Original Article

CITED2 Is a Novel Direct Effector of Peroxisome Proliferator-Activated Receptor γ in Suppressing Hepatocellular Carcinoma Cell Growth

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BACKGROUND: Previous reports from these authors found that activation of peroxisome proliferator-activated receptor gamma (PPARγ) suppressed hepatocellular carcinoma (HCC). This study sought to identify the molecular target of PPARγ and characterize its antitumor effect in HCC. **METHODS:** Optimal PPARγ binding activity was obtained using the PPARγ agonist rosiglitazone (100 μM) as determined by enzyme-linked immunosorbent assay. Under PPARγ activation, 114 PPARγ downstream targets associated with cancer development were identified by oligonucleotide microarray and Gene Ontology analysis. Among them, Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) was the most prominent PPARγ-bound target, as determined by chromatin immunoprecipitation–polymerase chain reaction. **RESULTS:** CITED2 messenger RNA and protein was significantly down-regulated in primary HCCs compared with their adjacent nontumor tissues. PPARγ induced expression of CITED2 in HCC cell lines after adenovirus-PPARγ transduction. The biological function of CITED2 was evaluated by loss- and gain-of-function assays. CITED2 knockdown in the hepatocyte cell line LO2 and HCC cell line Hep3B significantly increased cell viability and clonogenicity, and promoted G_1-S phase transition in both cell lines. In contrast, ectopic expression of CITED2 in HepG2 and BEL7404 HCC cell lines significantly suppressed cell growth. The tumor suppressive effect of CITED2 was associated with up-regulation of cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21^{Wat1/Cip1}$, $p27^{Kip1}$, antiproliferative regulator interferon alpha 1, proapoptotic mediators including tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), TNFRSF25, caspase-8, granzyme A, and the tumor suppressor gene maspin. CITED2 was also associated with the down-regulation of cell cycle regulator cyclin D1, oncogene telomerase reverse transcriptase, and proinvasion/metastasis gene matrix metallopeptidase 2. **CONCLUSIONS:** CITED2 is a direct effector of PPARγ for tumor suppression. ***Cancer*** **2013;119:1217-26.** © *2012 American Cancer Society.*

KEYWORDS: peroxisome profilerator-activating receptor gamma, CITED2, proliferation, apoptosis, hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) remains the third leading cause of cancer death worldwide.[1] Most patients with HCC are diagnosed at advanced stages, when treatment options are very limited. The prognosis of HCC is poor with an extremely high fatality rate (overall ratio of mortality to incidence of 0.93).[2] Although HCC can be eradicated by surgery at an early stage, there is a high incidence of recurrence following tumor resection.[3] Therefore, it is crucial to elucidate the molecular mechanisms underlying HCC for development of more specific and effective therapies.

The peroxisome proliferator-activating receptor gamma (PPARγ) is a ligand-activated transcription factor, activation of which mediates diverse biological processes including lipid metabolism, insulin sensitization, and anti-inflammation. It also inhibits proliferation and induces differentiation of cancer cells.[4,5] Recent evidence suggests that activation of PPARγ by agonists such as thiazolidinediones produces tumor suppressive effects in many human malignant cell types.[6] We and others previously showed that rosiglitazone, a thiazolidinedione-class drug, inhibited hepatocarcinogenesis in vitro and in vivo by inducing cell apoptosis and cell cycle arrest.[7-9] However, the PPARγ regulatory network and its molecular targets in liver cancer remain largely unknown. In this study, we identified the PPARγ downstream targets relevant to carcinogenesis, by using oligonucleotide microarray and gene ontology analysis. Among the identified targets and consistent with the major function of PPARγ in HCC suppression,[7-9] Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) was identified as a direct effector of PPARγ in HCC cells by chromatin immunoprecipitation (ChIP)-polymerase chain reaction (PCR). With the observation that CITED2 is significantly down-regulated in primary HCCs compared with paired adjacent normal tissues, we further demonstrated the tumor suppressive properties of CITED2 through gain- or loss-of-CITED2 function analyses, which are at least in part mediated

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by the suppression of cell proliferation and inhibition of cell cycle G_1-to-S phase transition. Moreover, the molecular basis of CITED2 as a potential tumor suppressor in HCC was also elucidated.

MATERIALS AND METHODS

Human Liver Cell Culture

Human HCC cell lines (BEL7404, Hep3B, HepG2, Huh7, PLC5, SKHep1, and SUN449) and a nontumorigenic hepatocyte cell line (LO2) were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Rockville, Md) supplemented with 10% fetal bovine serum (Gibco BRL).

PPARγ Binding Activity Assay

To determine the optimal PPARγ transcription factor DNA binding activity in HCC cells, the PPARγ agonist rosiglitazone was used to stimulate PPARγ/DNA binding activity. Hep3B cells (80% confluent) were exposed to 100 μM rosiglitazone for various times (3, 8, 12, or 24 hours). The PPARγ/DNA binding activity in nuclear extract was measured using an enzyme-linked immunosorbent assay (ELISA)-based assay (Cayman Chemical, Ann Arbor, Mich).

Oligonucleotide Microarray Procedure and Data Analysis

Gene expression profiles of Hep3B cells with or without rosiglitazone-induced PPARγ/DNA binding activity were analyzed using the Human Exonic Evidence-Based Oligonucleotide Chip (HEEBOChip) according to protocols from the Stanford Functional Genomics Facility (http://www.microarray.org). The DNA probe was prepared from 50 μg of total RNA labeled with cyanine 5 deoxyuridine triphosphate (Cy5-dUTP; red) or Cy3-dUTP (green) by reverse transcription (Amersham Biosciences, Piscataway, NJ). Two labeled complementary DNA (cDNA) samples were competitively hybridized to the microarray. A dye-swap microarray experiment was conducted in independent sample to exclude bias of dye label factor. Signal intensities were analyzed using a GenePix 4000A scanner (Axon Instruments, Molecular Devices, Palo Alto, Calif). Gridding was performed using GenePix Pro 5.0 (Axon Instruments), and array data were presented as the log base2 ratio of the Cy5/Cy3 signals.[10] Gene expression patterns between treated samples and controls were analyzed using unsupervised hierarchical clustering. Statistically significant differences were assessed by the *t* statistic using Significance Analysis of Microarrays (SAM) software.[11]

Chromatin Immunoprecipitation

A ChIP assay was performed on Hep3B cells treated with or without 100 μM rosiglitazone for 3 hours using the Red ChIP Kit (Diagenode, Liège, Belgium). Immunoprecipitation was performed using 5 μg of PPARγ antibody (sc-7196; Santa Cruz Biotechnology, Santa Cruz, Calif) for each aliquot (300 μL). The immunoprecipitated DNA was amplified with primers flanking the predicated PPARγ binding sites. The primer sequences are as follows: CITED2 (forward: 5′-GCAAAAACGGAAGGACTG GAA-3′, reverse: 5′-GCGCCGTAGTGTATGTGCT-3′), Kruppel-like factor 10 (KLF10) (forward: 5′-CCGGGAA CACCTGATTTTCAT-3′, reverse: TACAGTAGATGG CGCTGGTG-3′), growth arrest and DNA-damage–inducible protein 45 (GADD45A) (forward: 5′-GCCTG TGAGTGAGTGCAGAA-3′, reverse: CCCCACCTTAT CCATCCTTTC-3′). The final PCR products were resolved on 1% agarose gels by gel electrophoresis.

Adenovirus-Mediated PPARγ Gene Transfer

Recombinant adenovirus encoding PPARγ1 (Ad-PPARγ) and recombinant adenovirus encoding *E. coli* β-galactosidase (Ad-LacZ) as a control were generously provided by Dr. J. K. Reddy (Department of Pathology, Northwestern University, the Feinberg School of Medicine, Chicago, Ill). Recombinant adenovirus of 75 multiplicities of infection (MOI) was transduced into Hep3B cell lines as described.[9]

cDNA Synthesis, Quantitative PCR, and Reverse Transcription PCR

Total RNA was extracted using RNA Trizol reagent (Invitrogen, Carlsbad, Calif). The messenger RNA (mRNA) expression level of CITED2 was determined by quantitative real-time PCR (qPCR) or semiquantitative reverse transcription PCR (RT-PCR). The primer sequences are as follows: CITED2 (forward: 5′-GCAAAAACGGAAG GACTGGAA-3′, reverse: 5′-GCGCCGTAGTGTAT GTGCT-3′).

Western Blot Analysis

Total protein was extracted and protein concentration was measured by Bradford assay (DC protein assay; Bio-Rad Laboratories, Hercules, Calif). Protein lysate was resolved by sodium dodecyl polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and immunoblotted with antibodies to PPARγ (Santa Cruz Biotechnology), $p15^{INK4B}$, $p21^{Wat1/Cip1}$, $p27^{Kip1}$, cyclin D1 (Cell Signaling Technologies, Danvers, Mass), CITED2, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Hong Kong, China).

Construction of CITED2 Expression Vector

The CITED2 expression vector was generated by PCR cloning using the pcDNA3.1 TOPO TA Expression Kit (Invitrogen, Carlsbad, Calif). The CITED2 cDNA was obtained by RT-PCR using normal human liver RNA as a template (Ambion, Austin, Tex). The PCR products were subcloned into the pcDNA3.1 TOPO vector, and clones were screened and sequenced using vector-specific primers.

Short Hairpin RNA Knockdown

The RNA interference experiment was performed using a human short hairpin (shRNA) construct against CITED2 in a pGFP-V-RS vector (Origene, Rockville, Md). The sh-CITED2 was 29-base oligomer as follows: 5′-CTGTGCTAATAGTTATATTCAGTACATGA-3′. A noneffective scrambled shRNA was used as control (Origene).

Gene Transfection

Liver cell lines (LO2, Hep3B, HepG2, and BEL7404) were transfected with expression vector or shRNA construct using Lipofectamine LTX (Invitrogen), and were treated with G418 (0.4 mg/mL) for selection of stably transfected clones.

Cell Viability Assay

Cell viability was determined using CellTitre 96 aqueous one solution cell proliferation assay (Promega, Madison, Wis).

Colony Formation Assay

LO2 and Hep3B (2×10^5 cells/well) cells were transfected with 2.5 μg sh-CITED2 or sh-scramble vector using lipofectamine LTX (Invitrogen). Transfected cells were selected with G418 (0.4 mg/mL; Merck, Darmstadt, Germany) for 2 weeks. Surviving colonies (with > 50 cells per colony) were counted and analyzed after staining with gentian violet. The experiment was performed in 3 independent triplicates.

Cell Cycle Analysis

Transiently transfected cells were trypsinized, washed in phosphate-buffered saline, and fixed in ice-cold 70% ethanol in phosphate-buffered saline. DNA was labeled with propidium iodide. Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson, Franklin Lakes, NJ). Data acquisition and analysis were performed using the ModFitLT software (Becton Dickinson, San Diego, Calif).

cDNA Expression Array

Gene expression profiles in LO2 cells stably transfected with sh-CITED2 or sh-scramble vector were analyzed by the Human Cancer Pathway Finder RT2 Profiler PCR Array containing 84 functionally well-characterized genes involved in human tumorigenesis (SA Biosciences, Frederick, Md). A gene expression change ≥ 1.5 or ≤ 1.5 was considered to be of biological significance.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). The paired *t* test was performed to compare the CITED2 expression level in paired human liver tumors and their corresponding normal counterparts. The student *t* test was applied to analyze the functional effect of CITED2 on cell viability, clonogenicity, and cell cycle progression. All analyses were performed using SPSS software (version 13.0; SPSS, Chicago, Ill). Statistical significance was accepted at the level of $P < .05$.

RESULTS

Rosiglitazone Induces PPARγ/DNA Binding Activity

To test the effect of rosiglitazone-mediated stimulation of PPARγ/DNA binding affinity in liver cancer, we treated Hep3B cells with the PPARγ agonist rosiglitazone (100 μM)[12] for various time points. The results showed that PPARγ/DNA binding activity reached a maximal level after 3 hours of treatment, and remained elevated for at least 24 hours (Fig. 1A).

PPARγ Activation Modulates Cancer-Associated Genes and Networks

To identify novel PPARγ downstream targets, gene expression profiles of untreated and rosiglitazone-treated Hep3B cells were analyzed using an oligonucleotide microarray. Upon PPARγ stimulation, a total of 573 genes were differentially expressed ($\geq$ 4-fold changes), wherein 244 genes were up-regulated and 329 genes were down-regulated as identified by unsupervised hierarchical clustering.

We further classified the PPARγ-regulated gene candidates according to their biological functions by GoMiner and Gene Ontology analysis. The results showed that one-fifth (114 of 573) of the differentially expressed candidates are involved in tumorigenesis with functional roles in signal transduction (47.4%), cell cycle control (16.9%), apoptosis (16.9%), cell proliferation (11.7%), cell adhesion/metastasis (2.6%), and angiogenesis (4.5%). In addition, KEGG pathway analysis of these cancer-associated genes revealed their involvement in the P53 signaling pathway, MAPK (mitogen-activated protein kinase) signaling pathway, TGF-β (transforming growth factor beta) signaling pathway, and extracellular

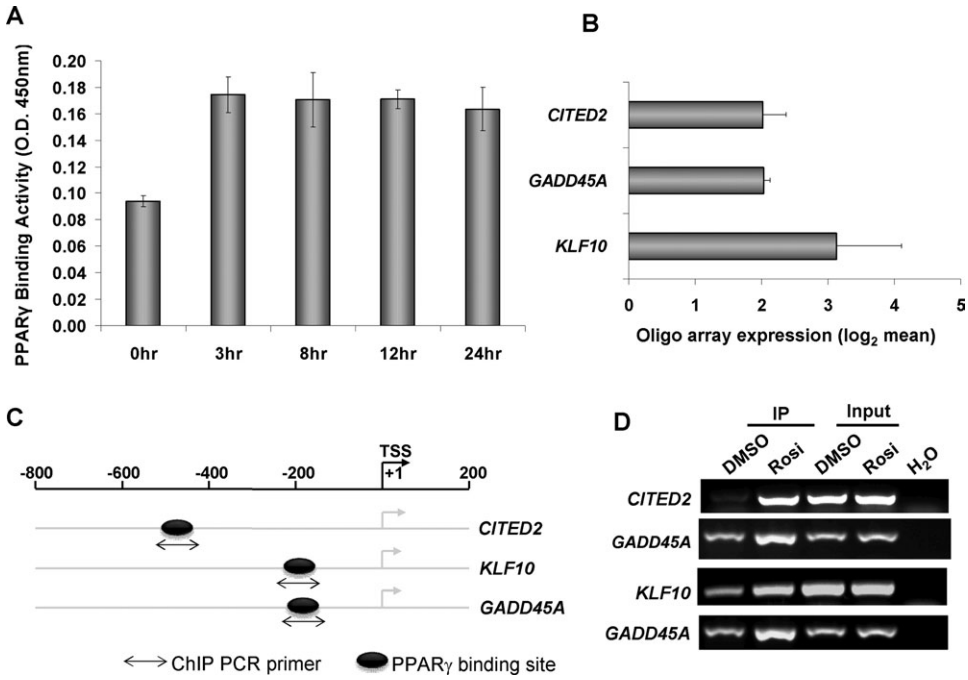

Figure 1. (A) Graph shows induction of PPARγ binding activity in Hep3B cells treated with PPARγ agonist rosiglitazone (100 μM) for 3, 8, 12, and 24 hours, as determined by ELISA-based assay. (B) Oligo array expression of cancer-associated candidates CITED2, GADD45A, and KLF10 was evaluated after treatment with 100 μM of rosiglitazone for 3 hours. (C) Computational predication of PPARγ binding sites. The locations of ChIP-PCR primers are indicated. The transcription start sites (TSS) are indicated by curved arrows. (D) ChIP-PCR analysis of PPARγ-binding gene promoters is shown in cancer-associated candidates after treatment with 100 μM of rosiglitazone (Rosi) for 3 hours. Input DNA was used as loading control. DMSO, dimethyl sulfoxide; IP, immunoprecipitated DNA.

matrix receptor interaction pathway. These data are consistent with the identified functions of PPARγ in diverse biological processes, and demonstrate the effects of PPARγ activation on specific cellular pathways relevant to cancer development.

CITED2 Is a Novel Direct Target Gene of PPARγ

To further explore the key direct targets of PPARγ among the tumor-associated candidates, we performed computational prediction of PPARγ binding sites of tumor-associated candidates using MatInspector. For each candidate promoter sequence, locations approximately 2 kilobases upstream of the transcription start site were analyzed. A potential PPARγ binding site was considered when both matrix similarity and core similarity scored > 0.8. Incidentally, *CITED2*, *GADD45A*, and *KLF10*, transcriptionally activated upon PPARγ stimulation (Fig. 1B), were predicted to have the binding sites of PPARγ in their promoter regions (Fig. 1C). For validation of direct interaction, ChIP-PCR was performed on untreated and rosiglitazone-treated Hep3B cells, using primers flanking the putative PPARγ binding sites of CITED2, GADD45A, and KLF10 (Fig. 1C). The results clearly demonstrate the direct interaction of PPARγ and the gene promoter of CITED2, GADD45A, and KLF10 (Fig. 1D). Given the significantly induced binding of PPARγ to the CITED2 promoter upon rosiglitazone treatment, *CITED2* was selected for further functional investigation in HCC.

Down-Regulation of CITED2 in Primary HCCs and HCC Cell Lines

The mRNA expression level of CITED2 was determined in a total of 30 paired liver tumor samples and 9 human cell lines. As shown by qPCR, the CITED2 mRNA level was significantly lower in tumors than in their adjacent nontumor tissues ($P < .0001$, Fig. 2A). Protein level of CITED2 was also detected in 10 paired HCC samples. The CITED2 protein expression is significantly downregulated in HCC tumors compared with the adjacent nontumor tissues ($P < .05$, Fig. 2B,C). Moreover, downregulation of CITED2 was observed in 7 HCC cell lines including BEL7404, Hep3B, HepG2, Huh7, PLC5, SKHep1, and SUN449 compared with that in normal liver tissue (Fig. 2D). On the basis of their expression level, we selected Hep3B, HepG2, and Huh7, which exhibit low CITED2 expression, for further validating the interplay of PPARγ and CITED2.

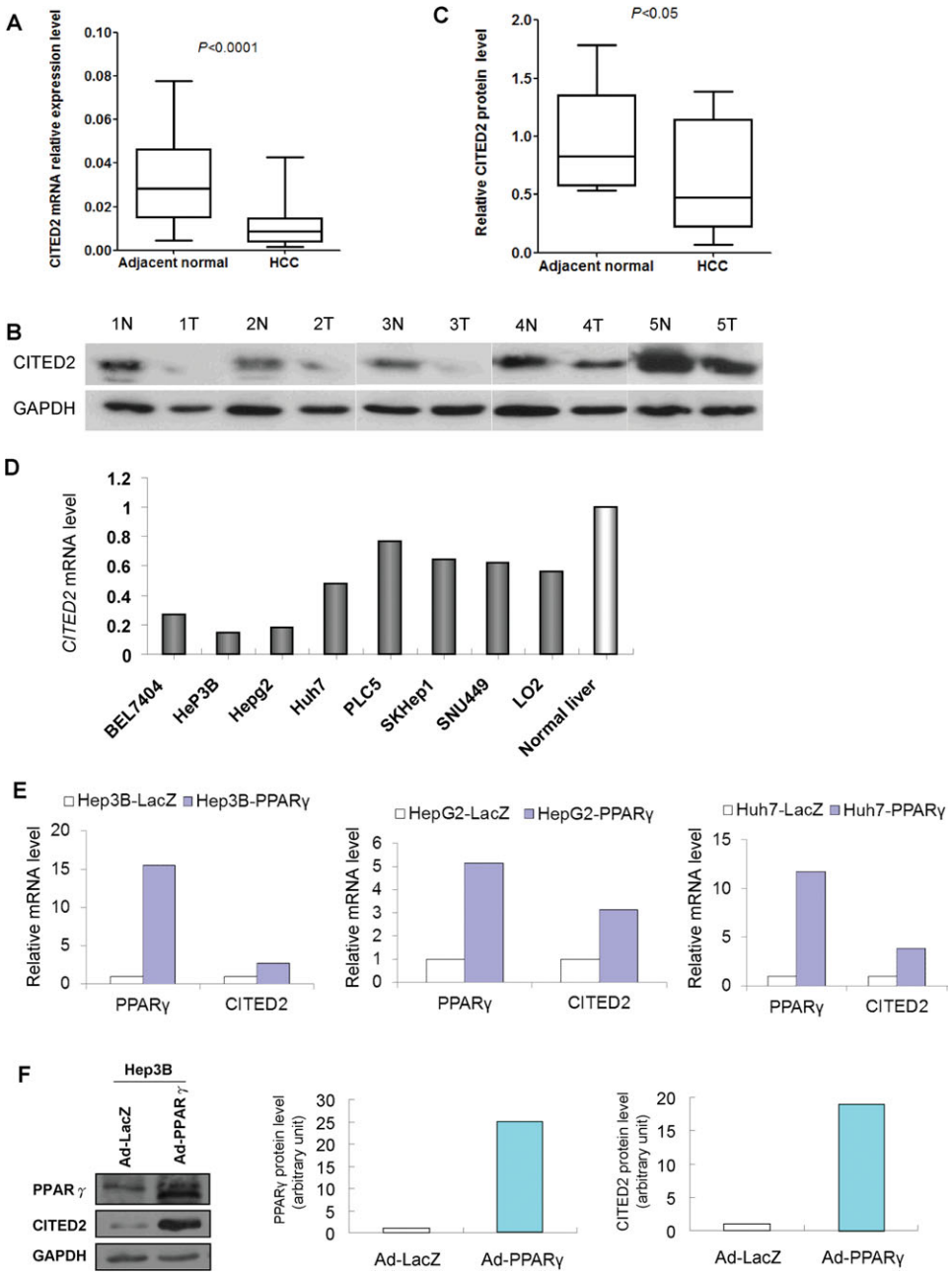

Figure 2. (A) CITED2 mRNA expression in 30 paired human HCC tumors with corresponding normal tissues was determined by qPCR. (B) Protein expression levels of CITED2 in 10 paired human HCC samples was determined using western blot analysis. N, normal; T, tumor. (C) The relative band intensity of CITED2 protein was compared in 10 paired HCC samples. (D) CITED2 mRNA expression in human cell lines was determined by quantitative PCR. (E) Induction of CITED2 mRNA expression in Hep3B, HepG2, and Huh7 cells transduced with Ad-PPARγ (750 MOI) were detected by qPCR. Ad-LacZ was used as negative vector control. (F) Protein level of PPARγ and CITED2 after PPARγ induction in Hep3B was detected by westernblot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was used as the internal control.

PPARγ Induces CITED2 Expression in HCC Cells

To examine whether CITED2 expression is directly regulated by PPARγ, we transduced adenovirus-PPARγ into Hep3B, HepG2, and Huh7 cells. Induction of PPARγ by adenovirus-PPARγ transduction dramatically increased CITED2 mRNA expression in all 3 HCC cell lines as determined by qPCR (Fig. 2E). Western blot also confirmed the up-regulation of CITED2 protein in HCC cells following PPARγ transduction (Fig. 2F). These results indicate that the up-regulation of CITED2 is regulated by PPARγ.

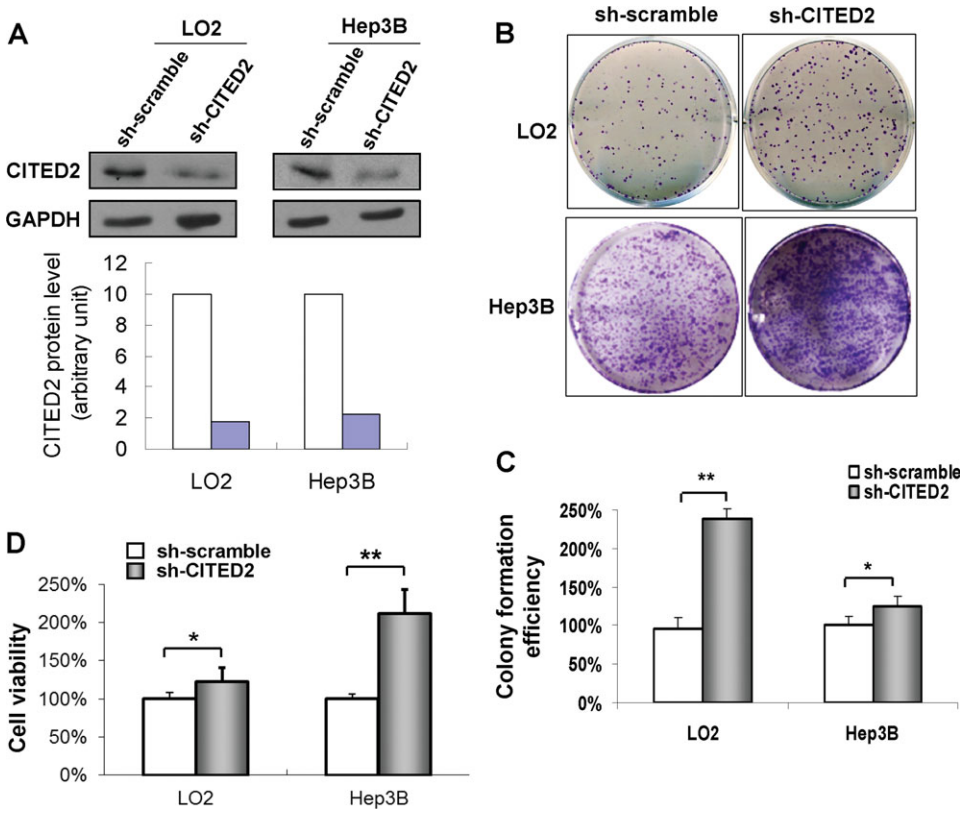

Figure 3. Knockdown of CITED2 increases cell viability and clonogenicity. (A) The nontumorigenic hepatocyte cell line (LO2) and HCC cell line (Hep3B) were transfected with sh-scramble and sh-CITED2 vector, respectively. Knockdown of CITED2 protein expression was confirmed by western blot. GAPDH was used as the internal control. (B) Colony formation assay. Representative dishes of LO2 and Hep3B cells stably transfected with sh-scramble or sh-CITED2. (C) Quantitative analysis of colony number (%) is shown as values of mean ± SD. The SD was measured from 3 independent triplicates. (D) Cell viability analysis was determined using LO2 and Hep3B cells stably transfected with sh-scramble (control) or sh-CITED2 (*$P < .05$, **$P < .01$).

Knockdown of CITED2 Induces Cell Proliferation

To evaluate the effect of CITED2 on cell growth, we stably transfected sh-CITED2 into LO2 and Hep3B cells, knockdown of CITED2 in these cells was evidenced by western blot (Fig. 3A). CITED2 depletion caused a significant increase of colony formation in LO2 cells (increase by 138%, $P < .01$) and in Hep3B cells (increase by 25%, $P < .05$) (Fig. 3B,C). The effect of CITED2 knockdown on promoting cell proliferation was further confirmed by a significant increase of cell viability in CITED2-ablated LO2 cells ($P < .05$) and Hep3B cells ($P < .01$) (Fig. 3D).

Knockdown of CITED2 Promotes Cell Cycle Progression

We examined the contribution of cell cycle to the observed growth induction of knockdown CITED2 in sh-*CITED2* transfected cells (Fig. 4A). FACS analysis of sh-*CITED2* transfected LO2 cells revealed a significant decrease in the number of cells accumulating in G_1 phase (66.5 ± 1.6 versus 70.5 ± 1.2, $P < .01$) compared to sh-scramble–transfected LO2 cells (Fig. 4B,C). Concomitant with this inhibition, there was a significant increase in the number of cells in S phase (25.2 ± 0.7 versus 22.6 ± 0.7, $P < .01$) (Fig. 4C) under the same conditions. Induction of the G_1-S phase transition was further confirmed by analysis, using western blot, of the key G_1-S phase regulators. Knockdown of CITED2 reduced the protein expression of negative cell cycle regulators $p15^{INK4B}$, $p21^{Wat1/Cip1}$, and $p27^{Kip1}$, but enhanced the level of cyclin D1 which is an important regulator to facilitate G_1-S phase transition (Fig. 4D,E).

Overexpression of CITED2 Suppresses HCC Cell Growth

To confirm the inhibitory role of CITED2 in cell growth, the effect of CITED2 overexpression was determined in HCC cell lines HepG2 and BEL7404. Ectopic expression of CITED2 in these cells was confirmed at mRNA (Fig. 5A) and protein levels (Fig. 5B) by RT-PCR and western blot, respectively. CITED2 caused a significant decrease in cell viability in both HepG2 cells ($P < .01$) and BEL7404 cells ($P < .05$) (Fig. 5C). In addition, the protein expression of $p21^{Wat1/Cip1}$ and $p27^{Kip1}$ was

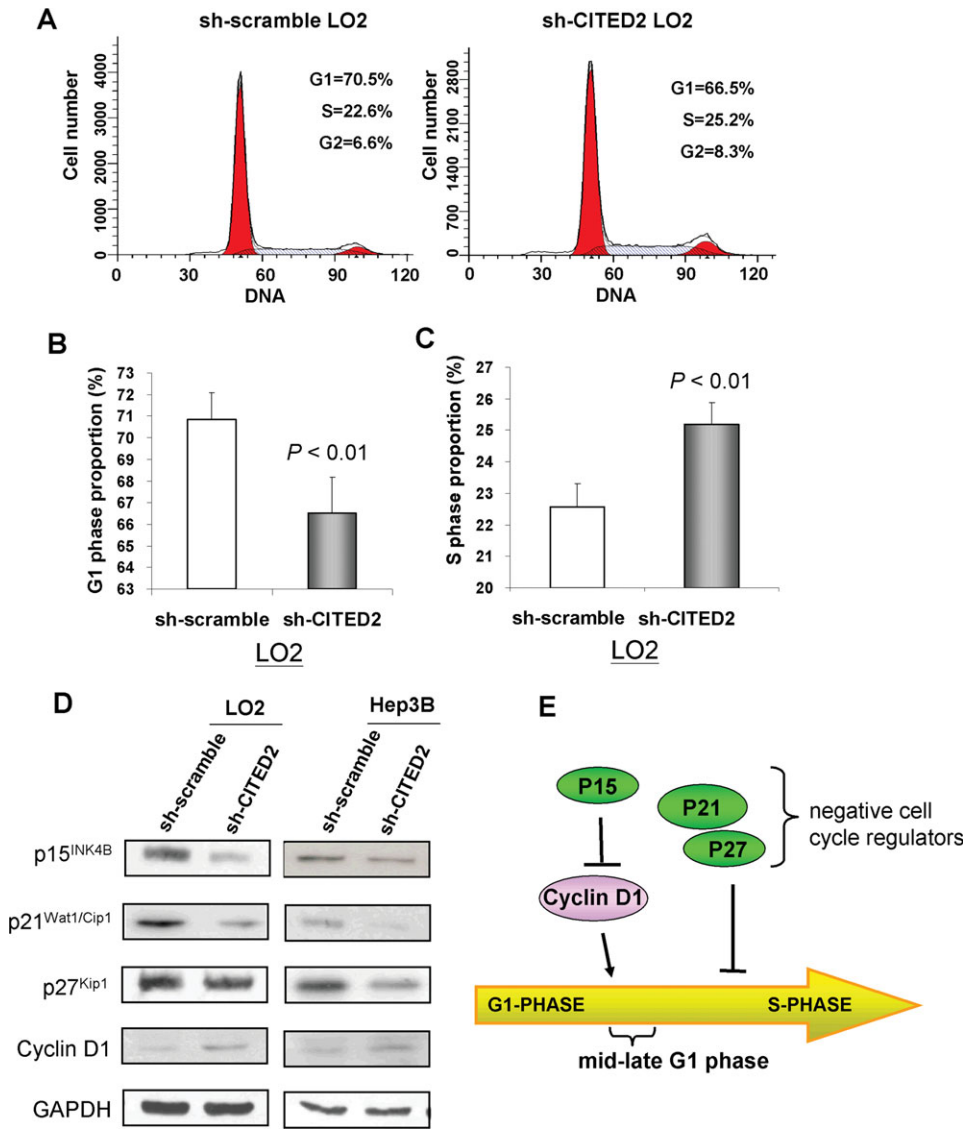

Figure 4. Knockdown of CITED2 promotes cell cycle progression. (A) The cell cycle distribution was analyzed by flow cytometry using LO2 cells transfected with sh-scramble or sh-CITED2. The fraction of cells in (B) G_1 and (C) S phases was indicated. (D,E) Effects of CITED2 knockdown on protein expression of cell cycle regulators $p15^{INK4B}$, $p21^{Wat1/Cip1}$, $p27^{Kip1}$, and cyclin D1 by western blot. GAPDH was used as loading control.

increased in CITED2-overexpressed HepG2 and BEL7404 cells (Fig. 5D).

Identification of Genes Modulated by CITED2

To gain insight into the molecular basis of CITED2 in HCC, gene expression profiles of the LO2 cells transfected with either sh-scramble or sh-CITED2 were analyzed by cDNA expression array. When compared with sh-scramble, CITED2 depletion by sh-CITED2 caused up-regulation of oncogene telomerase reverse transcriptase (TERT; 5.4-fold) and proinvasion/metastasis gene matrix metallopeptidase 2 (MMP2; 2.0-fold), but led to down-regulation of antiproliferative regulator interferon, alpha 1 (IFNA1; −1.6-fold), proapoptotic mediators including tumor necrosis factor receptor superfamily member 1A (TNFRSF1A; −1.5-fold), TNFRSF25 (−1.6-fold), caspase-8 (CASP8; −1.5-fold), granzyme A (GZMA; −1.5-fold), and a tumor suppressor gene maspin (SERPINB5; −1.9-fold) (Fig. 6A). The downstream effectors of CITED2, including IFNA1, TNFRSF1A, TERT, and MMP2, were verified by RT-PCR (Fig. 6B).

DISCUSSION

Activation of PPARγ has been shown to protect against HCC.[9,12,13] The precise mechanism and critical

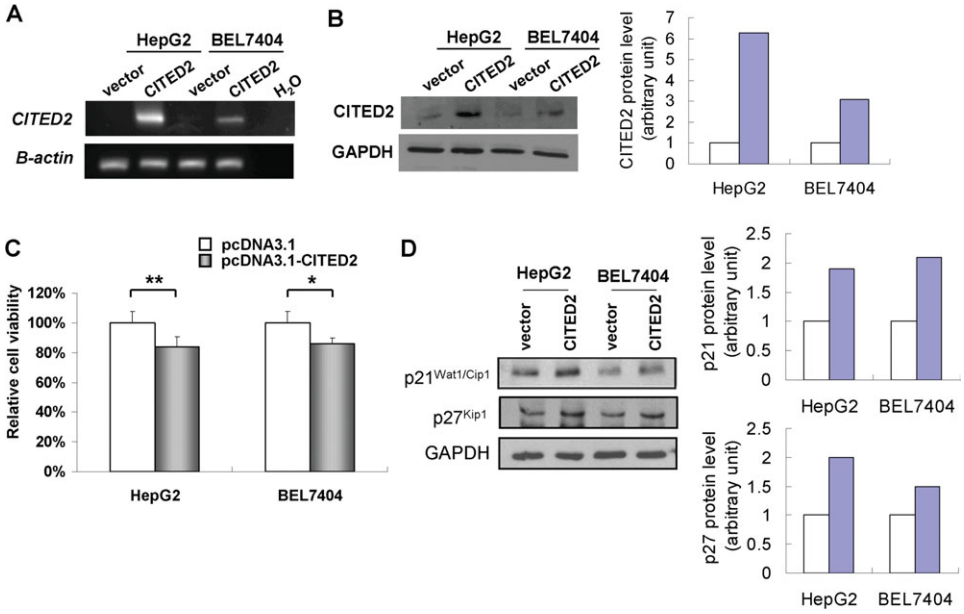

Figure 5. Ectopic expression of CITED2 suppresses cell growth of liver cancer cells. (A,B) HCC cell lines (HepG2, BEL7404) were transfected with pcDNA3.1 vector alone (vector) or pcDNA3.1-CITED2 (CITED2). Ectopic expression of CITED2 was confirmed by (A) RT-PCR and (B) western blot. (C) Ectopic expression of CITED2 in HepG2 and BEL7404 cells significantly suppressed cell viability after 72 hours (*$P < .05$, **$P < .01$). (D) Effects of CITED2 overexpression on levels of cell cycle regulators $p21^{Wat1/Cip1}$ and $p27^{Kip1}$ are shown by western blot. GAPDH was used as a loading control.

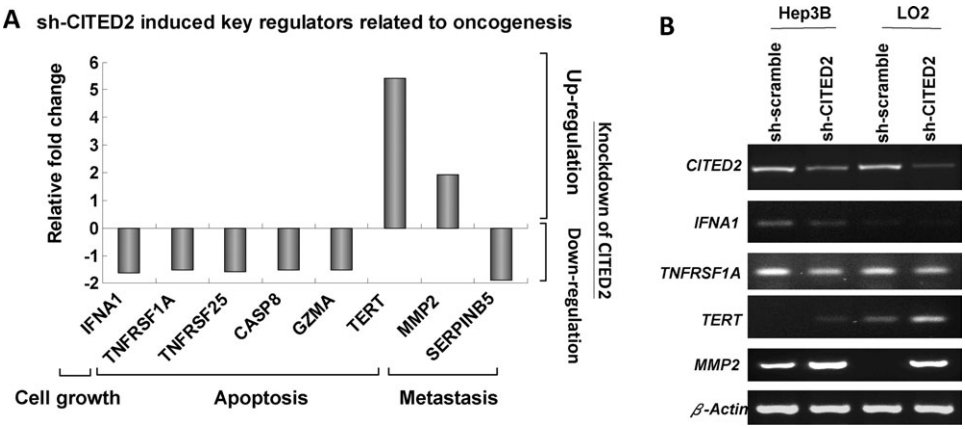

Figure 6. (A) Gene expression profile of the LO2 cells stably transfected with sh-CITED2 was analyzed by cDNA expression array. The identified important genes modulated by CITED2 are presented. (B) RT-PCR was performed to validate the result of cDNA expression array. The mRNA level of β-actin was used as internal control.

downstream effectors of PPARγ in relation to its therapeutic efficacy in HCC, however, remains poorly understood. In this study, potential target genes under the control of PPARγ in HCC were identified by microarray analysis of gene expression. Among the 573 differentially modulated genes (≥ 4-fold changes) by PPARγ activation, 114 genes are associated with cancer development. Gene Ontology analysis revealed that these candidates regulate cell proliferation, cell-cycle control, apoptosis, cell adhesion/metastasis, angiogenesis, and signal transduction in tumors. These findings support the tumor suppressive function of PPARγ in liver cancer and provide mechanistic insights for the anti-HCC effects upon PPARγ stimulation.

To further investigate the critical direct PPARγ targets, we applied an integrated approach combining oligonucleotide microarray and bioinformatic prediction of PPARγ binding sites, which enabled identification of PPARγ target genes that were not previously identified. Consistent with the critical role of PPARγ in tumor suppression, we identified several direct effectors of PPARγ including CITED2, GADD45A, and KLF10. The direct

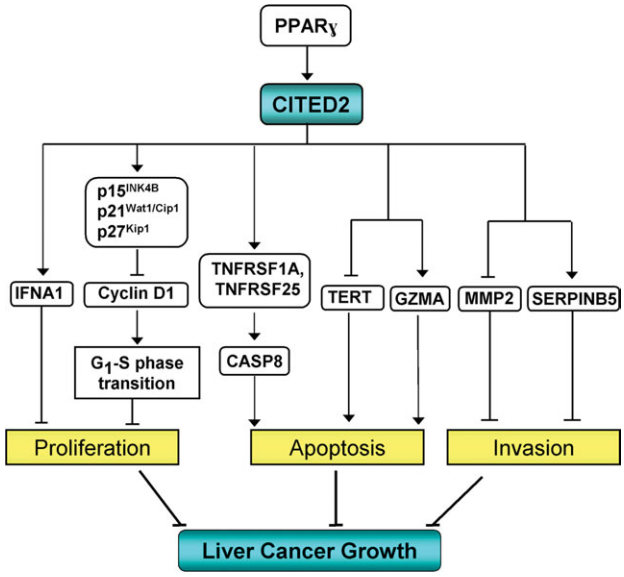

Figure 7. Schematic diagram showing the molecular roles of CITED2 as a direct effector of PPARγ in protection against hepatocarcinogenesis.

interaction between PPARγ and each of these effectors was confirmed by ChIP-PCR analysis (Fig. 1). Among these targets, CITED2 is the most significant novel PPARγ-bound gene. *CITED2* has been reported to play an essential role in liver development. CITED2-deficient mice display fetal liver hypoplasia with increased cell apoptosis and disrupted cell-cell contact.[14] CITED2 also plays a major role in shear-induced down-regulation of MMP-1 and MMP-13 via a TGF-β–dependent pathway.[15] CITED2 knockdown increases the invasiveness of colon cancer cell lines.[16] We examined CITED2 mRNA expression in paired primary HCC specimens and found that CITED2 transcription was significantly reduced in primary HCC tumors compared with their adjacent nontumor tissues, with the implication that CITED2 may function as a tumor suppressor in liver cancer development. To better define the effect of CITED2 in liver carcinogenesis, we examined the functional consequences of CITED2 knockdown and overexpression in the human liver cell lines. Depletion of CITED2 dramatically induced colony formation in both cell lines (Fig. 2). In agreement with the promoted clonogenicity, knockdown of CITED2 also caused an increase in cell viability (Fig. 2). On the other hand, ectopic expression of CITED2 in the HCC cell lines HepG2 and BEL7404 significantly suppressed the cell viability of both cell lines, confirming the antiproliferative effect of CITED2 in HCC. Collectively, these results demonstrate the importance of CITED2 as a novel tumor suppressor in liver cancer.

To investigate the mechanism by which CITED2 regulates cell growth, we performed FACS; cell cycle distribution analysis revealed that knockdown of *CITED2* significantly reduced the proportion of cells in G_1 phase, with a concomitant increase of cells in S phase, inferring that knockdown of CITED2 promoted cell cycle transition from G_1 to S phase. To explore the molecular mechanism underlying G_1-S phase transition, we examined the regulatory proteins that control the G_1-S checkpoint in cells. The increased G_1-S phase transition by CITED2 knockdown was associated with down-regulation of cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21^{Wat1/Cip1}$, and $p27^{Kip1}$, and up-regulation of cyclin D1 (Fig. 4D,E), thereby promoting cell proliferation. Conversely, overexpression of CITED2 in HepG2 and BEL7404 cells enhanced expression of $p21^{Wat1/Cip1}$ and $p27^{Kip1}$ (Fig. 5), thus inhibiting cell proliferation. The role of $p15^{INK4B}$, $p21^{Wat1/Cip1}$, $p27^{Kip1}$, and cyclin D1 as major players in G_1 arrest has been well accepted.[17] $p21^{Wat1/Cip1}$ and $p27^{Kip1}$ are potent inhibitors of cyclin D/Cdk4 and cyclin E/Cdk2 activities, $p15^{INK4B}$ is an important inhibitor of cyclin D/CDK4/6 activity,[17] whereas cyclin D1 protooncogene is an important regulator of G_1-S phase progression and serves as a cell cycle regulatory switch in actively proliferating cells.[18] Thus, results from assays of the loss- and gain-of-CITED2 function suggested that the mechanism of CITED2-mediated cell cycle arrest is most likely associated with deregulation of cell cycle regulators in HCC.

To further elucidate the molecular basis of the anti-HCC effect of CITED2, we conducted a cancer pathway cDNA array. The data showed that the growth inhibition by CITED2 in HCC was correlated with induction of the antiproliferative regulator IFNA1, proapoptotic mediators including TNFRSF1A, TNFRSF25, CASP8, and GZMA, and a tumor suppressor gene SERPINB5, but caused inhibition of the oncogene TERT and proinvasion/metastasis genes MMP2 (Fig. 7). IFNA1 has been shown to inhibit tumor growth and metastasis.[19] TNFRSF1A, TNFRSF25, and CASP8 have been reported to be key extrinsic proapoptotic mediators.[20] Therefore, CITED2 enhanced TNFRSF1A and TNFRSF25 expression, which can cause cleavage of downstream effector CASP8, resulting in apoptosis.[21] GZMA plays a pivotal role as an apoptotic inducer to trigger cell senescence in hematopoietic malignancy.[22,23] SERPINB5 is a tumor suppressor in human breast, prostate, and pancreatic cancers.[24-26] TERT is a direct target of c-Myc, an oncogenic transcription factor, and functions as a potential oncogene.[27] MMP2 has been well

documented to play an important role in promoting cancer invasion and metastasis.[28-30] Taken together, the anti-HCC effect of CITED2 is in part mediated through up-regulation of IFNA1, TNFRSF1A, TNFRSF25, CASP8, GZMA, and SERPINB5, and down-regulation of TERT and MMP2, which in turn suppresses HCC cell growth (Fig. 7).

In conclusion, we have applied an integrated approach to identify for the first time the direct effectors of PPARγ, and CITED2 plays a critical role in HCC as a novel tumor suppressor through regulating cell proliferation and cell cycle progression, which provides new mechanistic insight into the antitumor effect of PPARγ.

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CONFLICT OF INTEREST DISCLOSURE

The authors made no disclosure.

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