Wnt3a (50 ng/ml) for 24 hr. Whole cell extracts were prepared for Western blotting analyses. It was revealed that curcumin dose-dependently attenuated the stimulatory effect of Wnt3a on the abundance of LOX-1 in activated HSCs (Fig. 8C). Taken together, these results demonstrated that the activation of Wnt signaling induced *lox-1* expression in activated HSCs *in vitro*, which was dose-dependently eliminated by curcumin.

Curcumin interrupts canonical Wnt signaling in activated HSCs *in vitro*

To explore the mechanisms by which curcumin eliminated the stimulatory effect of Wnt signaling on the induction of *lox-1* expression, we assumed that the activation of PPAR γ by

curcumin antagonistically interacted with the Wnt signaling pathway and led to the interruption of canonical Wnt signaling in activated HSCs. To test the assumption, passaged HSCs were transfected with the plasmid TOPflash, or FOPflash. TOPflash was a canonical Wnt signaling luciferase reporter, which contained 8 copies of TCF/LEF binding sites³³. FOPflash was used as a control luciferase reporter, which contained 8 copies of *mutant* TCF/LEF binding sites³³. After recovery, cells were treated with curcumin at various concentrations (0-30 μ M) in DMEM with FBS (10%) for 24 hr. Luciferase activity assays in Fig. 9A demonstrated that curcumin caused a dose-dependent reduction in luciferase activities in cells transfected with TOPflash. However, curcumin had no impact on luciferase activities in cells transfected with FOPflash. These results suggested that curcumin interrupted canonical Wnt signaling in HSCs.

Canonical Wnt signaling is activated only when dephosphorylated β -catenin is translocated into the nucleus⁴². To verify the effect of curcumin on interrupting the Wnt signaling pathway, HSCs were treated with curcumin at various concentrations (0-30 μ M) for 24 hr. Total nuclear proteins were prepared and the abundance of nuclear β -catenin was evaluated by Western blotting analyses. It was revealed in Fig. 9B that curcumin dose-dependently reduced the abundance of nuclear β -catenin. The nuclear protein Histone H1 was used as an invariant control.

Nuclear β -catenin forms a complex with the transcription factor TCF/LEF to bind to the promoter of target genes and to stimulate target gene transcription^{42, 43}. EMSA were conducted to further verify the effect of curcumin on interrupting canonical Wnt signaling. Nuclear protein extracts from HSCs treated with curcumin at 0-30 μ M for 24 hr were incubated with biotin-labeled double-stranded oligonucleotide probe P(TCFwt). The probe P(TCFwt) contained the consensus TCF/LEF binding site found in the *lox-1* promoter. As shown in Fig. 9C, curcumin dose-dependently reduced the DNA binding activity of the transcription factor TCF/LEF to the probe. To examine the DNA binding specificity of TCF/LEF to the probe, competition assays were performed using a 10-, 50-, or 100-fold excess of the unlabeled probe P(TCFwt) (lanes 3-5 in Fig. 9D), or the unlabeled probe P(TCFmut) (lanes 6-8 in Fig. 9D). The probe P(TCFmut) contained the TCF/LEF binding site found in the *lox-1* promoter with site-directed mutations. It was found that the amount of TCF/LEF binding to the biotin-labeled probe P(TCFwt) was competitively reduced by the unlabeled probe P(TCFwt), but not by the mutant probe P(TCFwt). These results indicated that curcumin dose-dependently reduced the DNA binding activity of TCF/LEF to the promoter of LOX-1 gene in activated HSCs. Taken together, these results indicated that curcumin interrupted canonical Wnt signaling in activated HSCs by reducing the abundance of nuclear β -catenin and reducing the DNA binding activity of TCF/LEF.

The activation of PPAR γ by curcumin results in the interruption of canonical Wnt signaling in activated HSCs *in vitro*

Additional experiments were conducted to further test our above assumption. To evaluate the role of PPAR γ activation in the curcumin-caused interruption of canonical Wnt signaling, HSCs were transfected with the plasmid TOPflash or FOPflash. The cells were pre-treated with the PPAR γ antagonist PD68235 (20 μ M) for 30 min prior to the addition of

curcumin (20 μM) for additional 24 hr. Luciferase activity assays in Fig. 10A indicated that compared to the untreated control (the 1st black column on the left), curcumin reduced, as expected, luciferase activities in cells transfected with TOPflash (the 2nd black column). The pretreatment with PD68235 significantly attenuated the inhibitory effect of curcumin (the 4th black column). Curcumin with or without PD68235 caused no significant changes in luciferase activities in cells transfected with the control plasmid FOPflash (white columns). These results suggested that the activation of PPAR γ played a critical role in the curcumin-caused interruption of canonical Wnt signaling in HSCs.

To further confirm the inhibitory role of PPAR γ activation in the interruption of canonical Wnt signaling, HSCs were co-transfected with the plasmid TOPflash or FOPflash, plus the cDNA expression plasmid pPPAR γ . Transfected cells were cultured for 24 hr in media with 10% FBS, which contains enough agonists to activate PPAR γ in HSCs 19, 23, 24. Luciferase activity assays in Fig. 10B revealed that forced expression of PPAR γ dose-dependently reduced luciferase activities in cells transfected with TOPflash, but not in cells transfected with FOPflash, confirming that the activation of PPAR γ interrupted canonical Wnt signaling in HSCs. Additional experiments verified these observations and demonstrated that the activation of PPAR γ by PGJ₂ dose-dependently reduced luciferase activities in HSCs transfected with TOPflash (Fig. 10C). Taken together, these results indicated that the activation of PPAR γ by curcumin resulted in the interruption of canonical Wnt signaling in activated HSCs *in vitro*.

Discussion

NASH patients are often associated with high levels of lipid peroxidation products, such as ox-LDL 44. It has been suggested that the elevated levels of lipid peroxidation might make an important contribution to the pathogenesis of NASH 45, leading to hepatic fibrosis. In the current report, we demonstrated that ox-LDL stimulated the activation of HSCs *in vitro*, which was attenuated by curcumin by suppressing gene expression of LOX-1 via the activation of PPAR γ and the interruption of Wnt signaling.

The precise range of plasma ox-LDL concentrations in human remains unclear. Prior studies suggested that oxidized LDL was only a minor fraction of LDL ranging from 0.001% in healthy controls 46 to approximately 5% in patients with acute coronary events 47. A recent report indicated that the level of ox-LDL in healthy human was in 0.58 ± 0.23 ng/5 μg LDL protein 48. However, it bears emphasis that because the *in vivo* system is multi-factorial, directly extrapolating *in vitro* conditions and results, *e.g.* effective concentrations, to the *in vivo* system might be misleading. ox-LDL at 10 $\mu\text{g}/\text{ml}$ was chosen for most of our experiments. It showed no apparent cytotoxicity to cultured HSCs (data not presented here), though higher concentrations (>25 $\mu\text{g}/\text{ml}$) of ox-LDL might result in the reduction in the production of ECM components and even cytotoxicity to cultured HSCs 36.

Uptake and transport of ox-LDL into cells is mediated by a variety of scavenger receptors, including LOX-1, SR-AI/II, CD36 and SR-BI 49. Depending on cell types, these scavenger receptors display distinct expression profiles. For example, CD36 is highly expressed in macrophages, and plays a fundamental role in uptake of ox-LDL into macrophages 50, while

LOX-1 is the principal ox-LDL receptor in epithelia cells 7. CD36 is a multiligand scavenger receptor that recognizes and binds many ligands, including ox-LDL and long-chain free fatty acids 51. Our preliminary experiments indicated that compared to that of CD36, the expression of LOX-1 in activated HSCs is more inducible and highly expressed at both transcript and protein levels (unpublished observations), suggesting that LOX-1 might play a more important role in uptake and transport of extracellular ox-LDL into activated HSCs. We, therefore, focused our primary attention to the effects of curcumin on LOX-1 in this study. It bears emphasis that we do not exclude the roles of CD36 and other scavenger receptors in uptake and transport of ox-LDL into HSCs and their impacts on the ox-LDL-induced HSC activation. On the other hand, we could not exclude roles of curcumin in inhibiting gene expression of CD36 and other scavenger receptors in HSCs. Our preliminary experiments indicated that curcumin indeed suppressed gene expression of CD36 in activated HSCs *in vitro* (data not shown), which might collectively contribute to the curcumin-caused reduction in the level of intracellular ox-LDL observed in Fig. 3D.

During HSC activation, the abundance of PPAR γ is significantly reduced. We have previously shown that curcumin dramatically induces expression of endogenous PPAR γ gene and its activity in cultured HSCs, which is required for curcumin to inhibit HSC activation 23, 24, 27. We have also observed that although significantly reduced, PPAR γ is still detectable in cultured HSCs and responds to the stimulation with PGJ₂, a natural PPAR γ agonist 23, 38. To evaluate the role of PPAR γ activation in the curcumin-caused inhibition of LOX-1 gene expression in cultured HSCs, PPAR γ activation was blocked by its specific antagonist PD68235. Our results in Fig. 4 indicated that the blockade of PPAR γ activation apparently eliminated the role of curcumin in inhibiting gene expression of LOX-1 in cultured HSCs. Additional experiments revealed that forced expression of PPAR γ cDNA dose-dependently reduced the gene promoter activity of LOX-1 (Fig. 5A). On the other hand, forced expression of dominant negative PPAR γ cDNA dose-dependently eliminated the effect of curcumin on reducing the promoter activity of LOX-1 gene (Fig. 5B). Further more, the activation of PPAR γ by its natural agonist PGJ₂ dose-dependently suppressed gene expression of LOX-1 at both levels of transcription and translation (Fig. 5C & D). Taken together, our results demonstrated that the activation of PPAR γ played a critical role in the curcumin-caused inhibition of LOX-1 gene expression in activated HSCs *in vitro*. Our current observations are consistent with prior other reports 6, 52. The activation of PPAR γ by its ligand pioglitazone inhibited LOX-1 expression in coronary artery endothelial cells and fibroblasts 6, 52. In addition, PPAR γ activation inhibited TNF- α -induced LOX-1 expression in aortic endothelial cells 53. On the other hand, the stimulatory effect of PPAR γ on *lox-1* expression was also reported 54. PPAR γ bound to the peroxisome proliferator response elements (PPREs) in the *lox-1* promoter and stimulated LOX-1 gene transcription and expression in adipocytes 54. These observations indicate that the roles of PPAR γ in the regulation of *lox-1* expression are divergent and might greatly depend on cell types. Additional experiments are necessary to clarify the underlying mechanisms.

In the current study, we reported that the activation of PPAR γ by curcumin antagonized canonical Wnt signaling (Fig. 10), likely leading to the suppression of the *lox-1* expression and the inhibition of ox-LDL-induced HSC activation. To determine the role of the Wnt

signaling in the regulation of LOX-1 gene expression and in the curcumin-caused inhibition of LOX-1 gene expression in activated HSCs *in vitro*, we observed that the activation of the Wnt signaling by its agonist Wnt3a dose-dependently induced gene expression of LOX-1 in HSCs, including increasing the promoter activity (Fig. 8a) and elevating the abundance of LOX-1 protein (Fig. 8B). In addition, curcumin dose-dependently eliminated the stimulatory effect of the Wnt signaling activated by Wnt3a on expression of LOX-1 gene in cultured HSCs (Fig. 8C). Additional experiments revealed that curcumin interrupted Wnt signaling (Fig. 9A), reduced the abundance of nuclear β -catenin (Fig. 9B), decreased the DNA binding activity of the Wnt signaling target TCF (Fig. 9C). Taken together, our results demonstrated that the interruption of the Wnt signaling pathway played a key role in the curcumin-caused inhibition of LOX-1 gene expression in activated HSCs *in vitro*.

In contrast to non-canonical β -catenin-independent Wnt signaling, canonical Wnt signaling involves a series of events that is initiated by binding an extracellular Wnt protein, including Wnt3a, to a member of the family of Frizzled transmembrane receptors 42, 43. This interaction activates the Dishevelled protein by hyper-phosphorylation, which results in the prevention of the phosphorylation of β -catenin by the degradation complex (GSK-3, APC, and Axin) and the release of β -catenin from the degradation complex. Free β -catenin forms a complex with TCF/LEF and translocates into the nucleus, stimulating target gene transcription 42, 43. Although its effects remain controversial 55, accumulating evidence has indicated that activation of the canonical Wnt signaling pathway stimulates HSC activation and hepatic fibrogenesis 56, 57. We demonstrated in this report that curcumin significantly reduced the level of nuclear β -catenin in HSCs. In addition, the activation of PPAR γ by curcumin interrupted Wnt signaling, leading to the suppression of *lox-1* expression and the inhibition of ox-LDL-induced HSC activation. Our results are supported by prior observations that the activation of PPAR γ antagonistically interrupted Wnt signaling by inducing the degradation of β -catenin 58-60. In addition, the interruption of Wnt signaling inhibited HSC activation 15, 16. Other studies also showed the roles of the Wnt signaling pathway and the transcription factor TCF/LEF-1 in mediating curcumin actions. For example, curcumin treatment caused p53- and p21-independent G(2)/M phase arrest and apoptosis in colon cancer cell lines. Further experiments found that curcumin caused a caspase-3-mediated cleavage of β -catenin, decreased *trans*-activation of β -catenin/TCF, decreased promoter DNA binding activity of the β -catenin/TCF complex, and decreased levels of c-Myc protein in the colon cancer cells 61. Studies also indicated that curcumin and its derivatives attenuated the Wnt/ β -catenin pathway through down-regulation of the transcriptional coactivator p300 62. It was even suggested that curcumin and its derivative were excellent inhibitors of β -catenin/TCF signaling in cancer cell lines 63. The reduced β -catenin/TCF transcriptional activity was due to the decreased nuclear β -catenin and TCF-4 63.

Based on the present results, we propose a simplified action model for curcumin to inhibit ox-LDL-induced HSC activation (Fig. 11). Extracellular ox-LDL is transported into HSCs mediated by LOX-1, leading to the stimulation of HSC activation. This process is blocked by the curcumin-caused suppression of LOX-1 gene expression by activating PPAR γ and interrupting Wnt signaling. It bears emphasis that the underlying mechanisms are certainly

more complex than what is described here. In addition, our results do not exclude possible involvement of any other signaling pathways and mechanisms in the curcumin-caused suppression of LOX-1 gene expression and in the inhibition of ox-LDL-induced HSC activation. Our observations provide novel insights into the molecular mechanisms of curcumin in inhibiting ox-LDL-induced HSC activation and offer a natural anti-fibrotic candidate for the therapeutic treatment and prevention of T2DM & NASH-associated hepatic fibrosis.

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Abbreviations

α-SMA	alpha-smooth muscle actin
CTGF	connective tissue growth factor
DMEM	Dulbecco's modified Eagle's medium
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HSCs	hepatic stellate cells

LDL	low-density lipoprotein
LOX-1	lectin-like oxidized LDL receptor-1
NAFLD	nonalcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
ox-LDL	oxidized low-density lipoprotein
PDGF-βR	platelet-derived growth factor-beta receptor
TGF-βR	transforming growth factor-beta receptor

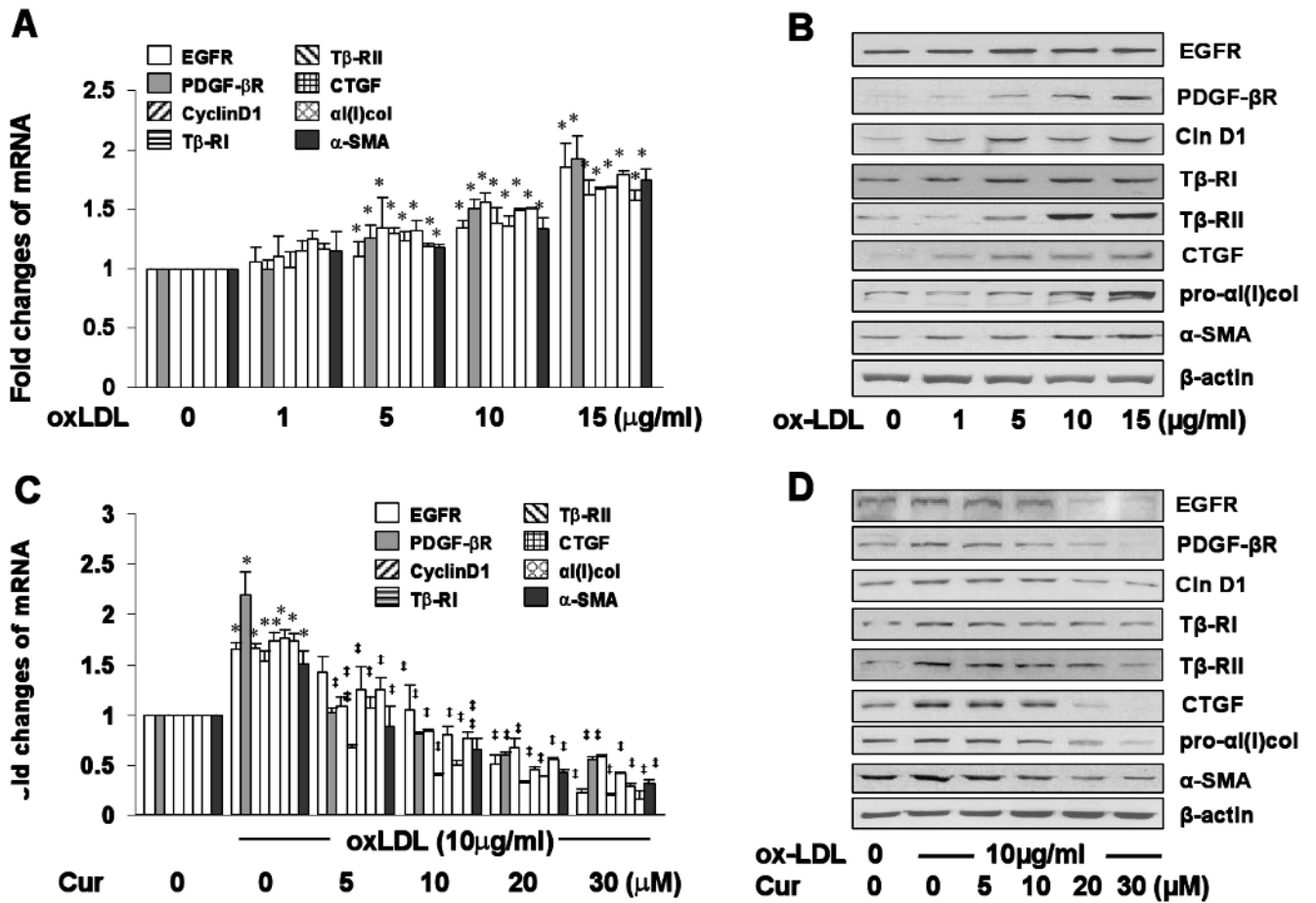


Figure 1. ox-LDL stimulates expression of genes relevant to HSC activation, which is dose-dependently eliminated by curcumin

Serum-starved HSCs were stimulated with ox-LDL at indicated concentrations in serum-depleted media for 24 hr in the absence (A & B) or presence (C & D) of curcumin at 0-30 μM. Total RNA and whole cell protein extracts were respectively prepared from the cells for real-time PCR (A & C) and Western blotting analyses (B & D). *P<0.05 versus cells without treatment (n=3) (1st corresponding column on the left); ‡P<0.05 versus cells treated with ox-LDL only (the 2nd corresponding column). β-actin was used in Western blotting analyses as an internal control for equal protein loading. Representatives were from three independent experiments.

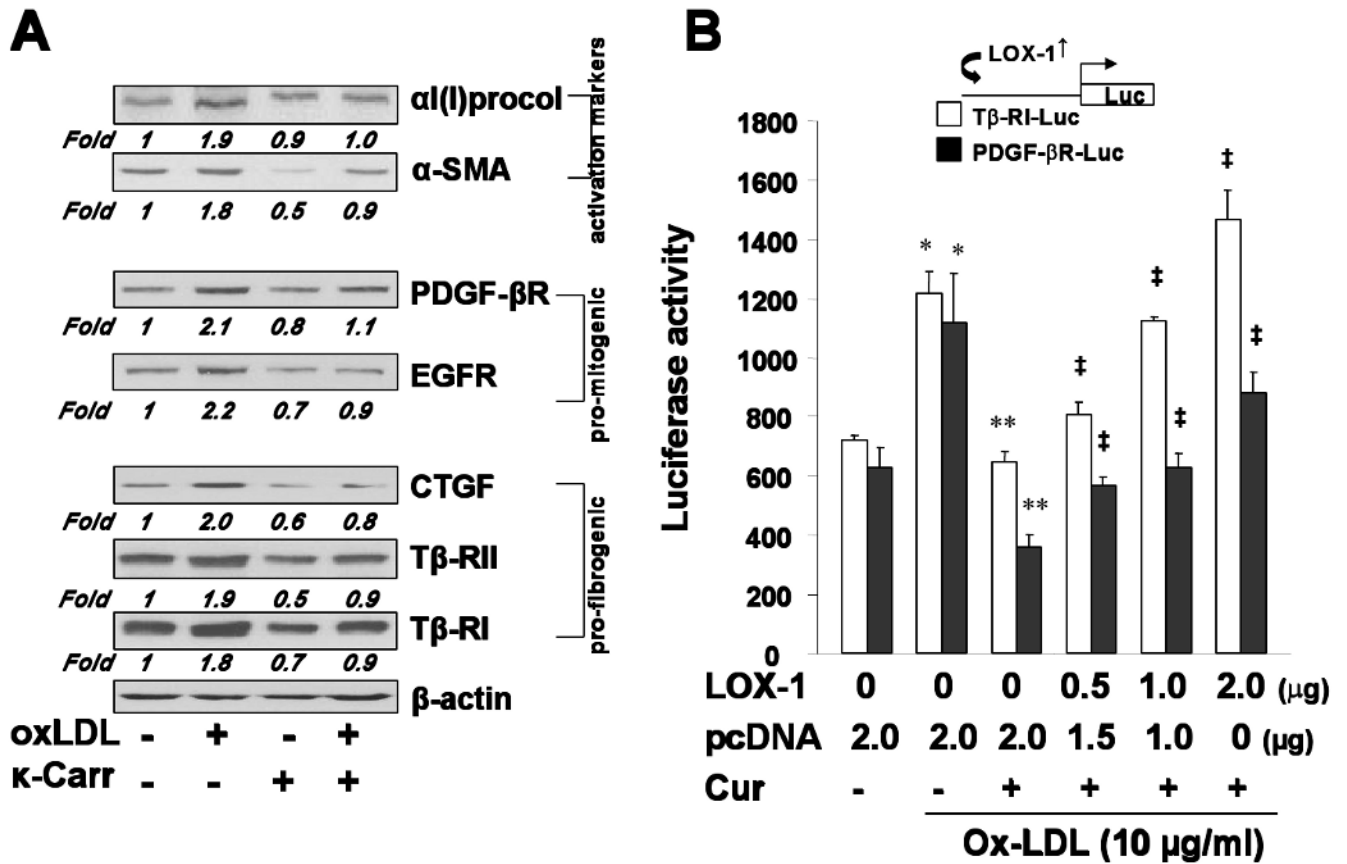


Figure 2. LOX-1 plays a mediating role in ox-LDL-induced HSC activation and in the curcumin elimination of the stimulatory effect of ox-LDL

(A). serum-starved HSCs were pre-treated with or without the LOX-1 antagonist κ-carrageenan at 250 μg/ml for 1 hr prior to the stimulation with or without ox-LDL (10 μg/ml) for additional 24 hr. Whole cell extracts were prepared for Western blotting analyses of genes relevant to HSC activation. β-actin was used in Western blotting analyses as an internal control for equal protein loading. Representatives were from three independent experiments. *Italic* numbers beneath blots were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with the internal invariable control β-actin. Because of the limited space, standard deviations were not presented.

(B). HSCs were co-transfected with LOX-1 cDNA expression plasmid pLOX-1 at indicated doses plus pPDGF-βR-Luc, or pTβ-RI-Luc. A total of 4.5 μg of plasmid DNA per well was used for co-transfection of HSCs in 6-well culture plates. It included 2 μg of pPDGF-βR-Luc or pTβ-RI-Luc, 0.5 μg of pSV-β-gal, and 2.0 μg of pLOX-1 at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were stimulated with or without ox-LDL (10 μg/ml) in the presence or absence of curcumin (20 μM) in serum-depleted media for 24 hr. Luciferase activities were expressed as relative units after β-galactosidase normalization (means ± s.d.; n 6). *P<0.05 versus cells without treatment (the corresponding 1st column); **P<0.05 versus cells treated only with ox-LDL (the corresponding 2nd column); ‡P<0.05

versus cells treated with ox-LDL plus curcumin (the corresponding 3rd column). The floating schema denoted the luciferase reporter construct pPDGF- β R-Luc or pT β -RI-Luc in use and forced expression of LOX-1 cDNA in the system.

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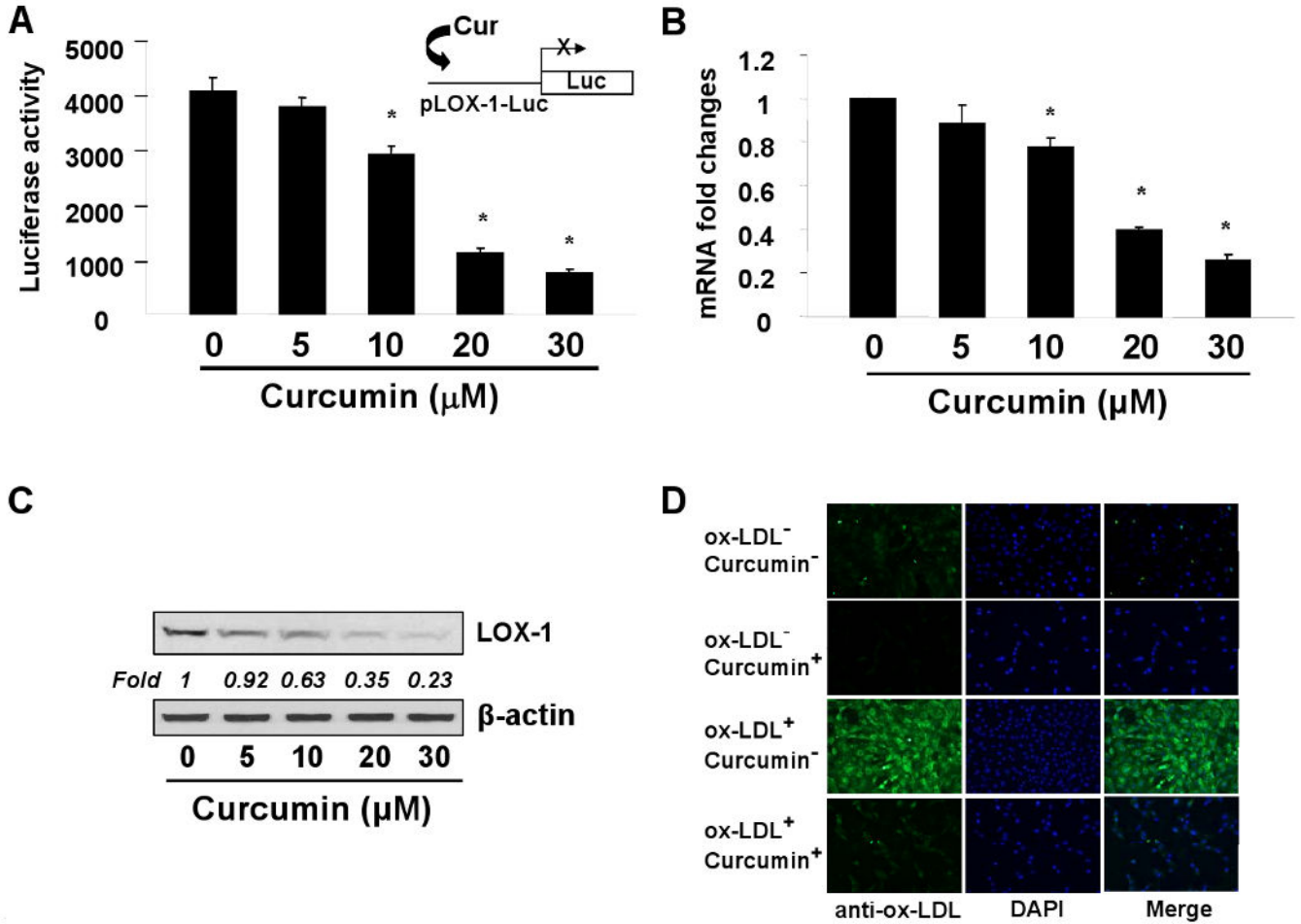


Figure 3. Curcumin suppresses *lox-1* expression in activated HSCs *in vitro*

(A). Luciferase activity assays of cells transiently transfected with the *lox-1* promoter luciferase reporter pLOX-1-Luc, and treated with curcumin after transfection. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.; n = 6). *P<0.05 versus cells without treatment (the first column on the left). The floating schema denoted the luciferase reporter construct pLOX-1-Luc in use and the application of curcumin to the system. (B). Real-time PCR analyses of the steady-state levels of LOX-1 mRNA in cells treated with curcumin. mRNA fold changes were calculated as stated in *Materials and Methods*. Values were expressed as means \pm s.d. (n = 3). *P<0.05 versus the untreated control (the first column on the left); (C). Western blotting analyses of LOX-1 in cells treated with curcumin at indicated concentrations. β -actin was used as an internal control for equal protein loading. Representative was from three independent experiments. *Italic* numbers beneath the blot were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with the internal invariable control β -actin. (D). Immuno-staining of cultured HSCs treated with or without ox-LDL (10 μ g/ml) plus or minus curcumin (10 μ M) for evaluating the impact of curcumin on the abundance of intracellular ox-LDL. DAPI in mounting solution was used for staining nuclei. Representative views were presented.

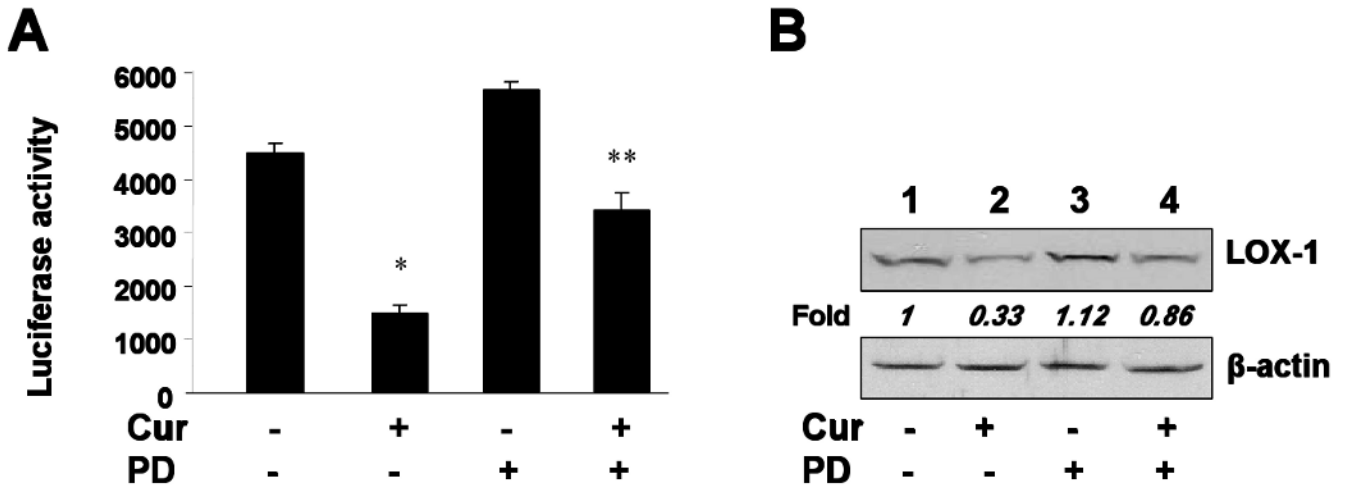


Figure 4. The activation of PPAR γ is required for curcumin to inhibit *lox-1* expression in activated HSCs *in vitro*

Semi-confluent HSCs were pretreated with or without the PPAR γ antagonist PD68235 (20 μ M) for 30 min prior to the addition of curcumin at 20 μ M for additional 24 hr. (A). Luciferase activity assays of HSCs transfected with the plasmid pLOX-1-Luc, followed by the above treatment. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.; n = 6). *P<0.05 versus cells with no treatment (the first column on the left). **P<0.05 versus cells treated with curcumin only (the second column). (B). Western blotting analyses of LOX-1 in cultured HSCs with the above treatment. β -actin was used as an internal control for equal protein loading. Representative was from three independent experiments. *Italic* numbers beneath the blot were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with β -actin. Because of the limited space, standard deviations were not presented.

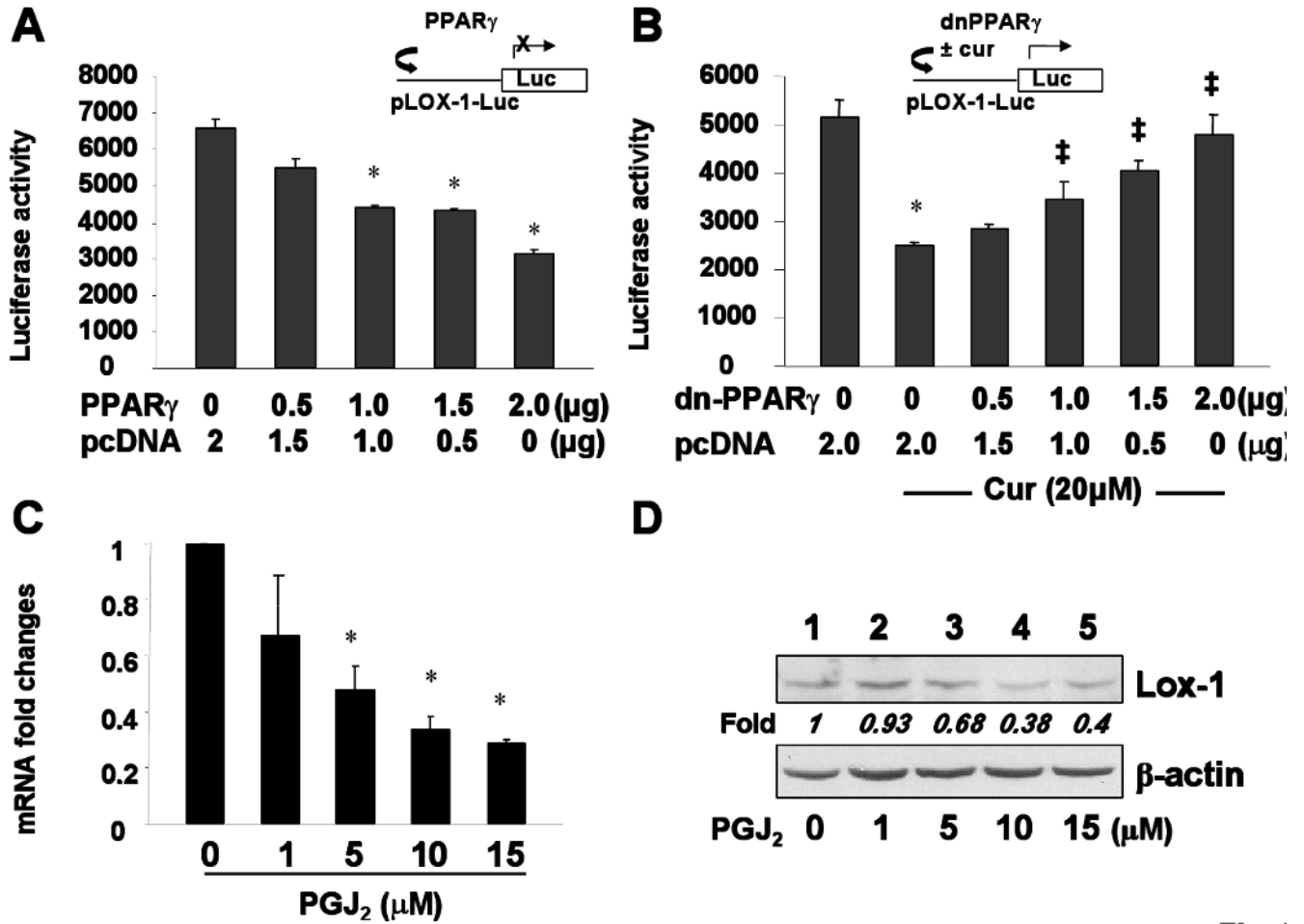


Figure 5. The activation of PPAR γ results in the suppression of *lox-1* expression in activated HSCs *in vitro*

(A) and (B): Semi-confluent HSCs were co-transfected with the plasmid pLOX-1-Luc and the plasmid pPPAR γ (A), or its mutant counterpart pdn-PPAR γ (B), at indicated doses. After recovery, cells were treated with or without curcumin (20 μ M) for 24 hr. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.; n = 6).

*P<0.05 versus cells without pPPAR γ , or pdn-PPAR γ , (the first column on the left).

‡P<0.05 versus cells transfected with no pdn-PPAR γ and treated with curcumin only (the second column).

The floating schema denoted pLOX-1-Luc in use and co-transfected plasmid pPPAR γ or pdn-PPAR γ \pm curcumin in the system. (C) and (D): Serum-starved HSCs were treated with the natural PPAR γ agonist PGJ₂ at 0-15 μ M for 24 hr. (C). Real-time PCR assays of LOX-1 mRNA. mRNA fold changes were expressed as means \pm s.d. (n = 3). *P<0.05 versus the untreated control (the first column on the left); (D). Western blotting analyses of LOX-1. β -actin was used as an internal control for equal protein loading.

Representative was from three independent experiments. *Italic* numbers beneath the blot were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with β -actin. Because of the limited space, standard deviations were not presented.

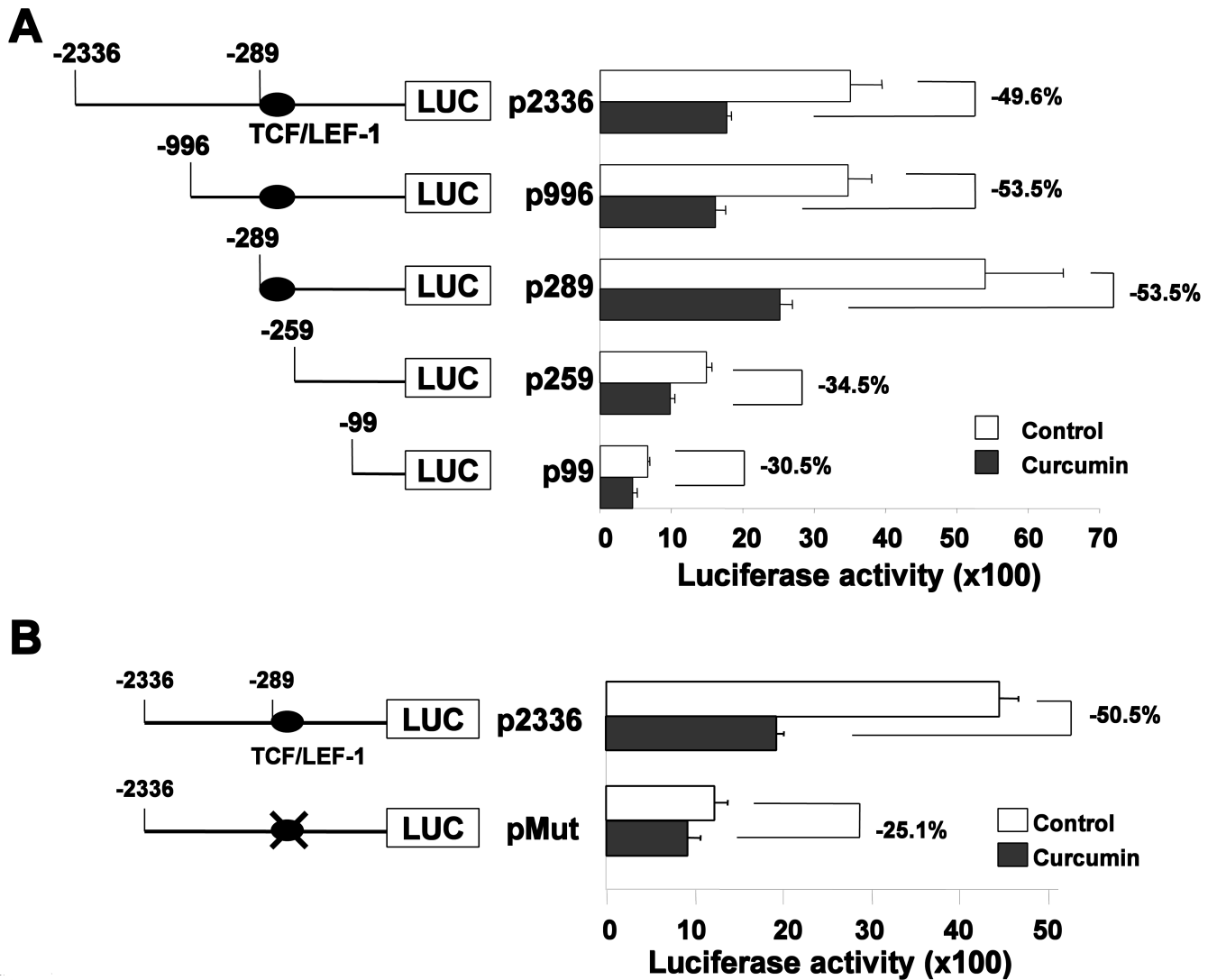


Figure 6. Identification of putative curcumin response elements in the *lox-1* promoter in HSCs Semi-confluent HSCs were transiently transfected with a group of LOX-1 promoter luciferase reporter plasmids. A total of 3.5 μ g of plasmid DNA per well was added to HSCs in 6-well culture plates. It included 3 μ g of a LOX-1 promoter reporter plasmid and 0.5 μ g of pSV- β -gal. After recovery, cells were treated with or without curcumin at 20 μ M for 24 hr. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.; n = 6). The percentages indicated the reduction in luciferase activities in cells treated with curcumin, compared to those in corresponding cells without curcumin treatment. **(A)** Luciferase activity assays of cells transfected with a group of plasmids with various lengths of the *lox-1* 5'-flanking promoter region. **(B)** Luciferase activity assays of HSCs transfected with the wild-type pLOX-1-Luc, or its mutant counterpart pLOX-1(mut)-Luc with site-directed mutations in the putative TCF/LEF-1 binding site.

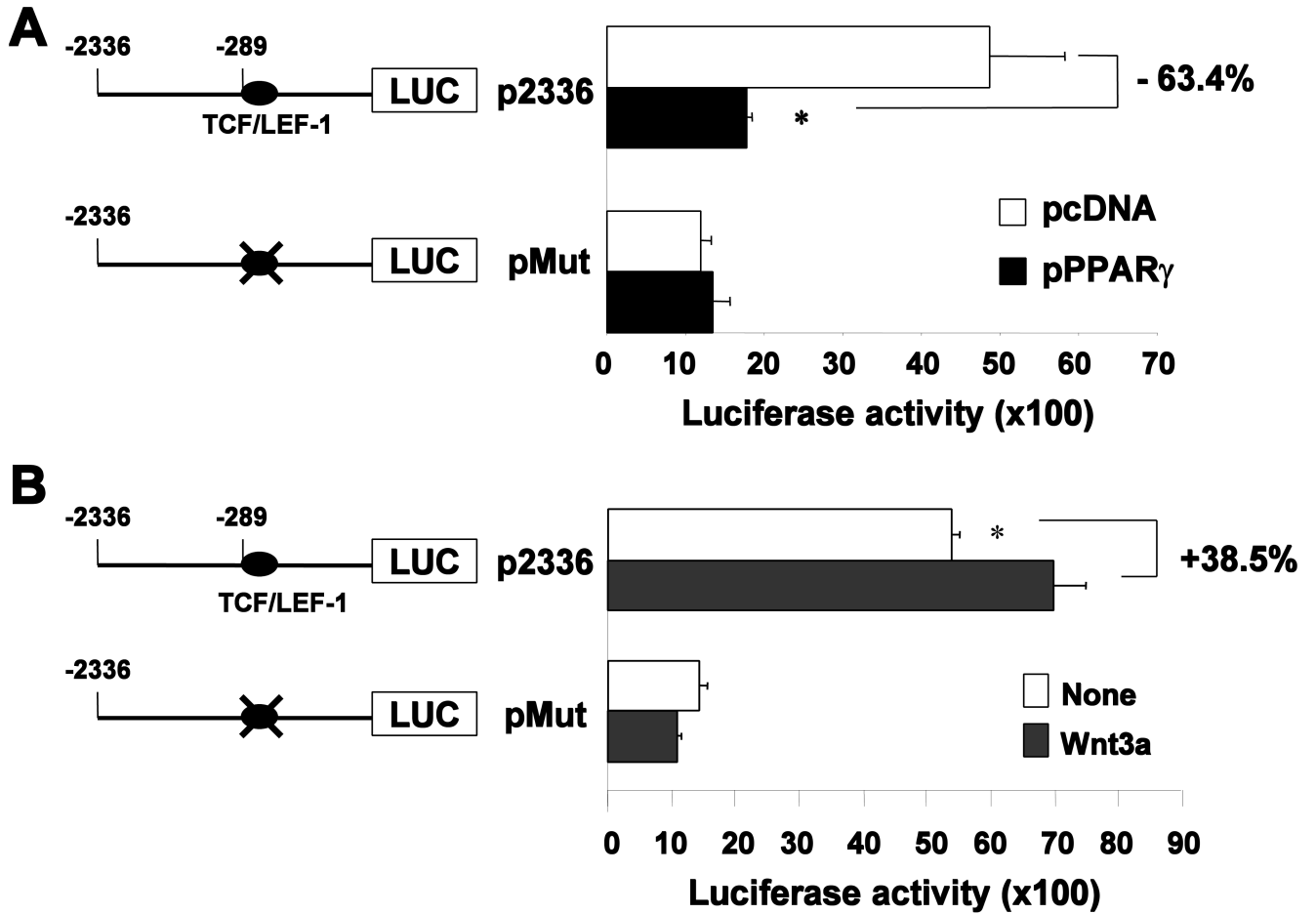


Figure 7. The TCF/LEF-1 binding site in the *lox-1* promoter mediates Wnt signaling and PPAR γ in the regulation of the *lox-1* promoter activity in HSCs

(A). Luciferase activity assays of HSCs co-transfected with pLOX-1-Luc, or pLOX-1(mut)-Luc, plus pPPAR γ , or the empty control vector pcDNA. A total of 4.5 μ g of plasmid DNA per well was used for co-transfection of HSCs in 6-well culture plates. It included 2 μ g of pLOX-1-Luc, or pLOX-1(mut)-Luc, 0.5 μ g of pSV- β -gal and 2.0 μ g of pPPAR γ or pcDNA. After recovery, cells were cultured in DMEM with FBS (10%) for 24 hr. (B). Luciferase activity assays of HSCs transfected with pLOX-1-Luc, or pLOX-1(mut)-Luc. Cells were treated with or without Wnt3a (50ng/ml) in serum-free medium for 24 hr. A total of 3.5 μ g of plasmid DNA per well was added to HSCs in 6-well culture plates. It included 3 μ g of pLOX-1-Luc, or pLOX-1(mut)-Luc, and 0.5 μ g of pSV- β -gal. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.; n = 6). The percentages indicated the changes in luciferase activities, compared to corresponding control cells (the white column).

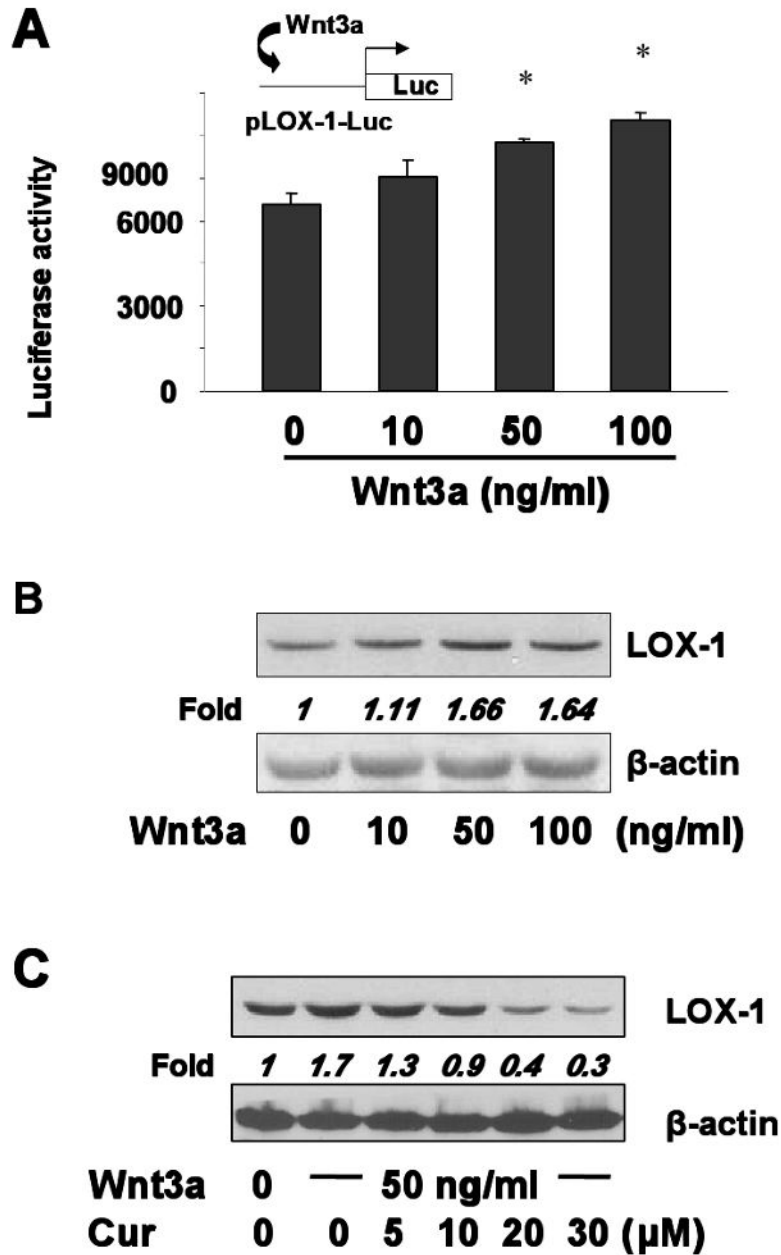


Figure 8. Activation of canonical Wnt signaling induces *lox-1* expression in activated HSCs, which is dose-dependently eliminated by curcumin
(A). HSCs were transiently transfected with pLOX-1-Luc and subsequently treated with Wnt3a at indicated concentrations in serum-free medium for 24 hr. * $P < 0.05$ vs. cells with no treatment (the first column). Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.) (n=6). The floating schema denoted pLOX-1-Luc in use and the application of Wnt3a to the system. **(B)** and **(C).** Western blotting analyses of LOX-1 in HSCs treated with Wnt3a at indicated concentrations in the absence **(B)** or

presence (C) of curcumin at various concentrations in serum-depleted media for 24 hr. β -actin was used as an internal control for equal protein loading. Representative was from three independent experiments. *Italic* numbers beneath the blot were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with β -actin. Because of the limited space, standard deviations were not presented.

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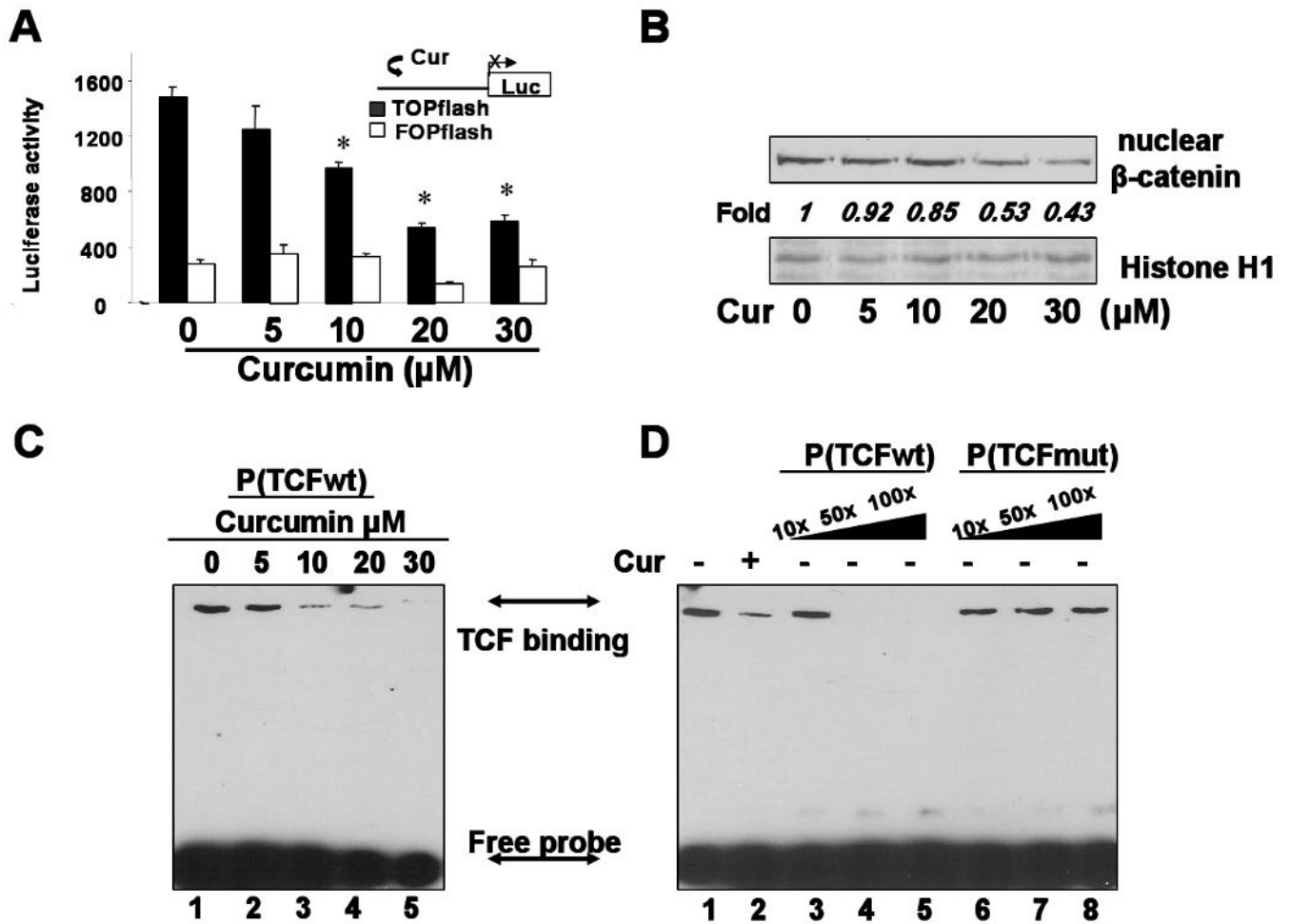


Figure 9. Curcumin interrupts canonical Wnt signaling in activated HSCs *in vitro*
 (A). Luciferase activity assays of HSCs transiently transfected with the plasmid TOPflash or FOPflash, followed by the treatment with curcumin at indicated concentrations for 24 hr. (n=6). * $P < 0.05$ vs. cells with no treatment (the first column). The floating schema denoted the canonical Wnt signaling luciferase reporter construct TOPflash or its mutant counterpart FOPflash in use and the application of curcumin to the system. (B). Semi-confluent HSCs were treated with curcumin at indicated concentrations for 24 hr. Total nuclear extracts were prepared for Western blotting analyses of β -catenin. Histone H1 was used as an invariant control for equal nuclear protein loading. Representative was from three independent experiments. *Italic* numbers beneath the blot were fold changes in the densities of the bands compared to the control without treatment in the blot (n=3), after normalization with Histone H1. Because of the limited space, standard deviations were not presented. (C). EMSA of nuclear protein extracts from HSCs treated with various concentrations of curcumin using the biotin-labeled probe P(TCFwt), which contained the consensus TCF/LEF binding site found in the *lox-1* promoter. (D). EMSA competition assays of nuclear protein extracts from HSCs treated with or without curcumin (Cur) at 20 μ M using the biotin-labeled probe P(TCFwt) and a 10-, 50-, or 100-fold excess of the unlabeled P(TCFwt) (lanes 3-5), or an unlabeled probe P(TCFmut) (lanes 6-8). The latter probe contained the consensus TCF/LEF

binding site found in the *lox-1* promoter with site-directed mutations. Representatives of EMSA were shown from 3 independent experiments.

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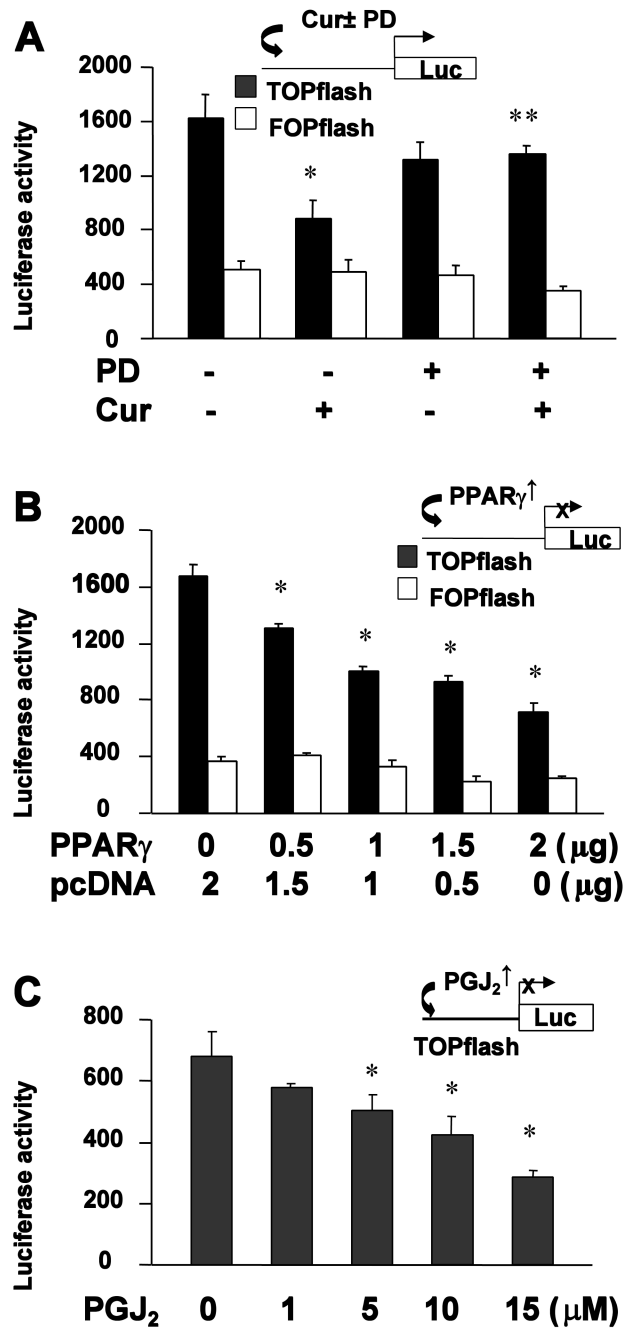


Figure 10. The activation of PPAR γ by curcumin interrupts canonical Wnt signaling in activated HSCs *in vitro*

(A) and (B): HSCs were transiently transfected with the plasmid TOPflash or FOPflash. After recovery, cells were incubated in DMEM with FBS (10%) with treatment for 24 hr. * $P < 0.05$ vs. cells with no treatment (the first column). Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.) (n=6). The floating schemas denote the plasmid TOPflash or its mutant FOPflash in use and the application of treatments to the system. (A). Luciferase activity assays of cells pretreated with PD68235

(20 μ M) for 30min prior to the addition of curcumin (20 μ M) for additional 24 hr. **(B)**. Luciferase activity assays of cells co-transfected with pPPAR γ at indicated doses. A total of 4.5 μ g of plasmid DNA per well was used for co-transfection of HSCs in 6-well culture plates. It included 2 μ g of TOPflash, or FOPflash, 0.5 μ g of pSV- β -gal and 2.0 μ g of pPPAR γ plus pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. **(C)**. HSCs were transfected with TOPflash and treated with PGJ₂ at indicated doses in serum-depleted medium for 24 hr. Luciferase activities were expressed as relative units after β -galactosidase normalization (means \pm s.d.) (n=6). * P < 0.05 *vs.* cells with no treatment (the first column). The floating schema denoted the plasmid TOPflash in use and the application of PGJ₂ to the system.

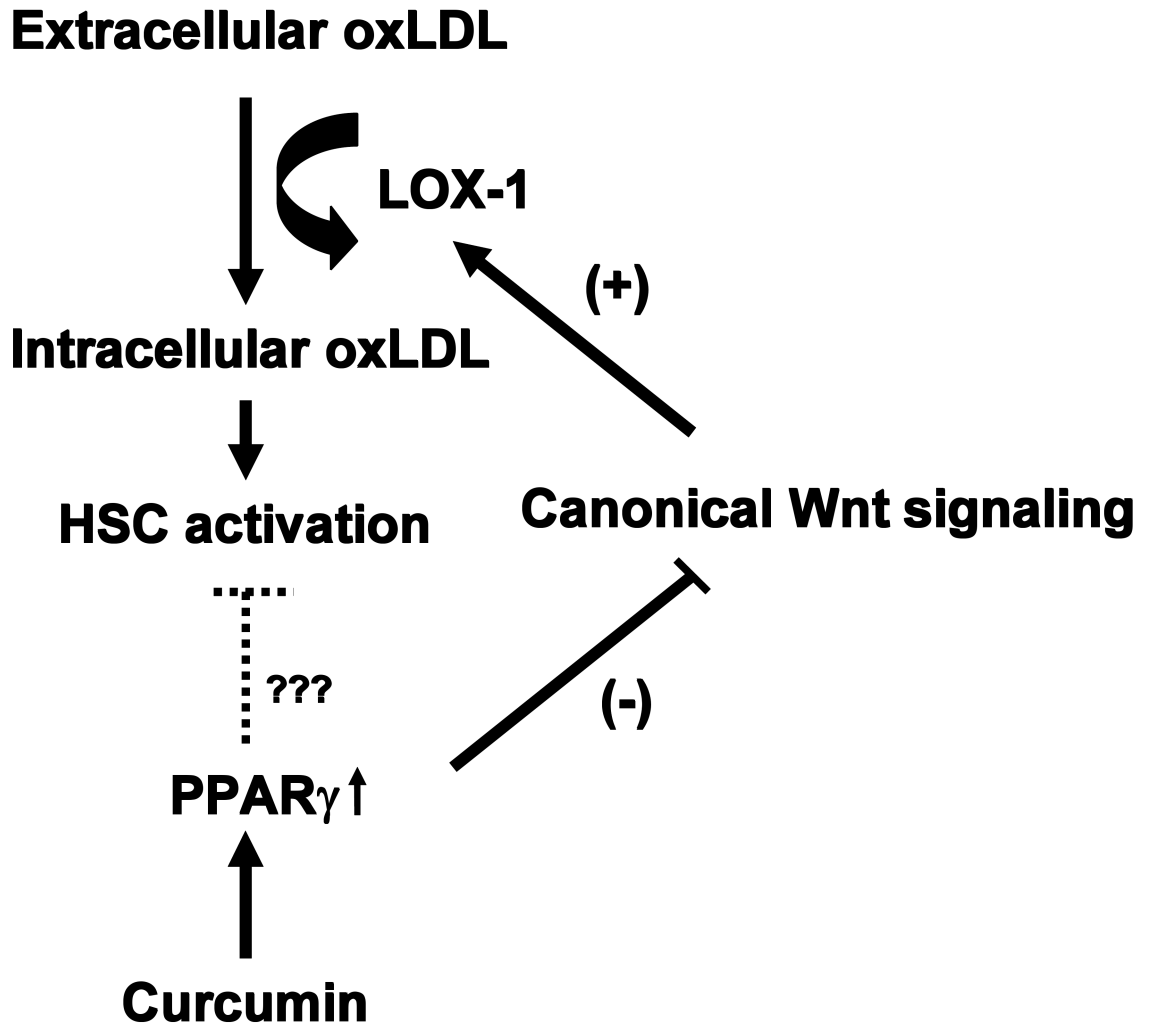


Figure 11. A simplified action model for curcumin to inhibit ox-LDL-induced HSC activation
 Extracellular ox-LDL is transported into HSCs, mediated by LOX-1, leading to the stimulation of HSC activation. This process is blocked by the curcumin-caused suppression of LOX-1 gene expression by activating PPAR γ and interrupting Wnt signaling.

Table 1

Antibodies use for Western blotting analyses

Company	Description	Catalog number
Santa Cruz Biotech. Inc.	Rabbit α -PDGF- β R antibody	sc-432
	Rabbit α -T β -RII antibody	sc-400
	Rabbit α -T β -RI antibody	sc-399
	Rabbit α -EGFR antibody	sc-03
	Rabbit α -CyclinD1 antibody	Sc-718P
	Goat α -CTGF antibody	sc-14939
	Goat α -Pro- α I(I)col antibody	sc-25974
	Bovine α -goat-IgG-HRP	sc-2350
	Goat α -mouse-IgG-HRP	sc-2005
	Goat α -rabbit-IgG-HRP	Sc-2004
Sigma Company	Mouse α -alpha-SMA monoclonal antibody	A2547
	Rabbit α - β -actin antibody:	A2066

Table 2

Primers for generating *lox-1* promoter luciferase reporter plasmids with various lengths of the promoter regions

Plasmid	Primers
Universe reverse primer	(R) 5-GAA GAT CTG AGT GAA GCA GTC ACG AAC TTC-3';
p-289/+36 Luc	(F) 5'-GGG GTA CCG TGT GAC TTA CTC TCT TTG AA T TTC AG-3'
p-259/+36-Luc	(F) 5'-GGG GTAC C CTG TCT CTG AAG AGT GGG TAC-3'
p-99/+36-Luc:	(F) 5'-GGG GTA CCC CAA TAT GAA GCA AAG CCT CTC-3';

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Table 3

Primers for site-directed mutagenesis and for real-time PCR assays

	Primers
Site-directed mutagenesis	(F). 5'-ATG TAG TGT GAC TTA CTC TCA TAG AAT TTC AGT TTC-3' (R). 5'-GAG AGT AAG TCA CAC TAC ATT TGT AAA AAA TGT GCC-3'.
LOX-1 PCR	(F) 5'-GTG CCC TGC TGC TGT GAC TCT G-3'; (R) 5'-GCT TTA GGA GGT CAG ATA CCT G-3'.
GAPDH PCR	(F) 5'-GGC AAA TTC AAC GGC ACA GT-3'; (R) 5'-AGA TGG TGA TGG GCT TCC C-3'.

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