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PPARα regulates tumor cell proliferation and senescence via a novel target gene carnitine palmitoyltransferase 1C

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Abstract

Carnitine palmitoyltransferase 1C (CPT1C), an enzyme located in the outer mitochondria membrane, has a crucial role in fatty acid transport and oxidation. It is also involved in cell proliferation and is a potential driver for cancer cell senescence. However, its upstream regulatory mechanism is unknown. Peroxisome proliferator activated receptor α (PPARα) is a ligand-activated transcription factor that regulates lipid metabolism and tumor progression. The current study aimed to elucidate whether and how PPARα regulates CPT1C and then affects cancer cell proliferation and senescence. Here, for the first time we report that PPARα directly activated CPT1C transcription and CPT1C was a novel target gene of PPARα, as revealed by dual-luciferase reporter and chromatin immunoprecipitation (ChIP) assays. Moreover, regulation of CPT1C by PPARα was p53-independent. We further confirmed that depletion of PPARα resulted in low CPT1C expression and then inhibited proliferation and induced senescence of MDA-MB-231 and PANC-1 tumor cell lines in a CPT1C-dependent manner, while forced PPARα overexpression promoted cell proliferation and reversed cellular senescence. Taken together, these results indicate that CPT1C is a novel PPARα target gene that regulates cancer cell proliferation and senescence. The PPARα-CPT1C axis may be a new target for the intervention of cancer cellular proliferation and senescence.

Introduction

Carnitine palmitoyltransferase 1C (CPT1C) is a member of the CPT family that catalyzes the acylation of long-chain fatty acids and their entries into mitochondria for β-oxidation (1–3). CPT1C is necessary for the regulation of energy homeostasis (3). CPT1C is involved in molecular pathways in the hypothalamus regulating food intake (3,4) and systemic energy use (5). Moreover, CPT1C is induced by metabolic stress situations such as glucose deprivation and hypoxia (6). Recently, it was revealed that CPT1C is related closely to fatty acid uptake and metabolism in metastatic progression and poor prognosis of human cancers (7,8). Most recently, we found that CPT1C is a key regulator of cellular

proliferation and senescence in cancer cell lines (unpublished data). However, the upstream regulatory mechanisms that lead to these CPT1C-dependent cell events have not been elucidated.

Peroxisome proliferator activated receptor alpha (PPARα) belongs to PPAR family which consists of PPARα, PPARδ and PPARγ. PPARα is a ligand-activated nuclear receptor that is activated by xenobiotics such as fibrate drugs and industrial plasticizers (9). PPARα is expressed in tissues with high fatty acid oxidation such as heart, liver, skeletal muscle and kidney (10), where it regulates genes involved in fatty acid transport, mitochondrial, peroxisomal β-oxidation and glucose metabolism

Abbreviations

CPT1C	carnitine palmitoyltransferase 1C
ChIP	chromatin immunoprecipitation
PBS	phosphate-buffered saline
PPARα	peroxisome proliferator activated receptor α
PPRE	peroxisome proliferator response element

(11–14). PPARα regulates genes encoding fatty acid binding proteins, transporters and β-oxidation genes such as medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase (VLCAD) (15). Most importantly, muscle-type carnitine palmitoyltransferase 1 called CPT1B has been identified as a PPARα target gene, which has a peroxisome proliferator response element (PPRE) in its first exon (16,17). Furthermore, PPARα agonist clofibrate stimulates liver-type carnitine palmitoyltransferase 1 called CPT1A through a classical direct repeat 1 motif while long chain fatty acids induce CPT1A via elements in the first intron of the gene (18). Since CPT1C is a member and isoform of the CPT1 family, the assumption was made that PPARα might also regulate the CPT1C gene.

Therefore, the current study aimed to elucidate whether and how PPARα regulates CPT1C and then regulates tumor cell proliferation and senescence in a CPT1C-dependent manner. In this study, depletion of PPARα resulted in low CPT1C expression as well as inhibition of proliferation and induction of cell senescence, while overexpression of PPARα promoted proliferation and reverses senescence of MDA-MB-231 and PANC-1 tumor cell lines. CPT1C was under the direct transcriptional control of PPARα which proved that CPT1C is a novel target gene of PPARα, as revealed by dual-luciferase reporter and ChIP assays. Moreover, regulation of CPT1C by PPARα was independent of p53. These results indicate that CPT1C is a novel PPARα target gene that regulates cancer cell proliferation and senescence. The PPARα-CPT1C axis may be a new target for the intervention of cellular proliferation and senescence.

Materials and methods

Cell culture

Human breast cancer cell line MDA-MB-231 (provided by Dr Jun Du at Sun Yat-sen University, Jan 2015) and pancreas cancer cell line PANC-1 (provided by Guangzhou Cellcook Biotech Company, Jan 2015) were cultured in Dulbecco's modified Eagle's medium (Corning) with 4.5 g/l glucose, L-glutamine and sodium pyruvate supplemented with 10% fetal bovine serum (Gibco), 1% penicillin sodium and streptomycin sulfate (Gibco) in a 5% CO_2 atmosphere with saturated humidity at 37°C. On 4 January 2016, these two cell lines, MDA-MB-231 and PANC-1 were authenticated using Short Tandem Repeat Authentication by authorizing Guangzhou Cellcook Biotech Company.

Transfections with siRNA and expression plasmids

For the RNA interference experiments, small interfering RNA (siRNA) (Guangzhou Ruibo Biotech Company) was used to decrease PPARα, PPARδ and PPARγ and CPT1C levels. MDA-MB-231 and PANC-1 cells were transfected with 5 nM siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with reduced serum medium Opti-MEM (Gibco). The sequence of CPT1C siRNA is 5′-GCCAUGAUCGCUGGUUUGA-3′ and the sequence of PPARα is 5′-GGAGCAUUGAACAUCGAAU-3′. Specific methods of transfection can be found in the manual of siRNA. RT-PCR analysis was used to determine the expression of *PPARα* or *CPT1C* to verify the effectiveness of three different siRNA chains in MDA-MB-231 and PANC-1 cell lines. Then the most effective one was chosen to finish our all experiments.

The human PPARα DNA was subcloned into the pGST vector (Addgene), and the plasmid was transfected at a concentration of 1 ug/10^6 cells using Lipofectamine2000 DNA Transfection Reagent (Invitrogen) with reduced serum medium Opti-MEM (Gibco).

Chemical agonist or inhibitor was also used to down-regulate or up-regulate PPARα. PPARα inhibitor GW6471 (Sigma) and its agonist WY14643 (Sigma) were used in this study.

Real-time PCR analysis

Total RNA from cultured cells were isolated by using Trizol reagent. About 1 ug RNA was reverse transcribed to cDNA by using Prime Script RT Reagent Kit with gDNA eraser (TaKaRa). Real-time PCR was performed by using SYBR Premix Ex-Taq II Kit (TaKaRa) in Applied Biosystems 7500 real-time PCR System and analyzed using the δδCt method. The sequences of primers are listed in Supplementary Table 1, available at *Carcinogenesis* Online.

Western blot analysis

Proteins from cultured cells were prepared using RIPA lysis buffer containing 1% 100 mM phenylmethanesulfonyl fluoride and quantified by BCA Protein Assay Kit (Thermo). Protein expression was analyzed by western blotting. Thirty micrograms protein was subjected to SDS-PAGE and transferred on to polyvinylidene fluoride membranes. After blocking, the membranes were immunoblotted with antibodies by overnight incubation at 4°C using antibodies against GAPDH (Cell Signaling Technology), PPARα (Abcam), CPT1C (Abcam) and p53 (Abcam). Secondary anti-rabbit or anti-mouse antibodies were applied on the following day. The ECL Detection Kit (Engreen Biosystem) was used to develop the blots. The intensity of protein bands was quantitated by Quantity One software (Bio-Rad Laboratories, Hercules, USA).

Luciferase activity assay

A series of different lengths of *CPT1C* promoter regions containing potential PPRE binding sites were PCR-amplified from genomic DNA and cloned into a pGL3-basic vector firstly. To detect the alteration of *CPT1C* promoter activity caused by PPARα, HEK-293T cells were plated in 96-well plates, and total plasmid DNA (120 ng luciferase reporter plasmid luc-pGL3-CPT1C, 60 ng overexpression plasmid pGST-PPARα, 5 ng internal control plasmid luc-TK) were transfected together in each well using Lipofectamine 2000 (Invitrogen). pGL3-basic empty vector was regarded as negative control and 10 μM GW6471 was utilized as inhibitor of PPARα. Cells were incubated for 24 h prior to lysis with 1× Passive Lysis Buffer. Luciferase enzymatic activity was measured by a commercial Dual-Luciferase Reporter Assay System Kit (Promega).

Chromatin immunoprecipitation-qPCR assay

Chromatin immunoprecipitation assay was performed using Pierce Agarose ChIP Kit (Thermo). Briefly, nuclear proteins were crosslinked to genomic DNA by 1% formaldehyde. Subsequently, cells were collected in cold phosphate-buffered saline (PBS) containing protease inhibitors. Following centrifugation, the pellets were resuspended in lysis buffer. Chromatin DNA fragments were precipitated with 5 μg anti-PPARα antibody (Abcam) overnight at 4°C. Protein G-sepharose beads were added then sequentially the resultant immune complexes were washed with a series of wash buffer. After centrifugation, the immune complexes were resuspended in elution buffers. Human genomic DNA was amplified using the primers shown in Supplementary Table 2, available at *Carcinogenesis* Online, by real-time qPCR according to the putative PPRE binding sites identified *in silico*. Input sample and IgG antibody were served as positive and negative control, respectively.

WST-8 assay and BrdU assay

Cell viability and proliferation capacity were analyzed by water-soluble tetrazolium-8 (WST-8) and bromodeoxyuridine (BrdU) assays, respectively. For both WST-8 and BrdU assays, cells were seeded into 96-well plates in a final volume of 100 μl/well. 48 h after transfection, the WST-8 activity was measured by addition of 10 μl highly sensitive water soluble tetrazolium salt reagent (Beyotime Biotech Company) to each well, incubation for 2 h at 37°C and absorbance was measured at 450 nm wavelength. BrdU incorporation was determined by use of a Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Company). In brief, cells were incubated for 2 h after adding 10 μl BrdU labeling solution into every well. Anti-BrdU-POD

working solution was served as specific antibody and absorbance was tested at 370 nm wavelength.

Cell cycle analysis

The flow cytometer was used to measure the cell cycle. Briefly, 48 h after transfection, the cells were washed with 1× PBS (Gibco), then centrifuged and fixed with 70% cold ethanol at 4°C overnight. Cells were then centrifuged and re-suspended in PBS. RNASE and 0.5 ml propidium iodide were added and incubated for 30 min at 37 °C in the dark. Samples were acquired by using flow cytometer at 488 nm wavelength and these data were processed with FlowJo 7.6.

Colony formation assay and β-gal assay

Cells were seeded with 2500 cells/well in six-well plates and incubated at 37°C for 14 days after transfecting for 48 h for the colony formation assay. The cells were fixed with 4% formaldehyde and stained with 1% crystal violet for 3 min.

To detect β-gal activity, cells after transfecting 48 h were fixed with gluteraldehyde in PBS for 15 min, then washed with PBS twice and stained for 12–16 h by using the Senescence β-galactosidase Staining Kit (Beyotime Biotech Company). Pictures were obtained by inverted fluorescence microscope (Olympus).

Statistical analysis

All values were expressed as mean ± SEM. Two-tailed Student's *t* tests and graphs were performed using GraphPad Prism v6.0c software (GraphPad Software, Inc). Significance is represented by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control, 'NS' means no significant difference between two groups.

Results

PPARα regulates CPT1C mRNA and protein expression

CPT1C mRNA and protein levels were measured in MDA-MB-231 cells and PANC-1 cells after down-regulating PPARα by siRNA or its inhibitor GW6471 and up-regulating PPARα with pGST-PPARα plasmid or its agonist WY14643. *CPT1C* mRNA was significantly down-regulated or up-regulated in a PPARα-dependent manner (Figure 1A). Similar results were obtained at the protein level as determined using western-blot assay (Figure 1B). The status of CPT1C protein in MDA-MB-231 cell line was also examined by immunohistochemistry and a similar correlation between PPARα expression and CPT1C expression was found. Positive staining of CPT1C decreased markedly when cells were transfected with a PPARα siRNA (Figure 1C). These data demonstrated that PPARα controls CPT1C expression.

PPARα directly activates CPT1C transcription

To determine whether there is direct regulatory effect of PPARα on *CPT1C*, dual-luciferase reporter gene and ChIP assays were performed. First, to detect whether PPARα can activate the transcriptional activity of *CPT1C* promoter, a dual-luciferase reporter gene assay was performed in HEK-293T cells. Luciferase activity assay revealed that a 3.0 kb region upstream of the CPT1C transcription start site and PPARα were required for PPARα regulation for CPT1C (Figure 2A). Through bioinformatic analysis, two potential PPRE binding elements AGGTCA N AGGTCA motif-containing direct repeat (DR)n, DR1 and DR3 were located between 2.1 and 1.7 kb upstream of the *CPT1C* transcription start site (Figure 2C PPRE1 and PPRE2). A 2.1-kb length promoter containing both DRn regions and a 1.7-kb region in which these two DRn were deleted, were inserted into a luciferase reporter plasmid. As in Figure 2A, 2.1 kb CPT1C reporter gene was activated by the transfection of PPARα. Moreover, serial deletion analysis showed that the 1.7-kb promoter remained activated (Figure 2A). These results indicate that there were other potential cryptic PPREs

within 1.7 kb *CPT1C* promoter region that were not identified by *in silico* analysis. More experiments were conducted to find other possible PPREs, and further deletion analysis revealed that the transactivation was abolished in the 1.0 and 0.6 kb length *CPT1C* promoter reporter plasmid constructs (Figure 2A). This indicates that PPREs with promoter activity exist between 1.7 kb and 1.0 kb of the *CPT1C* promoter region (Figure 2B).

Furthermore, assessment of PPARα's binding to the *CPT1C* promoter was confirmed using ChIP assays in MDA-MB-231 cells. Amplification of the 10% input sample prior to immunoprecipitation was equivalent in all samples which were regarded as a positive control and those with absence of signal in the control IgG immunoprecipitate were considered as a negative control. Cells were transfected with pGST-PPARα plasmid 24 h before ChIP assay. The result showed efficient responsive recruitment of PPARα to PPRE1 and PPRE2 (Figure 2C and D left). It is worth mentioning that the overexpression PPARα treatment group displayed bright electrophoresis bands in the expected 120 or 150 bp positions containing these two new predicted PPRE sites between 1.7 and 1.0 kb promoter region (Figure 2C), called PPRE3 and PPRE4 (Figure 2C and D right). These results were consistent with the results of luciferase reporter gene assay, and indicate that PPARα binds to several different DRn sites on *CPT1C* promoter.

PPARα regulates tumor cells viability and proliferation

Recently, we found that CPT1C is a novel regulator of cancer cell proliferation and senescence. Therefore, experiments were conducted to determine whether PPARα can regulate tumor cell proliferation and senescence in a CPT1C-dependent manner. The WST-8 assay was used to detect viability of cancer cells, and BrdU incorporation in DNA was measured to directly determine the role of PPARα in cell proliferation. Both the viability and proliferation of MDA-MB-231 and PANC-1 cells decreased in the PPARα siRNA-treated group compared with the siControl group, while the viability and proliferation increased after overexpressing PPARα in these two cell lines (Figure 3A and B). Immunohistochemistry with Ki67 in MDA-MB-231 cells was carried out to measure the cell multiplication capacity. Compared with the siControl group, positive staining for Ki67 was significantly reduced in the PPARα siRNA-treated cells, and there was an apparent tendency towards increased staining for Ki67 in the PPARα-overexpressing cells (Figure 3C). Based on these data, the assumption was made that the cell cycle may be affected in low PPARα-expressing cells. Indeed, cell cycle analysis showed an increased proportion in G2/M phase from 40% to 47% and in S-phase from 17% to 23% after treatment of MDA-MB-231 cells with PPARα siRNA. Similar results were observed in PANC-1 cells (Figure 3D). Thus, the cell cycle was arrested in G2/M and S-phase when the expression of PPARα was depleted.

PPARα affects cancer cells senescence

Furthermore, whether PPARα can regulate tumor cell senescence in a CPT1C-dependent manner was determined. Representative experiments were performed to validate cell senescence such as β-gal activity assay, colony formation assay, and senescence-associated secretory phenotype (SASP) factors detection. Colony formation assays confirmed that depletion of PPARα could inhibit the ability of tumor cells to form colonies, while overexpression PPARα promoted colony formation (Figure 4A). Consistent with this observation, positive staining for β-gal activity in cells suppressing PPARα siRNA was increased (Figure 4B), which indicated a substantial correlation between PPARα expression and senescence in tumor cells. There was no significant change in
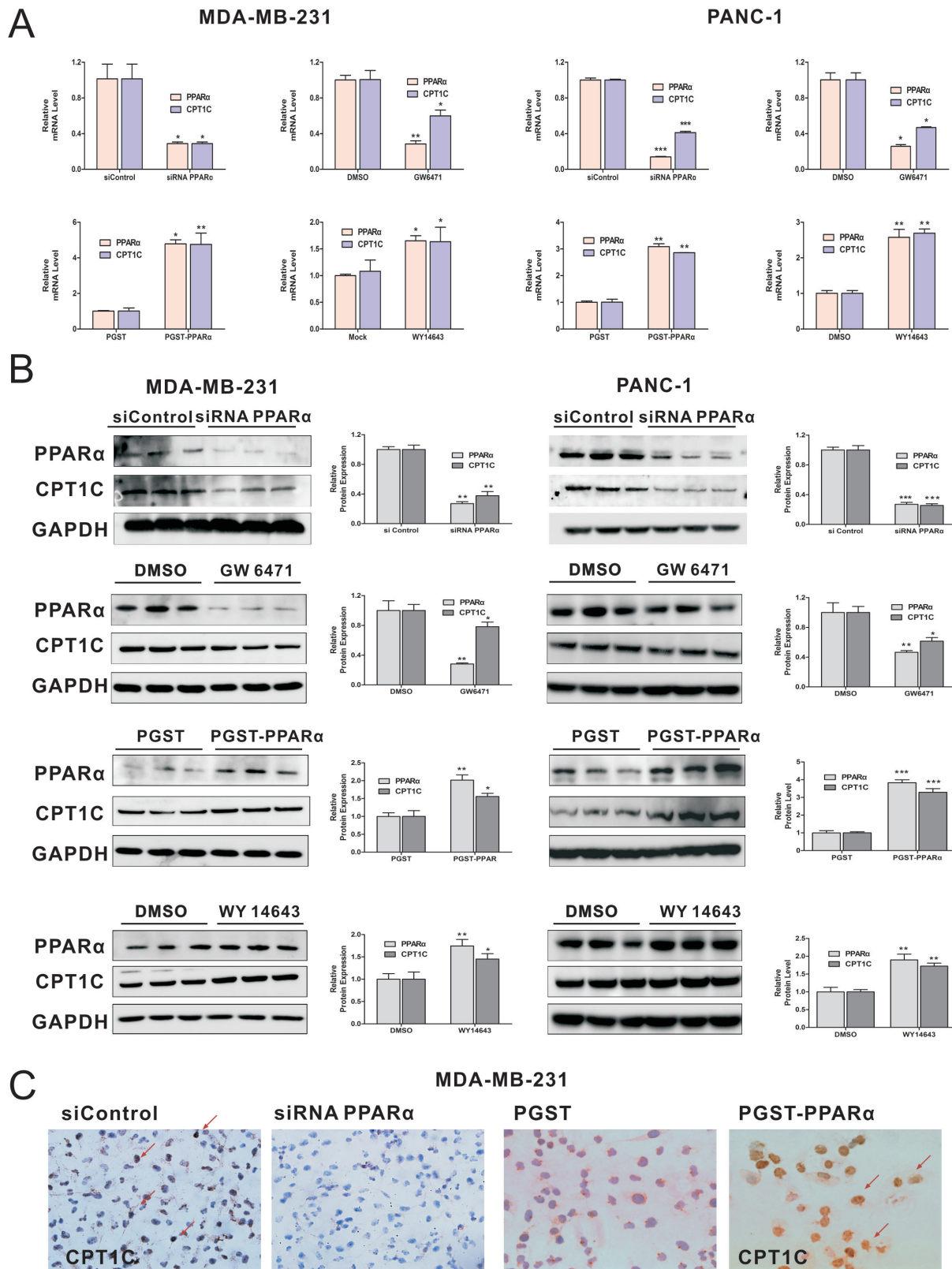
Figure 1. PPARα regulates CPT1C mRNA and protein expression. (**A**) RT-PCR analysis was used to determine the expression of *PPARα* and *CPT1C* in PANC-1 and MDA-MB-231 cells after down-regulating *PPARα* by siRNA or its inhibitor GW6471 (10 μM) and up-regulating *PPARα* with pGST-PPARα plasmid or its agonist WY14643 (100 μM). Data are the mean ± SEM (*n* = 4). (**B**) Western blot was used to measure protein level of PPARα and CPT1C as the same conditions described as above where GAPDH was used as a loading control. Gray scanning was evaluated by Quantity one. Data are the mean ± SEM (*n* = 3). (**C**) MDA-MB-231 cell line were stained for CPT1C by immunohistochemistry.
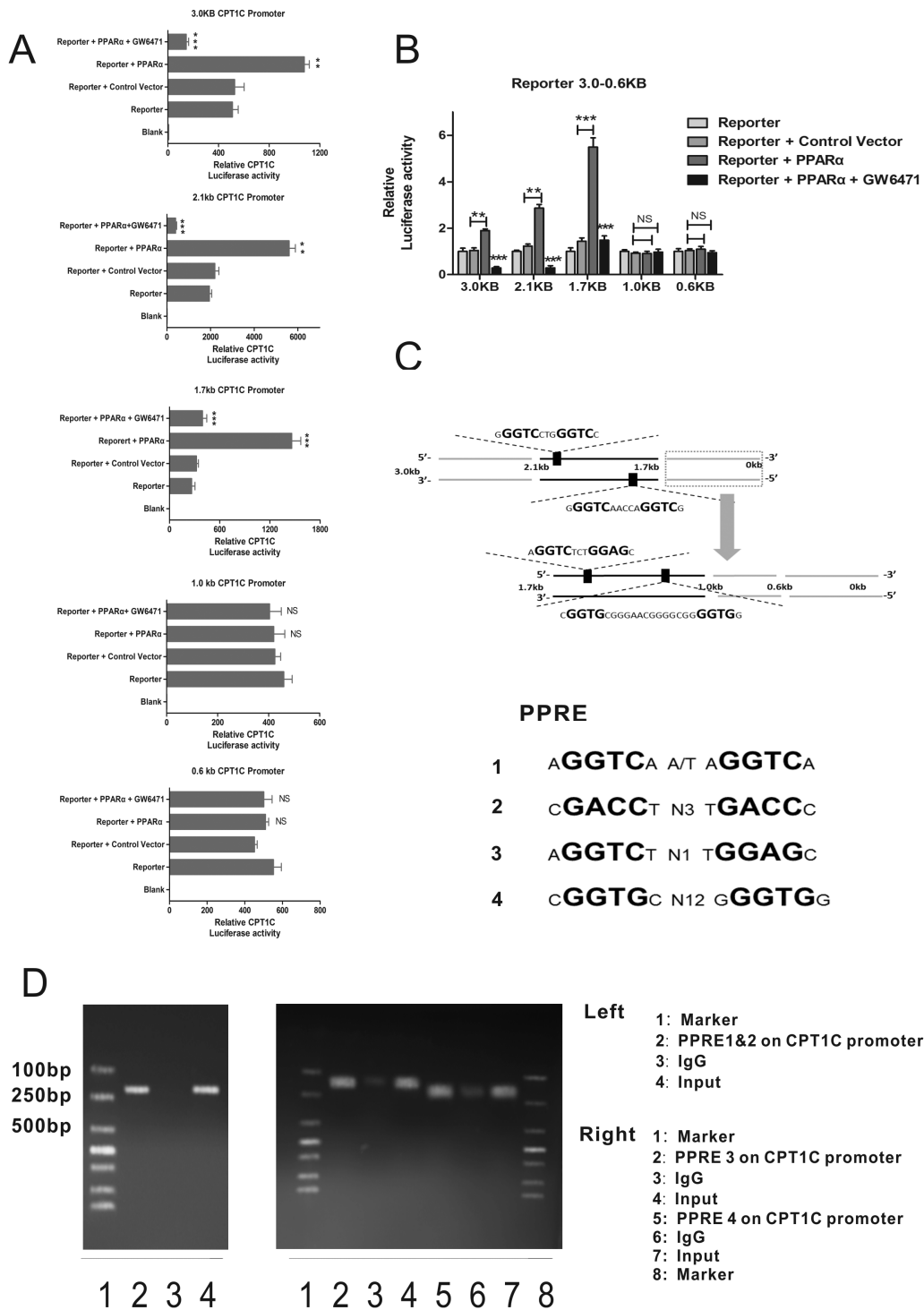
Figure 2. PPARα directly activates CPT1C transcription. (**A**) A series of dual-luciferase reporter gene assays were conducted in HEK-293T cells to compare reporter activities among plasmids with different lengths of CPT1C promoter regions. Data are the mean ± SEM (n = 6). (**B**) Dual-luciferase reporter gene assays results after integrating together. Data are the mean ± SEM (n = 6). (**C**) Four different PPRE regions called DRn were predicted in 3.0 kb CPT1C promoter region by bioinformatics. (**D**) MDA-MB-231 cells were treated with pGST-PPARα plasmid for 24 h then ChIP analysis was performed. DNA samples after precipitation reaction were purified and amplified through qPCR.

β-gal staining in the PPARα overexpressing group compared to the pGST group (Figure 4B). To further confirm these findings, more than ten types of representative SASP factors (19,20) were measured. The secretion of the major SASP components such as IL-6, IL-8, IL-1β, TNFα and MMP3 was markedly increased after PPARα siRNA treatment in these two cell lines (Figure 4C), although there were subtle differences in types of changed factors between MDA-MB-231 and PANC-1 cells.

The effect of PPARα on CPT1C is in a p53-independent way

It was reported that p53 protects tumor cells from metabolic stress via induction of CPT1C which was shown to be a p53 target gene (6). Since other studies indicated that PPARα had a regulatory effect on p53 (21,22), it should be further determined whether PPARα regulation of CPT1C is dependent on p53 signaling. MDA-MB-231 cells were
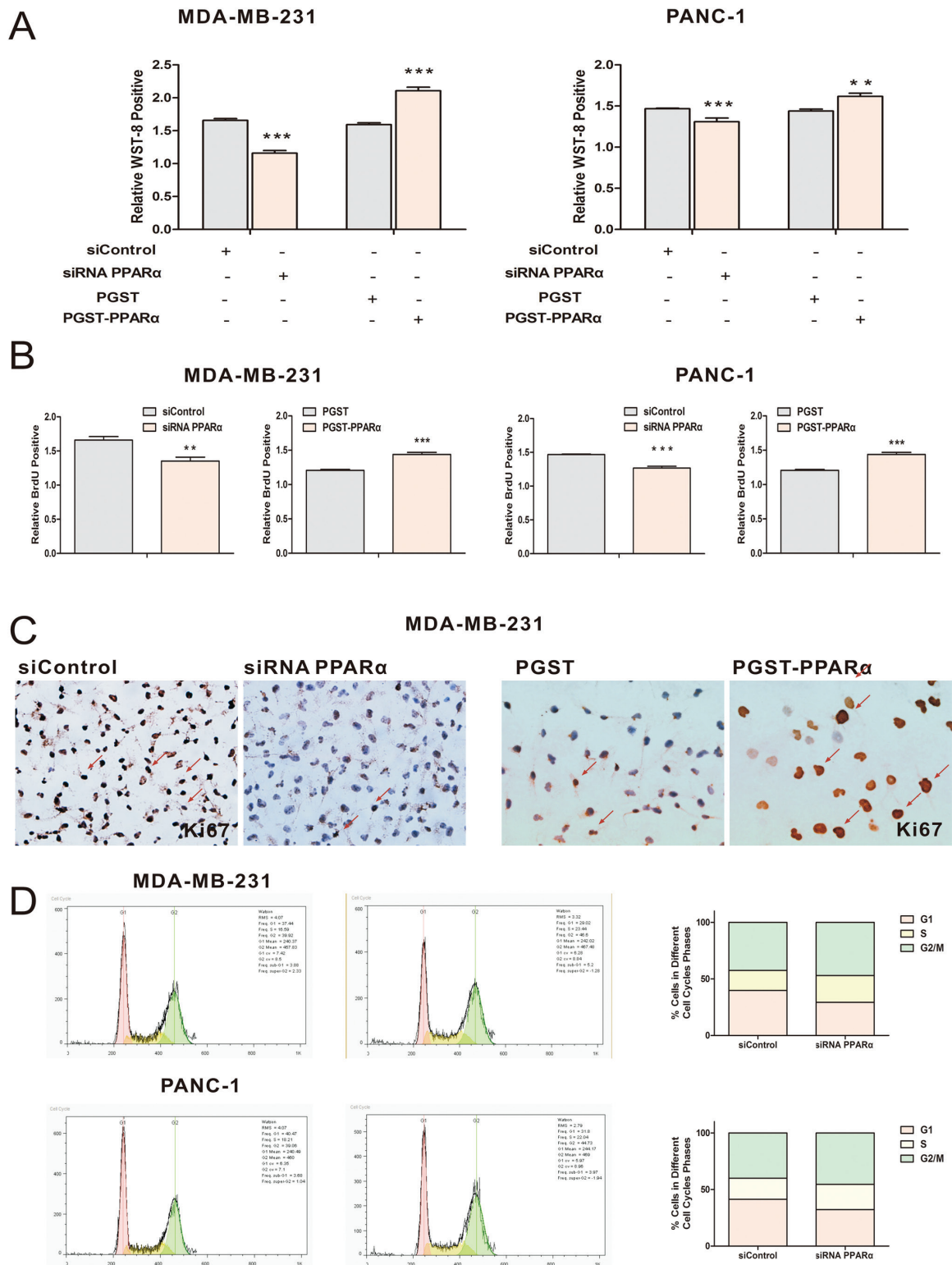
Figure 3. PPARα modulates cancer cell viability and proliferation. (**A**) WST-8 assay of MDA-MB-231 and PANC-1 cells were performed to examine cell viability after knocking down or overexpressing PPARα. (**B**) BrdU activity measured proliferation capacity of cells with treatment in siRNA PPARα or pGST-PPARα plasmid. (**C**) MDA-MB-231 cell lines were stained for Ki67 by means of immunohistochemistry. (**D**) Cell cycles of two cell lines were determined by flow cytometry when depletion of PPARα. Data are the mean ± SEM, P < 0.05 versus siControl or vehicle.

transfected with PPARα siRNA or the pGST-PPARα vector and treated with the PPARα agonist WY14643 or the PPARα antagonist GW6471. Knockdown or inhibition of PPARα expression decreased p53 expression at both the mRNA and protein levels (Figure 5A and B) while regulation of CPT1C by PPARα is independent of p53 (Figure 5C). These results indicate that CPT1C is directly regulated by PPARα in a p53-independent manner.
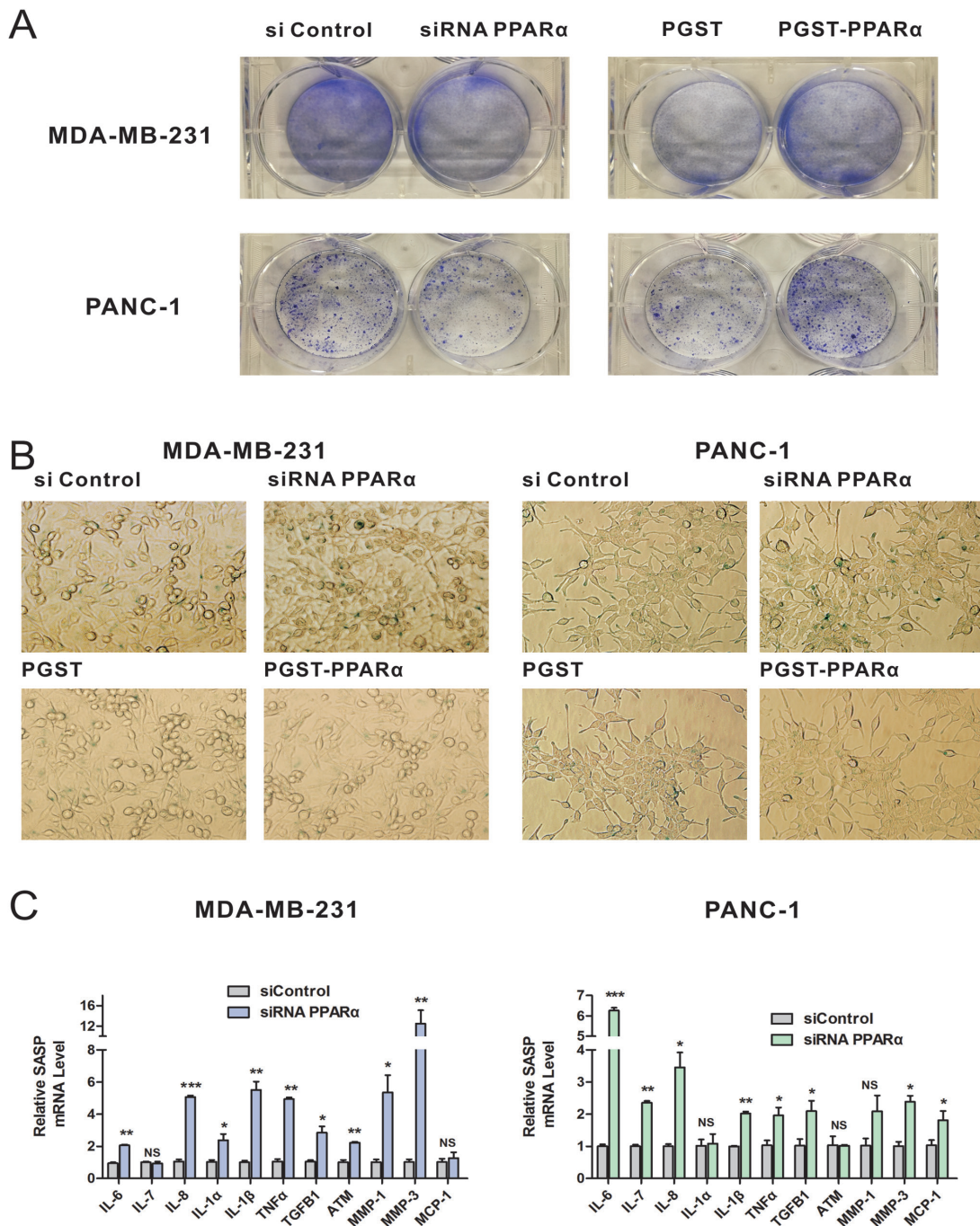
Figure 4. PPARα silencing induces cancer cell senescence. (**A**) Cells were stained with crystal violet for colony formation after being cultured for an additional 14 days. (**B**) SA-β-gal activity was measured to represent degree of senescence of tumor cells. (**C**) RT-PCR analysis was used to determine the expression of SASP factors such as *IL-6, IL-7, IL-8, IL-1α, IL-1β, TNFα, TGFB1, ATM, MMP-1, MMP-3* and *MCP-1* in two cell lines. Data are the mean ± SEM ($n = 4$).

Discussion

This study revealed that *CPT1C* is a PPARα target gene as evidenced by dual-luciferase reporter gene assay, ChIP assay and measurement of CPT1C mRNA and protein levels after gain-of-function and loss-of-function in PPARα. Moreover, PPARα modulates the proliferation and senescence of tumor cells via regulating expression of CPT1C. This PPARα-CPT1C axis may be a new target for the intervention of cancer cell proliferation and senescence.

CPT1 family catalyzes the reversible transesterification of carnitine and acyl-CoA esters to form acyl-carnitine esters and coenzyme A in the mitochondria membrane. CPT1A is expressed in liver and other tissues characterized with high rates of fatty acid synthesis, while CPT1B is expressed mainly in tissues with high rates of fatty acid oxidation, such as muscle and brown adipose tissue (23). CPT1C differs from both CPT1A and CPT1B, since it is constitutively expressed at low levels and induced under conditions of glucose deprivation and hypoxia in embryonic tissues (6,24). This suggests an important role for CPT1C in the
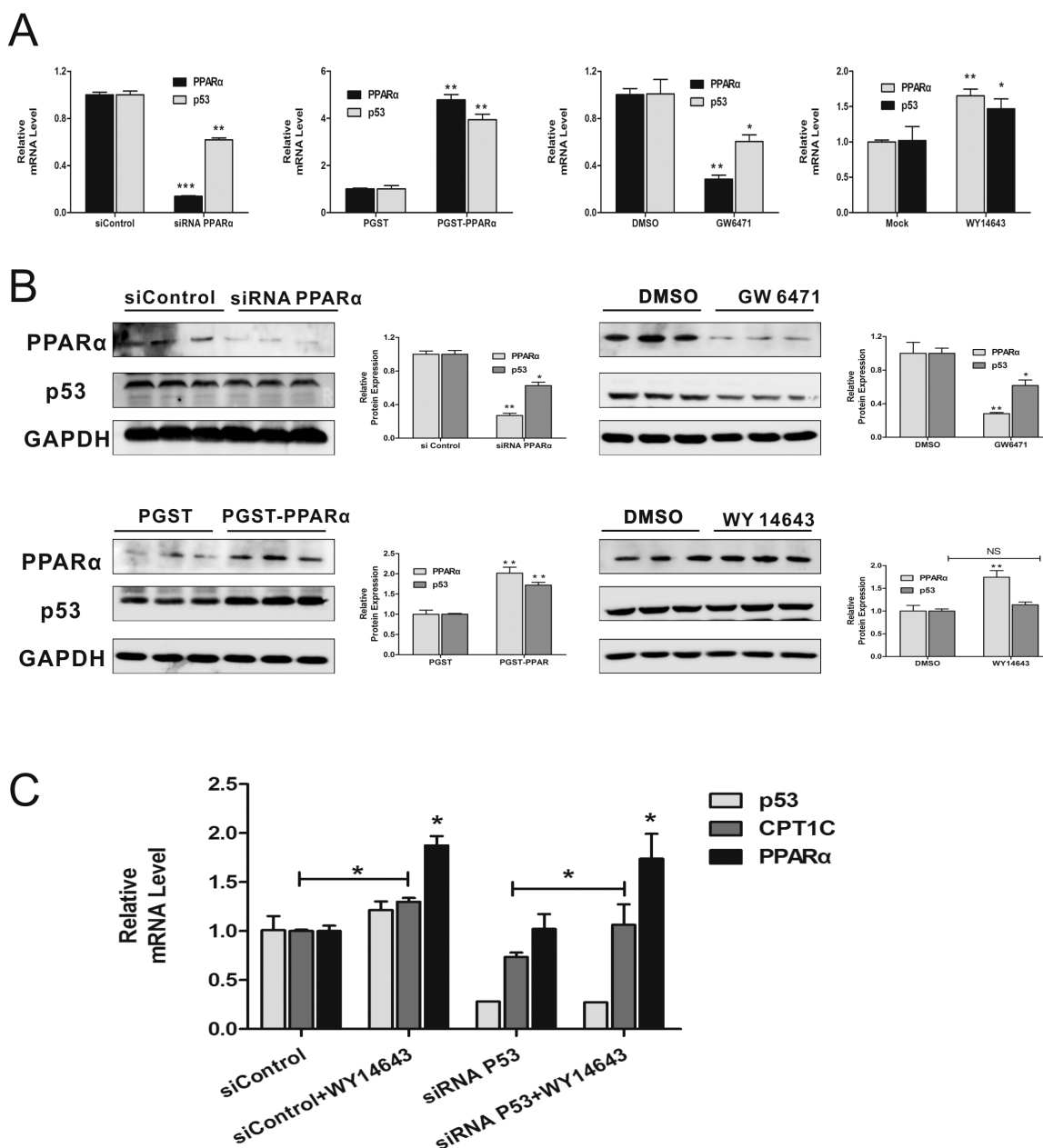
Figure 5. PPARα regulates CPT1C expression in a p53 independent way. (**A**) RT-PCR analysis was used to determine the expression of *PPARα* and *p53* mRNA levels in MDA-MB-231 cell line after down-regulating *PPARα* by siRNA or its inhibitor GW6471 (10 μM) and upregulating *PPARα* by overexpression plasmid pGST-PPARαor its agonist WY14643 (100 μM). Data are the mean ± SEM (*n* = 4). (**B**) Western blot was used to measure protein levels of PPARα and p53 as the same conditions as above by regarding GAPDH as internal references. Gray scanning was evaluated by Quantity one. Data are the mean ± SEM (*n* = 3). (**C**) RT-PCR analysis showed that the effect of *PPARα* on CPT1C expression still remained after silencing *p53* expression. Data are the mean ± SEM (*n* = 4).

adaptation to metabolic stress. Moreover, cancer cells are known to adapt to energy stress and develop a lipogenic phenotype which increases fatty acid synthesis (25–27). Some tumors maintain viability and growth under conditions of metabolic stress by consuming fatty acids (28,29), and many types of tumors display increased dependence on fatty acid oxidation as their primary sources of energy for proliferation and survival (30–32). Given that the entry of fatty acids into mitochondria is regulated by these long-chain acylcarnitine acyltransferases, CPT1s especially CPT1C have emerged as new potential therapeutic targets in various types of cancer. The expression of CPT1C is elevated in a wide array of human tumor types such as liver cancer, breast cancer and neuroblastoma (24,33). The relationship between human tumors and CPT1C expression needs to be thoroughly

examined, notably the mechanism by which CPT1C influences cell proliferation or senescence. Most recently, we found that CPT1C is a novel biomarker and key regulator of mitochondrial dysfunction-associated cellular senescence and proliferation (data unpublished), suggesting that inhibition of CPT1C may represent a new therapeutic strategy to suppress tumorigenesis. In these studies, low CPT1C expression and activity were observed in senescent PANC-1 cells by metabolomic analyses. Moreover, knocking down CPT1C in six tumor cell lines provoked mitochondrial dysfunction, caused growth suppression and cellular senescence, suppressed cell survival under metabolic stress and suppressed tumorigenesis in mice xenograft model. However, the upstream regulatory mechanisms that lead to these CPT1C-dependent cell events remain unknown.

The PPAR family is involved in the control of metabolism and metabolic regulation (34). Notably, PPARα, PPARβ/δ and PPARγ are regulators of energy homeostasis (35). PPARβ/δ is highly expressed in skeletal muscle and adipose tissue while PPARγ is abundant in the white and brown adipose tissues, where it promotes adipocyte differentiation and lipid storage. PPARα on the other hand, is expressed in tissues with a high rate of fatty acid catabolism, such as liver, heart, kidney and brown adipose tissue (14,36). In accordance with the function of PPARα in fatty acid transport and oxidation, PPARα controls the expression of *CPT1A and CPT1B* which have been identified as PPARα target genes (16–18). Through detecting the expression of *CPT1A* which is also a downstream gene of PPARα, a series of benzenesulfonimide compounds have been proved to play a role in antagonizing the transcriptional response induced by PPARα agonist (37). Since CPT1C is also an isoform of the CPT1 family, the assumption was made that PPARα might also regulate the CPT1C gene.

The current work confirms that PPARα regulates *CPT1C* at the transcriptional level. Dual-luciferase reporter gene assays revealed that the *CPT1C* promoter was activated by PPARα through two PPREs that are the typical direct repeat of the hexameric sequence AGGTCA separated by 1 (DR1) or 3 (DR3) base pairs (38). These were found between 1.7 and 2.1 kb of the *CPT1C* transcription start site. Reporter gene studies also revealed promoter activity within 1.7 kb promoter of the *CPT1C* transcription start site were no clear PPREs, suggesting the presence of other cryptic PPREs. No difference in reported gene activity was noted when 1.0 kb length or shorter upstream of the transcription start site was analyzed, thus indicating the absence of PPREs in the proximal promoter. These data indicate that PPARα binds to several different DRn sites on *CPT1C* promoter. PPARβ/δ and PPARγ were shown by a number of studies to affect cell proliferation (39,40), therefore, it should be further studied that whether PPARβ/δ and PPARγ can also regulate CPT1C to modulate cell proliferation. However, depletion of PPARβ/δ and PPARγ by siRNA did not alter expression of *CPT1C* (Supplementary Figure 1, available at *Carcinogenesis* Online), indicating that PPARβ/δ and PPARγ have no regulatory effect on CPT1C, and only PPARα, through modulation of *CPT1C*, influences tumor cell proliferation and senescence.

Furthermore, the AMPK-ACC-CPT1 pathway is a recognized FAO related pathway. Induction of CPT1C can be achieved by inhibition or elimination of ACC2 activity and through activation by transcription factors. In addition, CPT1C is also elevated by activation of AMPK in breast cancer cell, and *CPT1C* is a downstream target gene of ERRα via the AMPK/PGC-1β pathway (41). Moreover, expression of *CPT1C* can also be increased by direct or indirect effects of AMPK activation in a p53-dependent manner (42). AMPK induced p53 activation could affect a series of cellular events, it has been reported that p53 protects tumor cells from metabolic stress via induction of CPT1C which was shown to be a p53 target gene (6). These information indicate possible relationship between CPT1C and other upstream regulatory pathways such as p53. Since other studies indicated that PPARα had a regulatory effect on p53 (21,22) and p53 protects tumor cells from metabolic stress via induction of CPT1C which was shown to be a p53 target gene (6), we further determined whether PPARα regulation of CPT1C is dependent on p53 signaling. Knockdown or inhibition of PPARα expression decreased p53 expression at both the mRNA and protein levels while regulation of *CPT1C* by PPARα is independent of p53. These results indicate that CPT1C is directly regulated by PPARα in a p53-independent manner.

After confirming the direct regulatory effects of PPARα on *CPT1C*, additional studies were conducted to reveal that PPARα regulates cell proliferation and senescence in a CPT1C-dependent manner. WST-8 and BrdU assays, immunohistochemical for Ki67, colony formation assay, β-gal activity and the SASP test were used as a battery of frequently-used experiments to measure the cell proliferation and senescence after down-regulating PPARα by siRNA and up-regulating PPARα with pGST-PPARα plasmid. These results clearly demonstrate that PPARα regulates cell proliferation and senescence.

In order to further prove the cellular proliferation and senescence caused by PPARα were in a CPT1C-dependent manner, MDA-MB-231 cells and PANC-1 cells were transfected with siRNA CPT1C. It was found that depletion of CPT1C could inhibit the effects of PPARα agonist WY14643 or inhibitor GW6471 on cell proliferation and senescence (Supplementary Figure 2, available at *Carcinogenesis* Online), indicating that PPARα regulates cancer cell proliferation and senescence in a CPT1C-dependent manner.

In summary, this study demonstrates that PPARα directly activates CPT1C transcription and CPT1C is a novel target gene of PPARα, as revealed by dual-luciferase reporter and ChIP assays. Moreover, regulation of CPT1C by PPARα is p53-independent. Furthermore, depletion of PPARα results in low CPT1C expression and then inhibits proliferation and induced senescence of MDA-MB-231 and PANC-1 cancer cells in a CPT1C-dependent manner, while forced PPARα overexpression promotes cell proliferation and reverses cancer cellular senescence. Taken together, these results indicate that CPT1C is a novel PPARα target gene that regulates cancer cell proliferation and senescence. The PPARα-CPT1C axis may be a new target for the intervention of cancer cellular proliferation and senescence.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1 and 2 are available at *Carcinogenesis* online.

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