Sirtuin 6 promotes transforming growth factor-β1/H$_2$O$_2$/HOCl-mediated enhancement of hepatocellular carcinoma cell tumorigenicity by suppressing cellular senescence

Xin-Xia Feng, Jing Luo, Mei Liu, Wei Yan, Zhen-Zhen Zhou, Yu-Jia Xia, Wei Tu, Pei-Yuan Li, Zuo-Hua Feng and De-An Tian

1Department of Gastroenterology, Tongji Hospital, Wuhan; 2Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Key words
Cellular senescence, SIRT6, TGF-β1, tumor cells, tumorigenicity

Correspondence
De-An Tian, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel/Fax: +86-27-8366-2831;
E-mail: datian@tjh.tjmu.edu.cn

Funding Information
National Natural Science Foundation of China.

Received November 11, 2014; Revised January 20, 2015; Accepted February 4, 2015

Cancer Sci 106 (2015) 559–566
doi: 10.1111/cas.12632

T he ability to sustain uncontrolled proliferation represents the most fundamental trait of cancer cells. One of the barriers that limit uncontrolled cell proliferation is cellular senescence, which represents a natural tumor suppressor mechanism. Cancer cells need to overcome this obstacle to produce a clinically relevant tumor mass. Therefore, understanding the mechanisms through which tumor cells bypass senescence might allow for the development of effective approaches in tumor therapy.

H$_2$O$_2$ has been found to induce cellular senescence by promoting the expression of p21 and p16. Transforming growth factor-β1 (TGF-β1) also promotes the expression of p21, and has the potential to induce cellular senescence of tumor cells, including hepatocellular carcinoma (HCC) cells. However, our previous study showed that neither H$_2$O$_2$/HOCl (neutrophil-derived reactive oxygen species) nor TGF-β1 alone could induce the metastatic phenotype of HCC cells, but H$_2$O$_2$/HOCl could cooperate with TGF-β1 to induce the metastatic phenotype of HCC cells. Consistently, the higher density of intratumoral neutrophils in hepatocellular carcinoma has been found to promote tumor metastasis. These findings suggest that TGF-β1/H$_2$O$_2$/HOCl might not induce cellular senescence, as cellular senescence could reduce the clonogenicity and tumorigenicity of tumor cells.

Sirtuin 6 (SIRT6) has been found to negatively regulate cellular senescence. The average level of SIRT6 expression in HCC cells is lower than that in primary human hepatocytes. However, the increased expression of SIRT6 has been found in many human HCC samples, implying that SIRT6 expression in HCC cells might possibly be upregulated by the regulatory factors in tumor milieu. Importantly, TGF-β1 has been found to increase the expression of SIRT6. Therefore, the expression of SIRT6 might be upregulated by TGF-β1/H$_2$O$_2$/HOCl, thus playing a role in TGF-β1/H$_2$O$_2$/HOCl-mediated enhancement of HCC cell tumorigenicity.
The effects of SIRT6 on tumor cells are complicated and contradictory. The negative effect of SIRT6 on cellular senescence\(^{(12)}\) and the promoting effect of SIRT6 on clonogenicity of tumor cells\(^{(14)}\) suggest that SIRT6 might promote the tumorigeninity of HCC cells. However, SIRT6 has the potential to function as a tumor suppressor by suppressing aerobic glycolysis in tumor cells\(^{(13)}\) and increasing apoptosis sensitivity of tumor cells.\(^{(13)}\) To ascertain whether the upregulation of SIRT6 expression might be one of the reasons that TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl promoted the tumorigeninity of HCC cells, in this study we investigated whether H\(_2\)O\(_2\)/HOCl might cooperate with TGF-\(\beta\) to upregulate SIRT6 expression in HCC cells, and how SIRT6 might influence the tumorigeninity of HCC cells. Our data showed that TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl could promote SIRT6 expression in HCC cells, and that suppressing SIRT6 expression could abrogate TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl-mediated enhancement of HCC cell tumorigenicity.

Materials and Methods

Cells and reagents. Human HCC cell lines HepG2 and Huh7 were purchased from China Center for Type Culture Collection (Wuhan, China) and cultured according to their guidelines. H\(_2\)O\(_2\) and HOCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transforming growth factor-\(\beta\) was purchased from PeproTech (Rocky Hill, NJ, USA). SB203580, a selective inhibitor of p38 MAPK, was purchased from Calbiochem Frankfurter, Germany.

Senescence-associated \(\beta\)-gal assay. Senescence-associated \(\beta\)-gal (SA-\(\beta\)-gal) activity was analyzed with a Senescence \(\beta\)-Galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer’s instructions. The SA-\(\beta\)-gal\(^*\) cell ratios were determined in three wells. To avoid any non-specific staining due to confluence, SA-\(\beta\)-Gal cytotoxic staining was carried out on non-confluent cells.

Western blot assay. Western blot assay was carried out as described previously.\(^{(7)}\) Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology.

Measurement of lactate generation. After the indicated treatment, tumor cells were cultured in fresh medium (serum-free) for \(6\) h in the absence of stimuli. Lactate in the culture medium was determined with a Lactate Assay Kit (BioVision, Milpitas, CA, USA).

Assay of gene expression by real-time RT-PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen Frederick, MD, USA). The relative quantity of mRNA was determined by real-time RT-PCR as described previously.\(^{(7)}\) The relative expression of the SIRT6 gene was calculated using GeNorm software by using GAPDH, PPIA, and HPRT1 as reference genes.\(^{(7)}\) The primer sequences were as follows: SIRT6, sense 5\'-CATGAGGAGCGAGGTCT-3\'; antisense 5\'-GGCTTTACACTTGGCACA-3\'; GAPDH, sense 5\'-CTCATTTGACCTCAAATCATGGTTT-3\'; and antisense 5\'-GAAGAGTGTATTTGGAAGGTG-3\'; PPIA, sense 5\'-GTGACCCCCACCTGTGTTT-3\'; and antisense 5\'-CTGGACTCTTGGGACTGGTTTG-3\'; HPRT1, sense 5\'-GCTGAGAGGTTGAGGAAGGTG-3\'; and antisense 5\'-CAGAGGGTCTAACATGGTGAG-3\';

Cell transfection. To suppress the upregulation of SIRT6 expression, tumor cells were transduced with sh-SIRT6(1) and sh-SIRT6(2) lentiviral particles (GeneChem Shanghai, China) to express SIRT6 shRNAs targeting 5\'-GAAGATGGTGAGCAGGAGGTA-3\' and 5\'-GTCTCATTGTGGCTGGTCTG-3\', respectively. Control shRNA (sh-control lentiviral particle), not targeting any known gene, was used as the control. After selection with puromycin, the cells were used for further experiments.

Assay of anoikis. For the assay of anoikis, tumor cells were cultured (1 \times 10^5/well) for 48 h in 6-well plates pre-coated with poly(2-hydroxyethyl methacrylate) (10 mg/ml; Sigma-Aldrich). The cells were then stained with an annexin V-FITC/propidium iodide apoptosis detection kit (BD Biosciences, San Diego, CA, USA), and analyzed by flow cytometry.

Animal experiments. Athymic nude (nu/nu) mice (4–5 weeks old) were purchased from Beijing HFK Bio-Technology (Beijing, China). All mice received human care. The mice were maintained in the accredited animal facility of Tongji Medical College (Wuhan, China), and used for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College.

To analyze cellular senescence of tumor cells in tissue, 5 \times 10^6 tumor cells were injected into the right hind thigh of mice. The tissues at inoculation sites were harvested 3 days later. Frozen tissue sections were prepared and incubated in Senescence \(\beta\)-Galactosidase staining solution at 37°C overnight, then counterstained with eosin.\(^{(16)}\) Images were obtained using an Olympus Tokyo, Japan IX71 microscope at 20 \times 10 magnification. The density of senescent cells was defined as the number of SA-\(\beta\)-gal\(^*\) spots per microscopic field.

To test the tumorigenicity of HCC cells, 5 \times 10^6 tumor cells were injected into the right hind thigh of mice. Tumors were dissected and weighed on day 25 after inoculation. In other experiments, 5 \times 10^6 tumor cells were s.c. injected into the flank of mice. Tumor growth was monitored every 5 days. The length (L) and width (W) of tumors were measured. The volume of tumor (V) was determined by the formula: \(V = (L \times W^2)/2\).

Results

Cellular senescence of HCC cells induced by H\(_2\)O\(_2\)/HOCl but not TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl. Our previous study showed that TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl could promote anoikis-resistance of HCC cells;\(^{(7)}\) here we further investigated whether TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl might influence cellular senescence and/or aerobic glycolysis of HCC cells. For this purpose, we detected SA-\(\beta\)-gal activity after 10 days of treatment with TGF-\(\beta\), TGF-\(\beta\)/H\(_2\)O\(_2\)/HOCl, or H\(_2\)O\(_2)/HOCl. Interestingly, SA-\(\beta\)-gal\(^*\) cells were significantly increased by H\(_2\)O\(_2)/HOCl, but only slightly increased by TGF-\(\beta\) or TGF-\(\beta\)/H\(_2\)O\(_2)/HOCl (Fig. 1a). Consistently, the expression of p16 and p21 was upregulated only by H\(_2\)O\(_2)/HOCl, whereas TGF-\(\beta\)/H\(_2\)O\(_2)/HOCl failed to upregulate the expression of p16 and p21 (Figs. 1b, S1). However, treatment with either H\(_2\)O\(_2)/HOCl or TGF-\(\beta\)/H\(_2\)O\(_2)/HOCl could increase the production of lactate by HCC cells, indicating the augmentation of glycolysis (Fig. 1c). Taken together, these results and those of our previous study\(^{(7)}\) indicated that TGF-\(\beta\)/H\(_2\)O\(_2)/HOCl could promote anoikis resistance and glycolysis of HCC cells, but was inefficient in inducing cellular senescence.
Sirtuin 6 expression in HCC cells upregulated by TGF-β1/H2O2/HOCl. We then investigated whether TGF-β1/H2O2/HOCl could modulate the expression of SIRT6 in HCC cells. After 10 days of treatment, SIRT6 gene expression in HCC cells was not influenced by H2O2/HOCl, only slightly increased by TGF-β1, but remarkably upregulated by TGF-β1/H2O2/HOCl (Fig. 2a). SIRT6 mRNA was gradually increased after the prolonged stimulation with TGF-β1/H2O2/HOCl (Fig. 2b), which was consistent with the activation pattern of signaling pathways by these stimuli. Our previous study showed that either TGF-β1 alone or H2O2/HOCl only induced the transient, but not the sustained, activation of Smad, p38 MAPK, and ERK pathways. However, prolonged stimulation with TGF-β1/H2O2/HOCl could induce the sustained and gradually enhanced activation of these pathways. Therefore, we further analyzed TGF-β1/H2O2/HOCl-mediated upregulation of SIRT6 when the sustained activation of signaling pathways was inhibited with SIS3 (Smad3 inhibitor), PD98059 (inhibitor of ERK pathway), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), wortmannin (phosphatidylinositol 3-kinase inhibitor), and QNZ (nuclear factor-κB [NF-κB] inhibitor). The inhibitory effect of each inhibitor on the corresponding signaling pathway is shown in Figure S2. Inhibiting the upregulation of SIRT6 enabled TGF-β1/H2O2/HOCl to efficiently induce cellular senescence (Fig. 3b), concomitant with the upregulation of p16 and p21 expression (Figs. 3c,S3), suggesting that SIRT6 could suppress the induction of cellular senescence by TGF-β1/H2O2/HOCl. We then further investigated whether the upregulation of SIRT6 might suppress the glycolysis of HCC cells, as SIRT6 could suppress glycolysis by inhibiting hypoxia-inducible factor-1α (HIF-1α). Interestingly, SIRT6 shRNA augmented the promoting effect of TGF-β1/H2O2/HOCl on anoikis resistance of HCC cells (Fig. 3d), but did not significantly influence the effect of TGF-β1/H2O2/HOCl on glycolysis of HCC cells (Fig. 3e). Both HIF-1α and AMP-activated protein kinase (AMPK) could finally induce the phosphorylation of pyruvate dehydrogenase (PDH) to promote glycolysis. TGF-β1/H2O2/HOCl slightly promoted the expression of HIF-1α, but strongly activated AMPK and increased the phosphorylation level of PDH (Fig. S4a), suggesting that H2O2/HOCl or

Sirtuin 6 suppresses induction of cellular senescence by TGF-β1/H2O2/HOCl. We then wondered whether SIRT6 might influence TGF-β1/H2O2/HOCl-mediated induction of cellular senescence. As SIRT6 overexpression could induce massive apoptosis in cancer cells, we investigated the effect of SIRT6 on TGF-β1/H2O2/HOCl-mediated upregulation of SIRT6 expression with SIRT6 shRNA (Fig. 3a). Inhibiting the upregulation of SIRT6 enabled TGF-β1/H2O2/HOCl to efficiently induce cellular senescence (Fig. 3b), concomitant with the upregulation of p16 and p21 expression (Figs. 3c,S3), suggesting that SIRT6 could suppress the induction of cellular senescence by TGF-β1/H2O2/HOCl. We then further investigated whether the upregulation of SIRT6 might suppress the glycolysis of HCC cells, as SIRT6 could suppress glycolysis by inhibiting hypoxia-inducible factor-1α (HIF-1α). Interestingly, SIRT6 shRNA augmented the promoting effect of TGF-β1/H2O2/HOCl on anoikis resistance of HCC cells (Fig. 3d), but did not significantly influence the effect of TGF-β1/H2O2/HOCl on glycolysis of HCC cells (Fig. 3e). Both HIF-1α and AMP-activated protein kinase (AMPK) could finally induce the phosphorylation of pyruvate dehydrogenase (PDH) to promote glycolysis. TGF-β1/H2O2/HOCl slightly promoted the expression of HIF-1α, but strongly activated AMPK and increased the phosphorylation level of PDH (Fig. S4a), suggesting that H2O2/HOCl or

Cancer Sci | May 2015 | vol. 106 | no. 5 | S61

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SIRT6 contributes to inhibitory effect of ERK pathway on cellular senescence. The ERK pathway suppresses cellular senescence by suppressing the expression of p16 and p21.15,21 Transforming growth factor-β1/H2O2/HOCl could induce sustained and enhanced activation of the ERK pathway.15 Consistently, TGF-β1/H2O2/HOCl could efficiently induce cellular senescence, if the ERK pathway was inhibited (Fig. 4a). In this situation, the effect of TGF-β1/H2O2/HOCl on cellular senescence was also attenuated by inhibiting the NF-κB, Smad, and p38 MAPK pathways (Fig. 5a), which was consistent with the effect of suppressing the upregulation of SIRT6 (Fig. 4b). However, if SIRT6 expression was suppressed by shRNA, TGF-β1/H2O2/HOCl could similarly induce cellular senescence (Fig. 4b), even though the ERK pathway was activated (Fig. S2). These results suggest that SIRT6 might mediate the inhibitory effect of the ERK pathway on cellular senescence. This was further confirmed by analyzing the expression of p16 and p21. When the ERK pathway was inhibited, TGF-β1/H2O2/HOCl could upregulate the expression of p16 and p21 (Figs 5b, S5). If SIRT6 expression was suppressed by shRNA, TGF-β1/H2O2/HOCl could similarly induce the expression of p16 and p21. Further inhibiting the ERK pathway only slightly increased the effect of TGF-β1/H2O2/HOCl (Fig. 5b, S5).

Sirtuin 6 required for TGF-β1/H2O2/HOCl to promote tumorigenicity of HCC cells. Based on the above results, we then analyzed whether upregulation of SIRT6 is required for TGF-β1/H2O2/HOCl to enhance the tumorigenicity of HCC cells. When HCC cells were cultured under anchorage-independent conditions, the pretreatment with TGF-β1/H2O2/HOCl did not influence the size of colonies (Fig. S6a,b). Intriguingly, however, pretreatment with TGF-β1/H2O2/HOCl significantly enhanced the tumorigenicity of HCC cells.
increased number of colonies in soft agar (Fig. S6c). Sirtuin 6 shRNA did not influence the size of the colonies (Fig. S6b), but abrogated the promoting effect of TGF-β1⁄H2O2⁄HOCl on colony formation (Fig. S6c). These results suggest that TGF-β1⁄H2O2⁄HOCl-mediated upregulation of SIRT6 might promote the capability of clonogenicity of individual HCC cells by preventing cellular senescence.

To further confirm the requirement of SIRT6 for TGF-β1⁄H2O2⁄HOCl-mediated enhancement of tumorigenicity of HCC cells, we treated HCC cells with TGF-β1⁄H2O2⁄HOCl before inoculation of the cells to mice. After inoculation, senescent cells were observed in the untreated control group (Fig. 6a), suggesting that the microenvironment could induce the senescence of tumor cells. The senescent cells were significantly reduced if HCC cells were pretreated with TGF-β1⁄H2O2⁄HOCl (Fig. 6a), although the density of tumor cells in tissues were similar (Fig. S7a). Inhibiting the upregulation of SIRT6 gene expression with shRNA (Fig. S7b) abolished the effect of TGF-β1⁄H2O2⁄HOCl (Fig. 6a). Consistently, the pretreatment of HCC cells with TGF-β1⁄H2O2⁄HOCl treatment could not promote the development of tumor, indicating that the upregulation of SIRT6 is required for TGF-β1⁄H2O2⁄HOCl to promote the tumorigenicity of HCC cells, and that inhibiting the upregulation of SIRT6 could abrogate the promoting effect of TGF-β1⁄H2O2⁄HOCl on the tumorigenicity of HCC cells. Intriguingly, when untreated tumor cells were inoculated, SIRT6 expression in tumor cells was gradually increased (Fig. S7b).
as these factors could be produced by neutrophils (H$_2$O$_2$/HOCl) and other stromal cells (TGF-$\beta$1) in the tumor milieu. Simply inhibiting the upregulation of SIRT6 could hinder the development of tumors (Fig. 6b,c). Moreover, SIRT6 shRNA only slightly influenced HCC cell proliferation in vitro, but significantly suppressed HCC cell proliferation in the presence of TGF-$\beta$1/H$_2$O$_2$/HOCl (Fig. S8). Taken together, these results suggest that TGF-$\beta$1 and H$_2$O$_2$/HOCl in the tumor milieu might suppress the proliferation of HCC cells if the SIRT6 gene could not be upregulated.

Discussion

Although SIRT6 has the potential to function as a tumor suppressor, our data in this study showed that TGF-$\beta$1/H$_2$O$_2$/HOCl-mediated upregulation of SIRT6 in HCC cells was tumor promoting, but not tumor suppressing. Sirtuin 6 could efficiently suppress the inducing effect of TGF-$\beta$1/H$_2$O$_2$/HOCl on cellular senescence. Although SIRT6 could not abrogate the promoting effect of TGF-$\beta$1/H$_2$O$_2$/HOCl on the aerobic glycolysis and apoptosis resistance of HCC cells, TGF-$\beta$1/H$_2$O$_2$/HOCl failed to promote clonogenicity and tumorigenicity of HCC cells if the upregulation of SIRT6 expression was suppressed.

Transforming growth factor-$\beta$1/H$_2$O$_2$/HOCl could promote SIRT6 expression in HCC cells through the MAPK and Smad pathways. The activation of the ERK pathway was crucial for TGF-$\beta$1/H$_2$O$_2$/HOCl to upregulate SIRT6 expression. The Smad pathway was required for higher expression of SIRT6. These results are supported by published reports that c-Fos, which is activated by the ERK pathway, could induce the expression of SIRT6, and that Smad3 could cooperate with c-Fos in modulating gene expression. Nevertheless, TGF-$\beta$1 alone could not induce higher expression of SIRT6 in HCC cells. Our previous study showed that TGF-$\beta$1 could induce the transient activation of ERK and Smad pathways in HCC cells, but prolonged stimulation with TGF-$\beta$1/H$_2$O$_2$/HOCl was required for inducing the sustained and enhanced activation of these pathways. The sustained, but not transient, activation of the ERK and Smad pathways was required for upregulating SIRT6 expression, as shown by our data. Therefore, prolonged stimulation with TGF-$\beta$1/H$_2$O$_2$/HOCl was required for inducing higher expression of SIRT6.

Cellular senescence could be induced by the activation of different signaling pathways, including NF-$\kappa$B, Smad, and p38 MAPK. Transforming growth factor-$\beta$1 and H$_2$O$_2$/HOCl could activate these pathways, and therefore have the potential to induce cellular senescence. However, our data showed that both Smad and p38 MAPK might have different effects on the senescence of HCC cells. The Smad and p38 MAPK pathways could promote the senescence of HCC cells if SIRT6 expression was suppressed. When the sustained activation was induced, the Smad and p38 MAPK pathways cooperated with the ERK pathway to upregulate the expression of SIRT6, which in turn abrogated the inducing effect of NF-$\kappa$B, Smad, and p38 MAPK on cellular senescence. Therefore, TGF-$\beta$1/H$_2$O$_2$/HOCl-induced activation of Smad and p38 MAPK pathways has a negative effect on cellular senescence due to their promoting effect on SIRT6 expression.

Both Smad and p38 MAPK promote cellular senescence by upregulating the expression of p16 and p21, whereas the ERK pathway suppresses cellular senescence by suppressing the expression of p16 and p21. When HCC cells were suppressed.
Sirtuin 6 (SIRT6) is required for transforming growth factor-β1 (TGF-β1)/H₂O₂/HOCl (T/H/H) to promote the tumorigenicity of hepatocellular carcinoma cells. HepG2 and Huh7 cells, non-transfected or transfected with sh-SIRT6(1), were untreated or treated for 10 days with T/H/H. The cells were then inoculated into mice. Senescent cells in tissue at the inoculation site were identified by senescence-associated (SA)-β-gal staining 3 days after tumor inoculation (left panels, bar = 50 μm). The density of the senescent cells was determined (right panel) as the number of senescence-associated (SA)-β-gal+ spots per microscopic field. (b,c) Mice were inoculated with the size of tumors (n = 8, each group) was measured at indicated time points (b), or the tumors (n = 8, each group) were dissected and weighed on day 25 after inoculation (c). *P < 0.05, **P < 0.01.

Fig. 6. Sirtuin 6 (SIRT6) is required for transforming growth factor-β1 (TGF-β1)/H₂O₂/HOCl (T/H/H) to promote the tumorigenicity of hepatocellular carcinoma cells. HepG2 and Huh7 cells, non-transfected or transfected with sh-SIRT6(1), were untreated or treated for 10 days with T/H/H. The cells were then inoculated into mice. Senescent cells in tissue at the inoculation site were identified by senescence-associated (SA)-β-gal staining 3 days after tumor inoculation (left panels, bar = 50 μm). The density of the senescent cells was determined (right panel) as the number of senescence-associated (SA)-β-gal+ spots per microscopic field. (b,c) Mice were inoculated with the size of tumors (n = 8, each group) was measured at indicated time points (b), or the tumors (n = 8, each group) were dissected and weighed on day 25 after inoculation (c). *P < 0.05, **P < 0.01.

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Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81070333, 81270507, and 81001066).

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Disclosure Statement

The authors have no conflict of interest.

Supporting Information

Additional supporting information may be found in the online version of this article:

Doc. S1. Supplementary methods: soft agar assay, immunofluorescence, tumor cell proliferation assay, and isolation of tumor cells from tissues.

Fig. S1. Upregulation of p16 and p21 genes by H2O2/HOCl but not transforming growth factor-β1 (TGF-β1)/H2O2/HOCl.

Fig. S2. Inhibitory effect of inhibitors on signaling pathways.

Fig. S3. Sirtuin 6 (SIRT6) suppresses upregulation of p16 and p21 genes by transforming growth factor-β1 (TGF-β1)/H2O2/HOCl.

Fig. S4. Sirtuin 6 (SIRT6) does not significantly influence the phosphorylation of AMP-activated protein kinase (AMPK) or pyruvate dehydrogenase (PDH).

Fig. S5. Sirtuin 6 (SIRT6) suppresses the expression of p16 and p21 genes.

Fig. S6. Transforming growth factor-β1 (TGF-β1)/H2O2/HOCl increases the number of hepatocellular carcinoma colonies in soft agar.

Fig. S7. Expression of the SIRT6 gene in tumor cells in tissues after inoculation.

Fig. S8. Effect of sirtuin 6 (SIRT6) on the proliferation of hepatocellular carcinoma cells.