Sirtuin 1 Protects the Mitochondria in Hepatocellular Carcinoma Cells via Suppressing Hypoxia-induced Factor-1 Alpha Expression

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Research Article

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Abstract

Background: We hypothesized that Sirtuin 1 (SIRT1) might attenuate the Warburg effect in tumor cells by modulating hypoxia-induced factor-1 alpha (HIF-1α) expression. This study aimed to explore the role and the underlying mechanism of SIRT1 in protecting the mitochondrial functions in hepatocellular carcinoma (HCC) cells.

Methods: Quantitative real-time PCR and western blot analysis were conducted to determine gene expression in HCC cells. Co-immunoprecipitation (co-IP), chromatin immunoprecipitation (ChIP), and luciferase reporter assays were performed to examine DNA-protein interactions. Colony formation and MTT assays were carried out to explore the role of SIRT1 in HCC cell proliferation *in vitro*. PLC5 and Huh7 tumor xenografts were generated in mice to investigate the role of SIRT1–HIF-1α signaling in HCC development *in vivo*.

Results: In different HCC cell lines, overexpression of SIRT1 promoted oxidative phosphorylation-associated gene expressions, ATP production, cell proliferation, and apoptotic protein expression while attenuating VEGF expression. In mice, overexpression of SIRT1 resulted in significant reductions in the weights of PLC5 and Huh7 tumor xenografts. Knockdown of SIRT1 exhibited opposite effects. Mechanistically, overexpression of SIRT1 promoted HIF-1α deacetylation, VHL-mediated HIF-1α degradation, and AMPK expression. Furthermore, SIRT1 interfered with the HIF-1α–c-Myc interaction to stimulate the transcription of a mitochondrial biogenesis enhancer mitochondrial transcription factor A (TFAM). Overexpression of HIF-1α completely reversed the effects of SIRT1.

Conclusions: SIRT1 protects the mitochondria of HCC cells via suppressing HIF-1α expression, suggesting that SIRT1 may exert antitumor activity in HCC by reducing the Warburg effect.

Background

Hepatocellular carcinoma (HCC) is the sixth most prevalent of all cancers and the fourth leading cause of cancer-related deaths globally. Despite the recent advances in diagnosis and treatment procedures, the overall survival rate of patients with HCC remains poor[1]. Therefore, a better understanding of the pathogenesis of HCC is critically important.

The Warburg effect, or aerobic glycolysis, refers to the high consumption of glucose and preferential production of lactate, despite the availability of oxygen[2]. The Warburg effect has been observed in different types of cancer cells, including HCC cells[3–6]. Warburg hypothesized that defective mitochondrial functions in cancer cells could lead to impaired aerobic respiration and increased dependence on glycolytic metabolism[7]. HCC progression and metastasis have been closely linked to mitochondrial dysfunction[8]. In cancer cells, the Warburg effect has been attributed to the high expression of hypoxia-induced factor-1 alpha (HIF-1α) to adapt to the hypoxic microenvironment[9] physiologically. The activated HIF-1α can induce the transcription of at least 70 of its target genes in

mammalian cells, including vascular endothelial growth factor (VEGF)[10]. In addition, the mechanistic crosstalk between HIF-1α and c-Myc regulates cancer cell metabolism[11]. c-Myc promotes mitochondrial biogenesis by binding to the mitochondrial transcription factor A (TFAM) gene promoter, which is blocked by HIF-1α-mediated inhibition of c-Myc activity under hypoxic conditions [12]. Thus, targeting HIF-1α may interfere with the Warburg effect by improving mitochondrial function and serve as a promising therapeutic strategy for HCC as well[11]. However, the upstream regulators in the HIF-1α signaling in HCC cells remain unclear to date.

The NAD^+-dependent histone deacetylase silent mating-type information regulation 2 homolog 1 (SIRT1) modulates cell survival by deacetylating proteins involved in the cell cycle and apoptosis[13]. Thus, decreased SIRT1 expression may cause mitochondrial dysfunction and activated HIF-1α accumulation in the mitochondria, resulting in enhanced aerobic glycolysis and cellular senescence[14]. Likewise, SIRT1 might potentially modulate the Warburg effect through HIF-1α regulation, but the underlying mechanism needs to be delineated further.

In this study, we investigated the mechanistic roles of SIRT1 in regulating mitochondrial function in HCC cells. Our findings suggest that SIRT1 supports the maintenance of normal mitochondrial functions in HCC cells by suppressing HIF-1α expression, thereby reducing the Warburg effect and inhibiting cancer cell growth at the same time.

Materials And Methods

Cell culture

Human HCC cell lines PLC5, HepG2, and Huh7 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were grown in Dulbecco's modified Eagle's (DMEM) medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin (100 units/mL) and streptomycin (100 g/mL) at 37 °C in a humidified chamber with 5% CO_2.

Tumor xenografts

Cancer cells (1×10^6 cells/mice) were subcutaneously injected into the right flank of six-week-old male nude mice. Tumor size was measured using Vernier calipers. Tumors were collected and weighed two weeks after inoculation. The animals were treated according to NIH Guide for the Care and Use of Laboratory Animals (revised 1985). The animal study was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

Transduction with lentiviral vectors expressing shRNAs targeting SIRT1, HIF-1α, or AMPK

Lentiviral particles harboring scramble shRNA or shRNAs targeting SIRT1, HIF-1α, or AMPK were generated in HEK-293T cells using the pLKO.1-puro vector and packaging plasmids pLP1, pLP2, and pLP/VSVG in the BLOCK-iT lentiviral RNAi expression system (Invitrogen, Carlsbad, CA, USA). Lentiviruses were concentrated using the PEG-it virus precipitation solution (System Biosciences, Palo Alto, CA, USA). One day before transduction, 0.5×10^6 HCC cells were seeded in the antibiotic-free medium in a 6-well plate. Hexadimethrine bromide (8 μg/mL) was added to the cells when cells reached 80% confluency, followed by the addition of corresponding lentiviral particles. After overnight incubation, the medium was replaced with a puromycin-containing medium (2.5 μg/mL) to select stably transduced cells.

Transduction with retroviral vectors expressing SIRT1 or HIF-1α genes

Human SIRT1 or HIF-1α cDNAs were cloned into a retroviral vector PLXRN (Clonetech Bio, Shiga, Japan). GP293 cells were transduced with the vector expressing SIRT1 or HIF-1α using FuGENE 6 transfection reagent (Roche Applied Science, Penzberg, Germany). The medium was changed at 6 h after transduction. The retroviruses in the medium were collected at 24 h and 48 h after transduction, followed by virus filtration. HCC cells were seeded at approximately 60% confluency 12 h before transduction. The medium was refreshed at 24 h after transduction, and stably transduced cells were selected using G418 (1 M; Invitrogen).

Cell proliferation and colony formation assays

Cells were seeded in a 96-well plate at a density of 2,000 cells per well after transfection, followed by an MTT assay to determine cell viability. The optical density was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

In colony formation assay, cells were seeded into a six-well plate at 500 cells/well after transfection and cultured in a medium supplemented with 10% FBS at 37 °C for 14 days. After fixing with methanol/acetic acid, cells were stained with 0.5% crystal violet. The number of colonies containing more than 50 cells was counted under an inverted microscope.

Measurement of intracellular ATP concentration

ATP concentration was measured using an ATP assay kit (Beyotime, Shanghai, China), according to the manufacturer's protocol. Briefly, cells were lysed and centrifuged. The supernatant was incubated with 100 μL of ATP working solution for 3–5 min at room temperature. The luminescence was measured using a luminometer (Promega, Madison, WI, USA) and normalized to the protein amount.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen), following the manufacturer's instructions, followed by cDNA synthesis using the Superscript III reverse-transcription kit (Invitrogen). PCR amplification was performed at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. GAPDH was used as an internal reference. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total proteins were isolated using the M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). After the separation in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with primary antibodies for 1–2 h at room temperature or overnight at 4 °C, followed by incubation with the secondary antibody at room temperature for 1 h. Proteins were detected using an enhanced chemiluminescence kit (AbFrontier Co. Ltd., Seoul, South Korea) and exposed to X-ray films. Band intensities were measured using ImageJ v1.43 (NIH, Bethesda, MD, USA). GAPDH was used as an internal reference.

Chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (Co-IP)

ChIP assay was conducted using a ChIP assay kit (Upstate, Lake Placid, NY, USA) as previously described [8]. Protein samples were crosslinked using 1 M MDSP (Pierce Biotechnology, Waltham, MA, USA). Cells were lysed in low-stringency IP buffer containing protease inhibitor cocktail (Roche) and endonuclease (Pierce Biotechnology). The antibodies were coated with A/G magnetic beads (Pierce Biotechnology). HIF-1α was immunoprecipitated with anti-HIF-1α antibody (Cayman, Ann Arbor, MI, USA). Anti-rabbit IgG antibody (Millipore) was used as a control. The immunoprecipitated complex was washed with the lysis buffer, eluted in SDS-PAGE buffer containing 50 mM DTT, and boiled for 10 min, followed by SDS-PAGE separation under reducing conditions. After transferring onto a PVDF membrane (Perkin-Elmer), the proteins were incubated with the anti-HIF-1α (Cayman) or anti-c-Myc (Abcam, Cambridge, UK) antibody overnight at 4 °C. The immune complex was detected using Clean-Blot IP detection reagent (Pierce Biotechnology) and visualized using the Amersham ECL detection reagents (GE Healthcare, Chicago, IL, USA).

Luciferase reporter assay

Oligonucleotides containing the target site of genes were ligated into the pGL3-basic vector (Promega). The ligation was verified by *KpnI* and *HindIII* restriction digestions (Promega) and Sanger sequencing. HEK293T cells were co-transfected with the vector or control using Lipofectamine 2000 (Invitrogen). The

luciferase activity was measured at 48 h after transfection using a dual-luciferase reporter assay system (Promega).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the unpaired two-sided Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

Results

SIRT1 is required for normal mitochondrial function in HCC cells.

To explore the role of SIRT1 in mitochondrial function modulation, we measured the mRNA levels of representative genes encoding the components of the oxidative phosphorylation (OXPHOS) system[15], and the intracellular ATP concentrations in SIRT1 overexpressed or inhibited HCC cells. First, we confirmed that retroviral transduction-mediated SIRT1 overexpression in HepG2, PLC5, and Huh7 cells and significantly downregulated SIRT1 levels in lentiviral SIRT1 shRNA transduced cells (Supplementary Figure 1A). The mRNA levels of representative mitochondrial (*ND1*, *CytB*, *COX1*, and *TP6*) and nuclear (*NDUFS8*, *SDHB*, *UQCRCL*, *COX5*, and *ATP5a*) OXPHOS genes were significantly increased in SIRT1 overexpressing HCC cell lines, while decreased in SIRT1 downregulated cells (Supplementary Figures 1B and C). The same trends were also observed in the intracellular ATP levels (Supplementary Figure 1D). These results thus suggest that SIRT1 is required for normal mitochondrial function in HCC cells.

SIRT1 inhibits HCC cell growth both in vitro and in vivo.

Since SIRT1 is required for maintaining the normal mitochondrial function of HCC cells, we hypothesized that dysregulation of SIRT1 expression might affect the behavior of HCC cells. As expected, MTT and colony formation assays showed that overexpression of SIRT1 significantly inhibited the growth of HCC cells, whereas knockdown (KD) of SIRT1 significantly promoted the cell growth, compared with corresponding control-treated samples (Figures 1A and B). Besides, caspase-3 and caspase-9 protein levels were markedly increased by SIRT1-overexpression but decreased in SIRT1 KD cells (Figure 1C), suggesting that SIRT1 overexpression may induce apoptosis of HCC cells. Conversely, VEGF protein expression was substantially attenuated in SIRT1-overexpressing HCC cells but enhanced in SIRT1 silenced cells (Figure 1D), indicating that SIRT1 might inhibit angiogenesis of HCC tumors. Moreover, overexpression of SIRT1 resulted in significant reductions in the PLC5 and Huh7 tumor xenograft weight and size. Likewise, SIRT1 KD exhibited reverse effects on the xenograft models (Figure 1E). Together, these data suggest that SIRT1 suppresses HCC tumor growth likely by promoting cancer cell apoptosis and inhibiting tumor angiogenesis.

SIRT1 supports mitochondrial function via suppressing HIF-1α expression in HCC cells.

Given the involvement of HIF-1α upregulation in the Warburg effect[9], we determined the levels of HIF-1α in SIRT1 overexpressing and SIRT1 silenced HCC cells, which showed that both mRNA and protein levels of HIF-1α were remarkably reduced in SIRT1-overexpressing HCC cells and elevated upon SIRT1-silencing (Figure 2A). Furthermore, compared with overexpression of SIRT1 alone, co-overexpression of SIRT1 and HIF-1α significantly enhanced HIF-1α protein expression without changing SIRT1 protein expression in HCC cells (Figure 2B). Thus, these data suggest that HIF-1α is a downstream effector in the SIRT1-mediated regulatory pathway. Furthermore, in contrast to the SIRT1 overexpression alone, co-overexpression of SIRT1 and HIF-1α attenuated mRNA levels of representative mitochondrial- and nuclear-encoded OXPHOS genes (Figure 2C) and significantly reduced intracellular ATP levels in HCC cells (Figure 2D). These data demonstrated that overexpression of HIF-1α abolished the protective effects of SIRT1 on mitochondrial function and further impaired mitochondrial OXPHOs mechanism, suggesting that SIRT1 supports normal mitochondrial function via suppressing HIF-1α expression.

SIRT1 inhibits HCC cell growth via suppressing HIF-1α expression.

Next, we investigated the role of SIRT1–HIF-1α signaling axis in the regulation of HCC cell growth *in vitro* and *in vivo*. We observed that co-overexpression of SIRT1 and HIF-1α significantly promoted HCC cell proliferation (Figures 3A and B), attenuated caspase-3 and caspase-9 protein expressions (Figure 3C), and increased VEGF protein level (Figure 3D), compared with that of the control, which was opposite to the effects of SIRT1 overexpression alone. Moreover, compared with the corresponding controls, co-overexpression of SIRT1 and HIF-1α in HCC cells resulted in a significant increase in the volumes and weights of tumor xenografts in mice, whereas double KD of SIRT1 and HIF-1α exhibited an opposite effect (Figure 3E). These data suggest that SIRT1 may inhibit HCC development via suppressing HIF-1α expression.

SIRT1 suppresses HIF-1α expression via multiple mechanisms.

Considering the deacetylase activity of SIRT1[13] and the involvement of AMP-activated protein kinase (AMPK) in HIF-1α signaling in the mitochondria[16], we determined the levels of acetylated HIF-1α (Lys674-HIF-1α) and AMPK in HCC cells in response to altered SIRT1 expression to unveil the underlying mechanism of SIRT1-mediated HIF-1α suppression. We found that Lys674-HIF-1α protein expression was significantly attenuated in SIRT1-overexpressing PLC5 and Huh7 cells but enhanced in SIRT1 silenced cells (Figure 4A), suggesting that SIRT1 might suppress HIF-1α expression via deacetylation. Conversely,

AMPK protein expression was significantly enhanced in SIRT1-overexpressing HCC cells but attenuated in SIRT1 silenced cells (Figure 4B). Importantly, compared with the control, AMPK KD completely reversed the suppressive effect of SIRT1 on HIF-1α expression and further enhanced HIF-1α protein level in SIRT1 overexpressing HCC cells (Figure 4C). These findings suggest that SIRT1 can suppress HIF-1α expression via the AMPK pathway.

Given that hydroxylation at proline 564 of HIF-1α is required for its binding to the Von Hippel–Lindau (VHL) protein and subsequent degradation[17, 18], we hypothesized whether SIRT1 expression could promote HIF-1α degradation via VHL. Interestingly, we found that VHL protein level was significantly upregulated in SIRT1 overexpressing HCC cells but reduced in SIRT1 silenced cells (Figure 4D). Moreover, the ChIP assay revealed that the VHL promoter sequence was significantly enriched with SIRT1 protein in SIRT1-overexpressing HCC cells but decreased upon SIRT1 silencing (Figure 4E). Luciferase reporter assay further confirmed that the activity of VHL promoter was substantially elevated in SIRT1 overexpressing HCC cells and reduced in SIRT1KD cells (Figure 4F). These results indicate that SIRT1 promotes VHL-mediated hydroxylation and subsequent degradation of HIF-1α protein. Collectively, these data suggest that SIRT1 suppresses HIF-1α expression via multiple mechanisms, including deacetylation, AMPK upregulation, and VHL-mediated degradation.

SIRT1 supports normal mitochondrial function through HIF-1α–c-Myc signaling axis.

Considering that the HIF-1α–c-Myc crosstalk interferes with the binding of c-Myc to the TFAM promoter and thus impairs mitochondrial biogenesis[11], we examined whether SIRT1 could regulate mitochondrial function via HIF-1α–c-Myc–TFAM signaling axis. Co-IP analysis showed that SIRT1 KD significantly enhanced HIF-1α/c-Myc interaction in PLC5 cells, compared with that of the control (Figure 5A). The luciferase reporter assay demonstrated that the c-Myc promoter activity was substantially decreased in SIRT1 downregulated PLC5 cells compared with that in control cells (Figure 5B), suggesting that SIRT1 might be required for c-Myc activation in HCC cells. Furthermore, ChIP assay revealed that overexpression of SIRT1 significantly elevated the amount of the PCR products corresponding to the TFAM promoter region in HCC cell lysates immunoprecipitated by anti-HIF-1α antibody, whereas SIRT1 KD exhibited a contrasting effect (Figure 5C). Luciferase assay further demonstrated that the TFAM promoter activity was increased in SIRT1 overexpressing HCC cells and decreased in SIRT1KD cells (Figure 5D). Of note, co-overexpression of SIRT1 and HIF-1α significantly reduced the promoter activity of TFAM in HCC cells, which was contrary to the effect of SIRT1 overexpression alone (Figure 5E). These findings suggest that SIRT1 may interfere with HIF-1α–c-Myc interaction and subsequently stimulate TFAM transcription, thereby promoting mitochondrial biogenesis in HCC cells.

Discussion

SIRT1 acts as the key oncoprotein in several types of cancers, including HCC[19–23]; however, the exact role of SIRT1 in tumorigenesis remains controversial[24]. In this study, we demonstrated that SIRT1 suppressed HCC cell growth *in vitro* and *in vivo* by maintaining normal mitochondrial function via suppressing HIF-1α signaling. Mechanistically, SIRT1 could suppress HIF-1α expression via multiple mechanisms, including deacetylation, AMPK upregulation, and VHL-mediated degradation. Furthermore, SIRT1 could interfere with the HIF-1α–c-Myc interaction to stimulate the transcription of a mitochondrial biogenesis enhancer TFAM. Taken together, our results suggest that SIRT1 protects the mitochondria and reduces the Warburg effect via suppressing HIF-1α expression in HCC cells, providing a promising therapeutic strategy for HCC.

A shifting from OXPHOS to anaerobic glycolysis is a hallmark of cancer cell metabolism, resulting in mitochondrial dysfunction, inefficient OXPHOS, and aberrant activation of HIF-1α[9, 25]. This metabolic reprogramming, known as Warburg effect, involves different signaling pathways, including c-Myc and HIF-1α signaling cascades[26]. Although most OXPHOS genes are nuclear-encoded, thirteen of them are encoded by the mitochondrial genome. Thus, the formation of stoichiometric OXPHOS complexes requires precise communication between mitochondria and nucleus. This phenomenon induces the expression of nuclear-encoded proteins, like TFAM, to participate in the regulation of mitochondrial gene expression[27]. Here, we demonstrated that SIRT1 could potentially promote the expression of not only the mitochondria-encoded OXPHOS mRNAs (ND1, CytB, COX1, and TP6) but also nuclear-encoded OXPHOS mRNAs (NDUFS8, SDHB, UQCRC1, COX5, and ATP5a), along with an increase in intracellular ATP level. The protective role of SIRT1 in mitochondrial functions could attenuate the Warburg effect in HCC cells, thereby inhibiting cell growth. These data were in line with Michan's findings that the decrease in SIRT1 expression could result in mitochondrial dysfunction and HIF-1α accumulation, leading to enhanced aerobic glycolysis and cellular senescence[13].

SIRT1 has been shown to regulate HIF-1α expression via multiple mechanisms. For example, SIRT1 deacetylates HIF-1α at lysine 674, consequently suppressing HIF-1α target gene expression[28]. Gomes *et al.* have shown that inactivation of SIRT1 stabilizes HIF-1α by reducing VHL level in primary myoblasts[14]. Thus, we sought to explore the mechanism of SIRT1 in suppressing HIF-1α expression by detecting different signaling factors in the related pathways. First, we explored the relationship between SIRT1 and AMPK because SIRT1 expression had been shown to regulate hepatocellular lipid metabolism through AMPK signaling[29]. Our results revealed that HIF-1α overexpression could reverse SIRT1-mediated activation of AMPK, suggesting that the SIRT1–AMPK signaling axis was responsible for regulating HIF-1α expression in the HCC cell model. Studies have demonstrated substantial crosstalk between SIRT1 and AMPK signaling. AMPK can activate SIRT1 in myoblasts by increasing the NAD^+/NADH ratio [30]. On the other hand, in human embryonic kidney cells, SIRT1 activates AMPK by deacetylating serine/threonine kinase 11 (also called LKB1) at Lys48[31]. Together, this study and our previous report suggest that AMPK is activated by SIRT1 in HCC and that SIRT1 deacetylates LKB1, inducing AMPK phosphorylation and activation[32, 33]. We found that HIF-1α could be deacetylated by

SIRT1. Direct deacetylation of HIF-1α by SIRT1[28] has also been shown by Laemmle *et al.* by co-IP of endogenous SIRT1 and HIF-1α. It has been shown that loss of SIRT1 leads to hyperacetylation of HIF-1α[34]. Since VHL E3 ubiquitin ligase regulates HIF-1α protein expression by hydroxylating the proline residues on HIF-1α[35], we investigated whether SIRT1 could directly interact with VHL. Our results showed that SIRT1 directly interacted with VHL, suggesting that SIRT1 maintains mitochondrial homeostasis by recruiting VHL to degrade HIF-1α.

C-Myc increases mitochondrial biogenesis by promoting TFAM transcription. The HIF-1α–c-Myc interaction inhibits this effect, resulting in mitochondrial dysfunction[14, 36, 37]. Therefore, we interrogated whether SIRT1 could block the HIF-1α–c-Myc interaction. We found that SIRT1 KD promoted the binding of HIF-1α with c-Myc while reducing c-Myc reporter activity, consistent with a study showing that SIRT1 downregulation could reduce c-Myc expression[38]. ChIP and luciferase reporter assays revealed that the binding of c-Myc to the TFAM promoter was increased upon SIRT1 overexpression, which was reversed by HIF-1α overexpression. Conversely, the binding of c-Myc to the TFAM promoter was reduced upon SIRT1 silencing. These data suggest that SIRT1 expression may suppress the HIF-1α–c-Myc interaction and subsequently promote the binding of c-Myc to the TFAM promoter, thereby enhancing mitochondrial biogenesis in HCC cells. However, we could not investigate the alterations in the SIRT1-HIF-1α–c-Myc–TFAM signaling axis or the changes in angiogenesis/cellular apoptosis in tumor xenografts, which will be addressed in the future studies.

Emerging studies have demonstrated that SIRT1 expression aggravates cancer progression, including tumor growth in murine models of HCC. However, we found that overexpression of SIRT1 inhibited the growth of HCC tumor xenografts, while its loss exhibited opposite effects.

Conclusions

SIRT1 suppresses HIF-1α expression via multifaceted mechanisms, including HIF-1α deacetylation, promoting VHL-mediated HIF-1α degradation, and activation of AMPK signaling. SIRT1-mediated HIF-1α suppression was carried out by inhibiting the crosstalk between HIF-1α and c-Myc, thereby promoting mitochondrial function via TFAM transcription activation and attenuating the Warburg effect in HCC cells (**Supplementary Figure 2**). This study demonstrated that SIRT1 could exert both protective effects on the mitochondrial function as well as play suppressive roles in inhibiting the HCC tumor growth.

Declarations

Ethics approval and consent to participate:

The animals were treated according to NIH Guide for the Care and Use of Laboratory Animals (revised 1985). The animal study was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

Consent for publication:

Not applicable

Availability of data and materials:

The datasets generated during the current study are not publicly available due to further study but are available from the corresponding author on reasonable request.

Competing interests:

the authors have no relevant financial or non-financial interests to disclose.

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Authors' contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zun-Qiang Zhou, Jiao Guan, Shi-Geng Chen, Jian-Hua Sun, Zheng-Yun Zhang. The first draft of the manuscript was written by Zun-Qiang Zhou and Jiao Guan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Effects of SIRT1 on HCC cell growth and VEGF expression. (**A, B**) MTT (**A**) and colony formation (**B**) assays were performed to examine the cell proliferation capacity of SIRT1 overexpressing or SIRT1 silenced HepG2, PLC5, and Huh7 cells. (**C, D**) Western blot analysis was performed to determine the protein levels of caspase-3, caspase-9, and VEGF (**D**) in HCC cells. (**E**) PLC5 and Huh7 tumor xenografts were generated in nude mice. Tumor weights were measured two weeks after inoculation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. vector-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. scramble; n = 3. OE, overexpression; NC, negative control; sh, small hairpin RNA.

Figure 2

SIRT1 supports normal mitochondrial function via suppressing the HIF-1α expression in HCC cells. (**A**) Western blot analysis and qRT-PCR were performed to determine protein, and mRNA levels of HIF-1α in SIRT1 overexpressing or SIRT1 silenced HepG2, PLC5, and Huh7 cells. (**B**) Western blot analysis was carried out to determine HIF-1α protein levels in PLC5 and Huh7 cells overexpressing SIRT1 alone or with HIF-1α. (**C**) The mRNA levels of mitochondrial (*ND1, CytB, COX1,* and *TP6*) and nuclear (*NDUFS8, SDHB, UQCRCL, COX5*, and *ATP5a*) encoding OXPHOS-related genes were determined by qRT-PCR. (**D**) The intracellular ATP concentrations were measured in PLC5 and Huh7 cells overexpressing SIRT1 alone or co-overexpressing SIRT1 and HIF-1α. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. vector-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. OE-SIRT1; n = 3. OE, overexpression; NC, negative control; sh, small hairpin RNA.

Figure 3

SIRT1 regulates HCC growth and VEGF expression via suppressing HIF-1α activity. (**A and B**) MTT and colony formation assays were conducted to examine the proliferation rates of HepG2, PLC5, and Huh7 cells overexpressing SIRT1 alone or co-overexpressing SIRT1 and HIF-1α. (**C**) Western blot analysis was carried out to determine the levels of caspase-3 and caspase-9 in HepG2, PLC5, and Huh7 cells overexpressing SIRT1 or HIF-1α. (**D**) Western blot analysis and qRT-PCR were performed to determine VEGF expressions in HepG2, PLC5, and Huh7 cells overexpressing SIRT1 or HIF-1α. (**E**) SITR1 and HIF-1α were co-overexpressed or co-silenced in PLC5 and Huh7 cells. Tumor xenografts were generated in mice as indicated. Tumors were collected two weeks after inoculation, followed by tumor size measurement and cell mass weight recording. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. vector-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. OE-SIRT1 or scramble; n = 3. OE, overexpression; NC, negative control; sh, small hairpin RNA.

Figure 4

SIRT1 suppresses HIF-1α expression via multiple mechanisms. (**A and B**) Western blot analysis was conducted to determine Lys674-HIF-1α, and AMP-activated protein kinase (AMPK) levels in SIRT1 overexpressing or SIRT1 downregulated PLC5 and Huh7 cells. (**C**) Western blot analysis was performed to determine the expressions of SIRT1, AMPK, and HIF-1α in SIRT1 overexpressing or SIRT1 overexpressing plus AMPK silenced PLC5 and Huh7 cells. (**D and E**) Western blotting and qRT-PCR analyses showed the VHL levels in SIRT1 overexpressing or SIRT1 silenced PLC5 and Huh7 cells. (**F**) Luciferase reporter assay exhibited the activity of the VHL promoter in SIRT1 overexpressing or SIRT1 KD PLC5 and Huh7 cells. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vector-NC or control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. scramble; n = 3. OE, overexpression; NC, negative control; sh, small hairpin RNA.

Figure 5

SIRT1 interferes with the HIF-1α–c-Myc interaction. (**A**) Co-IP was performed to examine the HIF-1α–c-Myc interaction in SIRT1 silenced PLC5 cells. (**B**) Luciferase reporter assay was performed to examine the c-Myc promoter activity in SIRT1 silenced PLC5 cells. (**C**) The cell lysates of SIRT1 overexpressing or SIRT1 downregulated PLC5 cells were immunoprecipitated by anti-c-Myc antibody. ChIP assay was carried out to measure the TFAM promoter activity in these cells. (**D**) Luciferase assay was carried out to measure the activity of the TFAM promoter in SIRT1 overexpressing or SIRT1 KD PLC5 and Huh7 cells. (**E**) A luciferase assay was performed to measure the activity of the TFAM promoter in PLC5 and Huh7 cells overexpressing SIRT1 or co-overexpressing SIRT1 and HIF-1α. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vector-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $\#^{\#\#}P < 0.001$ vs. scramble; n = 3. OE, overexpression; NC, negative control; sh, small hairpin RNA.

Supplementary Files

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- Supplementaryfigure1.tif
- Supplementaryfigure2.tif