Cancer Science
Japanese Cancer Association JCA
Open Access

Sirtuin 6 promotes transforming growth factor-β1/H_2O_2/HOCl-mediated enhancement of hepatocellular carcinoma cell tumorigenicity by suppressing cellular senescence

Xin-Xia Feng,[1] Jing Luo,[2] Mei Liu,[1] Wei Yan,[1] Zhen-Zhen Zhou,[1] Yu-Jia Xia,[1] Wei Tu,[1] Pei-Yuan Li,[1] Zuo-Hua Feng[2] and De-An Tian[1]

[1]Department of Gastroenterology, Tongji Hospital, Wuhan; [2]Department 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

Sirtuin 6 (SIRT6) can function as a tumor suppressor by suppressing aerobic glycolysis and apoptosis resistance. However, the negative effect of SIRT6 on cellular senescence implies that it may also have the potential to promote tumor development. Here we report that the upregulation of SIRT6 expression was required for transforming growth factor (TGF)-β1 and H_2O_2/HOCl reactive oxygen species (ROS) to promote the tumorigenicity of hepatocellular carcinoma (HCC) cells. Transforming growth factor-β1/H_2O_2/HOCl could upregulate SIRT6 expression in HCC cells by inducing the sustained activation of ERK and Smad pathways. Sirtuin 6 in turn abrogated the inducing effect of TGF-β1/H_2O_2/HOCl on cellular senescence of HCC cells, and was required for the ERK pathway to efficiently suppress the expression of p16 and p21. Sirtuin 6 altered the effect of Smad and p38 MAPK pathways on cellular senescence, and contributed to the inhibitory effect of the ERK pathway on cellular senescence. However, SIRT6 was inefficient in antagonizing the promoting effect of TGF-β1/H_2O_2/HOCl on aerobic glycolysis and anoikis resistance. Intriguingly, if SIRT6 expression was inhibited, the promoting effect of TGF-β1/H_2O_2/HOCl on aerobic glycolysis and anoikis resistance was not sufficient to enhance the tumorigenicity of HCC cells. Suppressing the upregulation of SIRT6 enabled TGF-β1/H_2O_2/HOCl to induce cellular senescence, thereby abrogating the enhancement of HCC cell tumorigenicity by TGF-β1/H_2O_2/HOCl. These results suggest that SIRT6 is required for TGF-β1/H_2O_2/HOCl to enhance the tumorigenicity of HCC cells, and that targeting the ERK pathway to suppress the upregulation of SIRT6 might be a potential approach in comprehensive strategies for the therapy of HCC.

The ability to sustain uncontrolled proliferation represents the most fundamental trait of cancer cells.[1] One of the barriers that limit uncontrolled cell proliferation is cellular senescence, which represents a natural tumor suppressor mechanism.[2,3] Cancer cells need to overcome this obstacle to produce a clinically relevant tumor mass.[2,3] Therefore, understanding the mechanisms through which tumor cells bypass senescence might allow for the development of effective approaches in tumor therapy.

H_2O_2 has been found to induce cellular senescence by promoting the expression of p21 and p16.[2–4] 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.[5,6] However, our previous study showed that neither H_2O_2/HOCl (neutrophil-derived reactive oxygen species) nor TGF-β1 alone could induce the metastatic phenotype of HCC cells, but H_2O_2/HOCl could cooperate with TGF-β1 to induce the metastatic phenotype of HCC cells.[7] Consistently, the higher density of intratumoral neutrophils in hepatocellular carcinoma has been found to promote tumor metastasis.[8,9] These findings suggest that TGF-β1/H_2O_2/HOCl might not induce cellular senescence, as cellular senescence could reduce the clonogenicity and tumorigenicity of tumor cells.[10,11]

Sirtuin 6 (SIRT6) has been found to negatively regulate cellular senescence.[12] The average level of SIRT6 expression in HCC cells is lower than that in primary human hepatocytes.[13] However, the increased expression of SIRT6 has been found in many human HCC samples,[13] 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.[12] Therefore, the expression of SIRT6 might be upregulated by TGF-β1/H_2O_2/HOCl, thus playing a role in TGF-β1/H_2O_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 tumorigenicity of HCC cells. However, SIRT6 has the potential to function as a tumor suppressor by suppressing aerobic glycolysis in tumor cells[15] and increasing apoptosis sensitivity of tumor cells.[13] To ascertain whether the upregulation of SIRT6 expression might be one of the reasons that TGF-β1/H_2O_2/HOCl promoted the tumorigenicity of HCC cells, in this study we investigated whether H_2O_2/HOCl might cooperate with TGF-β1 to upregulate SIRT6 expression in HCC cells, and how SIRT6 might influence the tumorigenicity of HCC cells. Our data showed that TGF-β1/H_2O_2/HOCl could promote SIRT6 expression in HCC cells, and that suppressing SIRT6 expression could abrogate TGF-β1/H_2O_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_2O_2 and HOCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transforming growth factor-β1 was purchased from PeproTech (Rocky Hill, NJ, USA). SB203580, PD98059, SP600125, wortmannin, specific inhibitor of Smad3 (SIS3), and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from Merck4Biosciences (Calbiochem Frankfurter, Germany).

Senescence-associated β-gal assay. Senescence-associated β-gal (SA-β-gal) activity was analyzed with a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's instructions. The SA-β-gal$^+$ cell ratios were determined in three wells. To avoid any non-specific staining due to confluence, SA-β-Gal cytochemical 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′-CATGGAGGAGCGAGGTCT-3′ and antisense 5′-GCGTCTTACACTTGGCACA-3′; *GAPDH*, sense 5′-TCATTGACCTCAACTACATGGTTT-3′ and antisense 5′-GAAGATGGTGATGGGATTTC-3′; *PPIA*, sense 5′-GTCAACCCCACCGTGTTCTT-3′ and antisense5′-CTGCTGTCTTTGGGACCTTGT-3′; and *HPRT1*, sense 5′-GCTGAGGATTTGGAAAGGGTG-3′ and antisense 5′-CAGAGGGCTACAATGTGATGG-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′-GAAGAATGTGCCAAGTGTAAG-3′ and 5′-GTCTCACTTTGTTACTTGT-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 (1×10^6/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, 5×10^6 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 β-Galactosidase staining solution at 37°C overnight, then counterstained with eosin.[16] Images were obtained using an Olympus Tokyo, Japan IX71 microscope at 20 × 10 magnification. The density of senescent cells was defined as the number of SA-β-gal$^+$ spots per microscopic field.

To test the tumorigenicity of HCC cells, 5×10^6 tumor cells were injected into the right hind thigh of mice. Tumors were dissected and weighed on day 25 after inoculation. In other experiments, 5×10^6 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: $V = (L \times W^2)/2$.

Other methods. Other methods are described in Document S1, including soft agar assay, immunofluorescence, tumor cell proliferation assay, and isolation of tumor cells from tissues.

Statistics. Data were pooled from three independent experiments with a total of eight samples in each group. Results were expressed as mean value ± SD and interpreted by one-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

Results

Cellular senescence of HCC cells induced by H_2O_2/HOCl but not TGF-β1/H_2O_2/HOCl. Our previous study showed that TGF-β1/H_2O_2/HOCl could promote anoikis-resistance of HCC cells;[7] here we further investigated whether TGF-β1/H_2O_2/HOCl might influence cellular senescence and/or aerobic glycolysis of HCC cells. For this purpose, we detected SA-β-gal activity after 10 days of treatment with TGF-β1, TGF-β1/H_2O_2/HOCl, or H_2O_2/HOCl. Interestingly, SA-β-gal$^+$ cells were significantly increased by H_2O_2/HOCl, but only slightly increased by TGF-β1 or TGF-β1/H_2O_2/HOCl (Fig. 1a). Consistently, the expression of p16 and p21 was upregulated only by H_2O_2/HOCl, whereas TGF-β1/H_2O_2/HOCl failed to upregulate the expression of p16 and p21 (Figs. 1b,S1). However, treatment with either H_2O_2/HOCl or TGF-β1/H_2O_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-β1/H_2O_2/HOCl could promote anoikis resistance and glycolysis of HCC cells, but was inefficient in inducing cellular senescence.

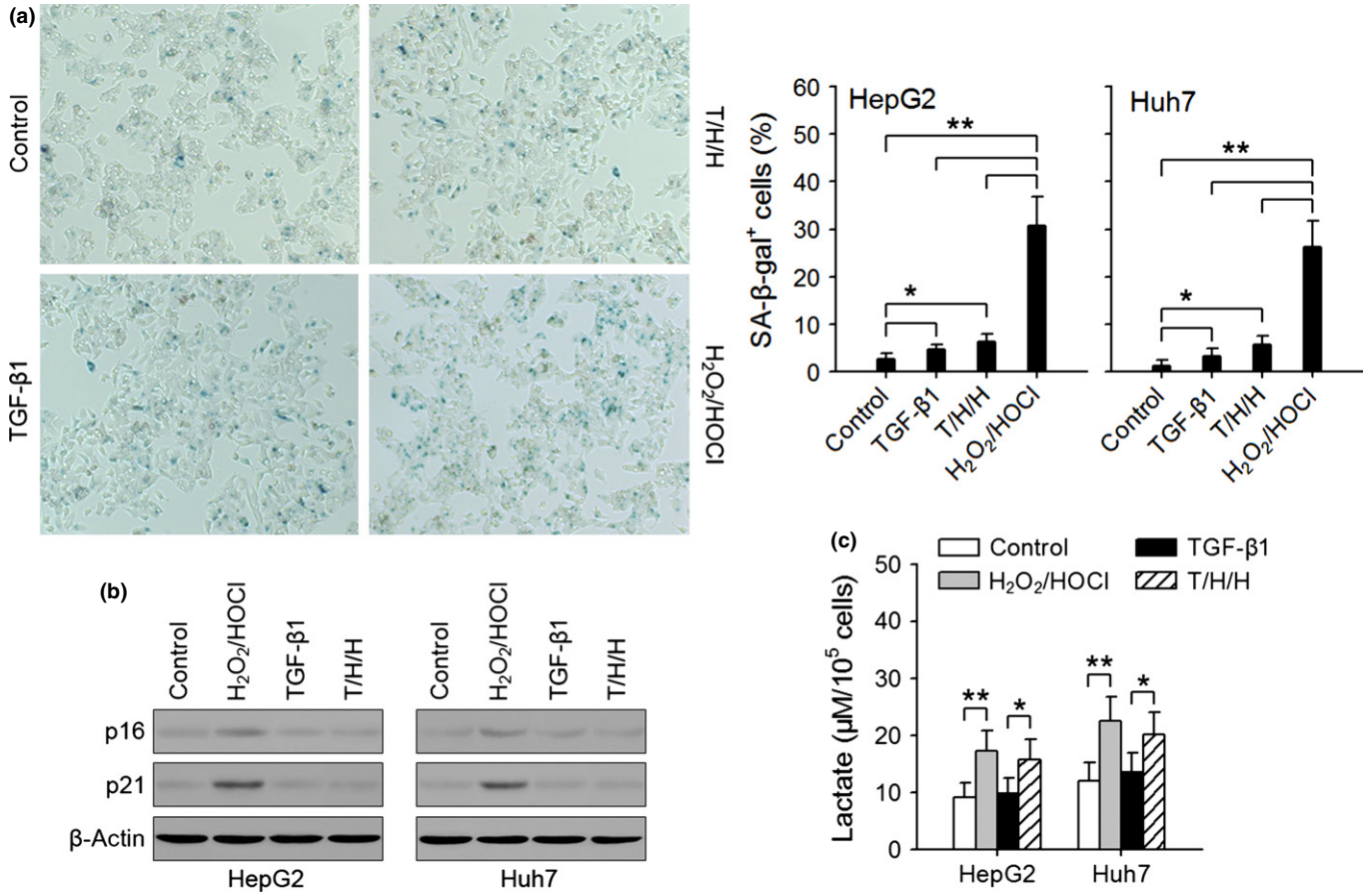

Fig. 1. Transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H) promotes aerobic glycolysis but not cellular senescence. HepG2 and Huh7 cells were untreated or treated for 10 days with TGF-β1 (5 ng/mL), T/H/H, or H_2O_2 (100 μM)/HOCl (50 μM). (a) Cells were stained for the observation of senescence-associated (SA)-β-gal$^+$ cells (left). The percentage of SA-β-gal$^+$ cells was calculated (right). (b) The expression of p16 and p21 was analyzed by Western blot. (c) The cells were used for the assay of lactate generation. $*P < 0.05$, $**P < 0.01$.

Sirtuin 6 expression in HCC cells upregulated by TGF-β1/H_2O_2/HOCl. We then investigated whether TGF-β1/H_2O_2/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 H_2O_2/HOCl, only slightly increased by TGF-β1, but remarkably upregulated by TGF-β1/H_2O_2/HOCl (Fig. 2a). *SIRT6* mRNA was gradually increased after the prolonged stimulation with TGF-β1/H_2O_2/HOCl (Fig. 2b), which was consistent with the activation patter of signaling pathways by these stimuli. Our previous study showed that either TGF-β1 alone or H_2O_2/HOCl only induced the transient, but not the sustained, activation of Smad, p38 MAPK, and ERK pathways. However, prolonged stimulation with TGF-β1/H_2O_2/HOCl could induce the sustained and gradually enhanced activation of these pathways.[7] Therefore, we further analyzed TGF-β1/H_2O_2/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 ERK pathway abrogated the upregulation of *SIRT6* by TGF-β1/H_2O_2/HOCl (Fig. 2c). Smad3 inhibitor and p38 MAPK inhibitor also suppressed the expression of *SIRT6*.

Sirtuin 6 suppresses induction of cellular senescence by TGF-β1/H_2O_2/HOCl. We then wondered whether SIRT6 might influence TGF-β1/H_2O_2/HOCl-mediated induction of cellular senescence. As SIRT6 overexpression could induce massive apoptosis in cancer cells,[17] we investigated the effect of SIRT6 by suppressing TGF-β1/H_2O_2/HOCl-mediated upregulation of SIRT6 expression with SIRT6 shRNA (Fig. 3a). Inhibiting the upregulation of SIRT6 enabled TGF-β1/H_2O_2/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/H_2O_2/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).[18,19] Interestingly, SIRT6 shRNA augmented the promoting effect of TGF-β1/H_2O_2/HOCl on anoikis resistance of HCC cells (Fig. 3d), but did not significantly influence the effect of TGF-β1/H_2O_2/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.[20] H_2O_2/HOCl or TGF-β1/H_2O_2/HOCl slightly promoted the expression of HIF-1α, but strongly activated AMPK and increased the phosphorylation level of PDH (Fig. S4a), suggesting that H_2O_2/HOCl or

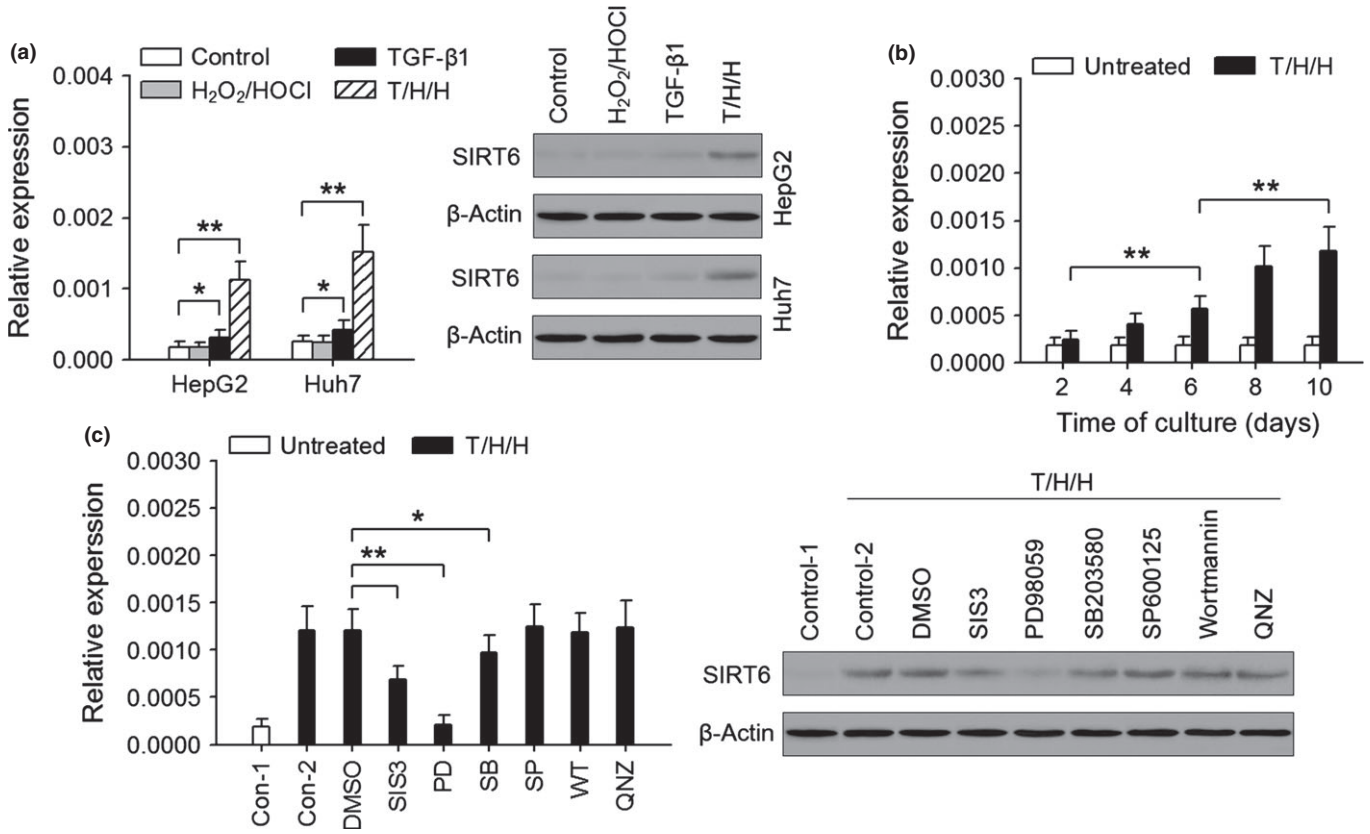

Fig. 2. Transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H) upregulates expression of sirtuin 6 (SIRT6) in hepatocellular carcinoma cells. (a) HepG2 and Huh7 cells were untreated or treated for 10 days with H_2O_2 (100 μM)/HOCl (50 μM), TGF-β1 (5 ng/mL), or T/H/H. Expression of the *SIRT6* gene was detected by real-time RT-PCR and Western blot. (b) HepG2 cells were untreated or treated with T/H/H. *SIRT6* expression was detected by real-time RT-PCR at the indicated time points. (c) HepG2 cells were untreated or treated for 10 days with T/H/H in the absence or presence of specific inhibitor of Smad3 (SIS3; 2 μM), PD98059 (PD; 10 μM), SB203580 (SB; 10 μM), SP600125 (SP; 10 μM), wortmannin (WT; 40 nM), and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ; 40 nM). *SIRT6* expression was detected by real-time RT-PCR and Western blot. $*P < 0.05$, $**P < 0.01$. Con-, control.

TGF-β1/H_2O_2/HOCl could enhance the aerobic glycolysis. Sirtuin 6 shRNA did not influence the activation of AMPK (Fig. S4b), and only slightly increased the phosphorylation level of PDH (Fig. S4c), which might be the reason that SIRT6 could not efficiently suppress the enhancing effect of TGF-β1/H_2O_2/HOCl on aerobic glycolysis.

Upregulation of SIRT6 alters the effect of Smad and p38 MAPK pathways on cellular senescence. Consistent with the promoting effect of Smad and p38 MAPK pathways on the expression of SIRT6, inhibiting each of these pathways enabled TGF-β1/H_2O_2/HOCl to induce the cellular senescence of HCC cells (Fig. 4a). Interestingly, however, if SIRT6 expression was suppressed by shRNA, the inducing effect of TGF-β1/H_2O_2/HOCl on cellular senescence was attenuated by inhibiting the NF-κB, Smad, and p38 MAPK pathways, but not by inhibiting the ERK pathway (Fig. 4b). These results suggest that TGF-β1/H_2O_2/HOCl could induce the cellular senescence of HCC cells through the NF-κB, Smad, and p38 MAPK pathways, but their effect was suppressed by TGF-β1/H_2O_2/HOCl-mediated upregulation of SIRT6 expression.

Sirtuin 6 contributes to inhibitory effect of ERK pathway on cellular senescence. The ERK pathway suppresses cellular senescence by suppressing the expression of p16 and p21.[6,21] Transforming growth factor-β1/H_2O_2/HOCl could induce sustained and enhanced activation of the ERK pathway.[7] Consistently, TGF-β1/H_2O_2/HOCl could efficiently induce cellular senescence, if the ERK pathway was inhibited (Fig. 4a). In this situation, the effect of TGF-β1/H_2O_2/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/H_2O_2/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/H_2O_2/HOCl could upregulate the expression of p16 and p21 (Figs 5b,S5). If SIRT6 expression was suppressed by shRNA, TGF-β1/H_2O_2/HOCl could similarly induce the expression of p16 and p21. Further inhibiting the ERK pathway only slightly increased the effect of TGF-β1/H_2O_2/HOCl (Fig. 5b,S5).

Sirtuin 6 required for TGF-β1/H_2O_2/HOCl to promote tumorigenicity of HCC cells. Based on the above results, we then analyzed whether upregulation of SIRT6 is required for TGF-β1/H_2O_2/HOCl to enhance the tumorigenicity of HCC cells. When HCC cells were cultured under anchorage-independent conditions, the pretreatment with TGF-β1/H_2O_2/HOCl did not influence the size of colonies (Fig. S6a,b). Intriguingly, however, pretreatment with TGF-β1/H_2O_2/HOCl significantly

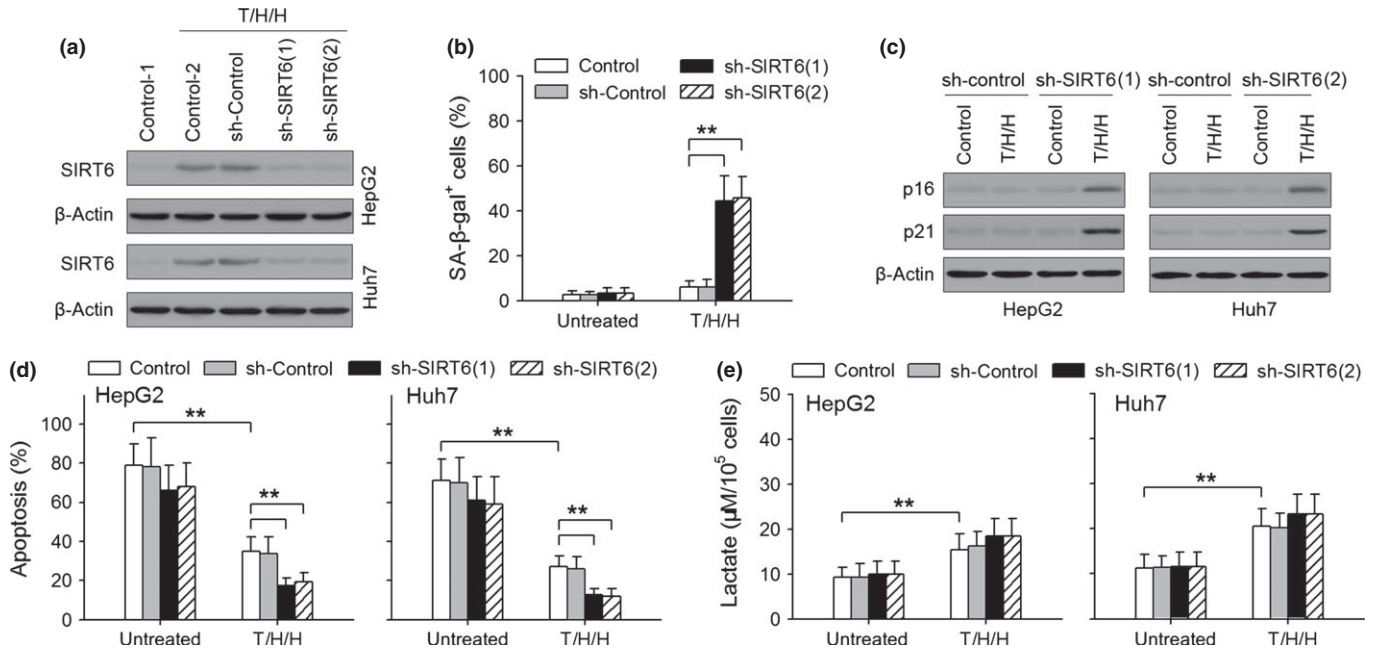

Fig. 3. Sirtuin 6 (SIRT6) suppresses the effect of transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H) on cellular senescence but not anoikis resistance or aerobic glycolysis. HepG2 and Huh7 cells, non-transfected or transfected with the indicated vectors, were untreated or treated for 10 days with T/H/H. (a) SIRT6 expression was detected by Western blot. (b) HepG2 cells were stained for the observation of senescence-associated (SA)-β-gal$^+$ cells. The percentage of SA-β-gal$^+$ cells was calculated. (c) Expression of p16 and p21 was analyzed by Western blot. (d) Cells were incubated under anchorage-independent conditions for 48 h. Apoptosis was analyzed by flow cytometry. (e) Cells were used for the assay of lactate generation. $^{**}P < 0.01$.

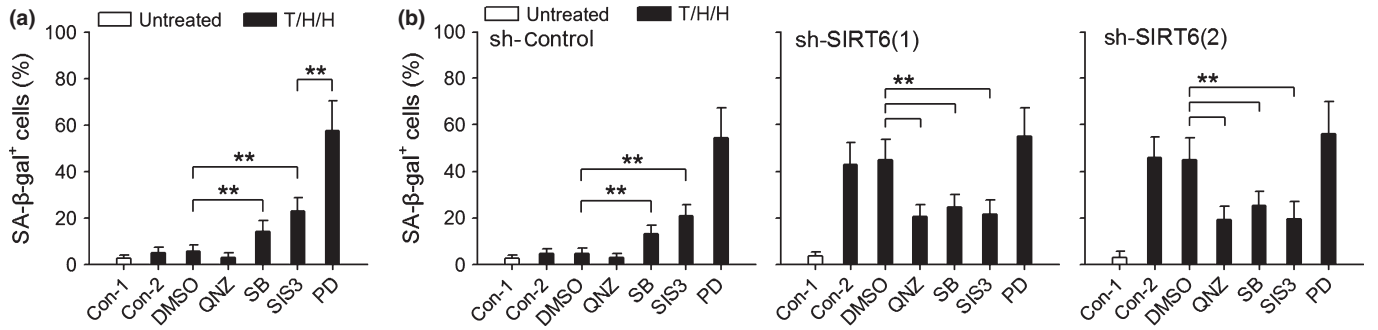

Fig. 4. Sirtuin 6 (SIRT6) alters the effect of Smad and p38 MAPK pathways on cellular senescence in the presence of transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H). HepG2 cells, non-transfected (a) or transfected with the indicated vectors (b), were untreated or treated for 10 days with T/H/H in the absence or presence of 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ; 40 nM), SB203580 (SB; 10 μM), specific inhibitor of Smad3 (SIS3; 2 μM), and PD98059 (PD; 10 μM). The cells were then stained for the observation of senescence-associated (SA)-β-gal$^+$ cells. The percentage of SA-β-gal$^+$ cells was calculated. $^{**}P < 0.01$. Con-, control.

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/H_2O_2/HOCl on colony formation (Fig. S6c). These results suggest that TGF-β1/H_2O_2/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/H_2O_2/HOCl-mediated enhancement of tumorigenicity of HCC cells, we treated HCC cells with TGF-β1/H_2O_2/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/H_2O_2/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/H_2O_2/HOCl (Fig. 6a). Consistently, the pretreatment of HCC cells with TGF-β1/H_2O_2/HOCl promoted the development of tumor (Fig. 6b,c). If the upregulation of *SIRT6* gene expression was inhibited with SIRT6 shRNA, TGF-β1/H_2O_2/HOCl treatment could not promote the development of tumor, indicating that the upregulation of SIRT6 is required for TGF-β1/H_2O_2/HOCl to promote the tumorigenicity of HCC cells, and that inhibiting the upregulation of SIRT6 could abrogate the promoting effect of TGF-β1/H_2O_2/HOCl on the tumorigenicity of HCC cells. Intriguingly, when untreated tumor cells were inoculated, *SIRT6* expression in tumor cells was gradually increased (Fig. S7b). The expression of the *SIRT6* gene in these tumor cells might be upregulated by TGF-β1/H_2O_2/HOCl in the tumor milieu after inoculation,

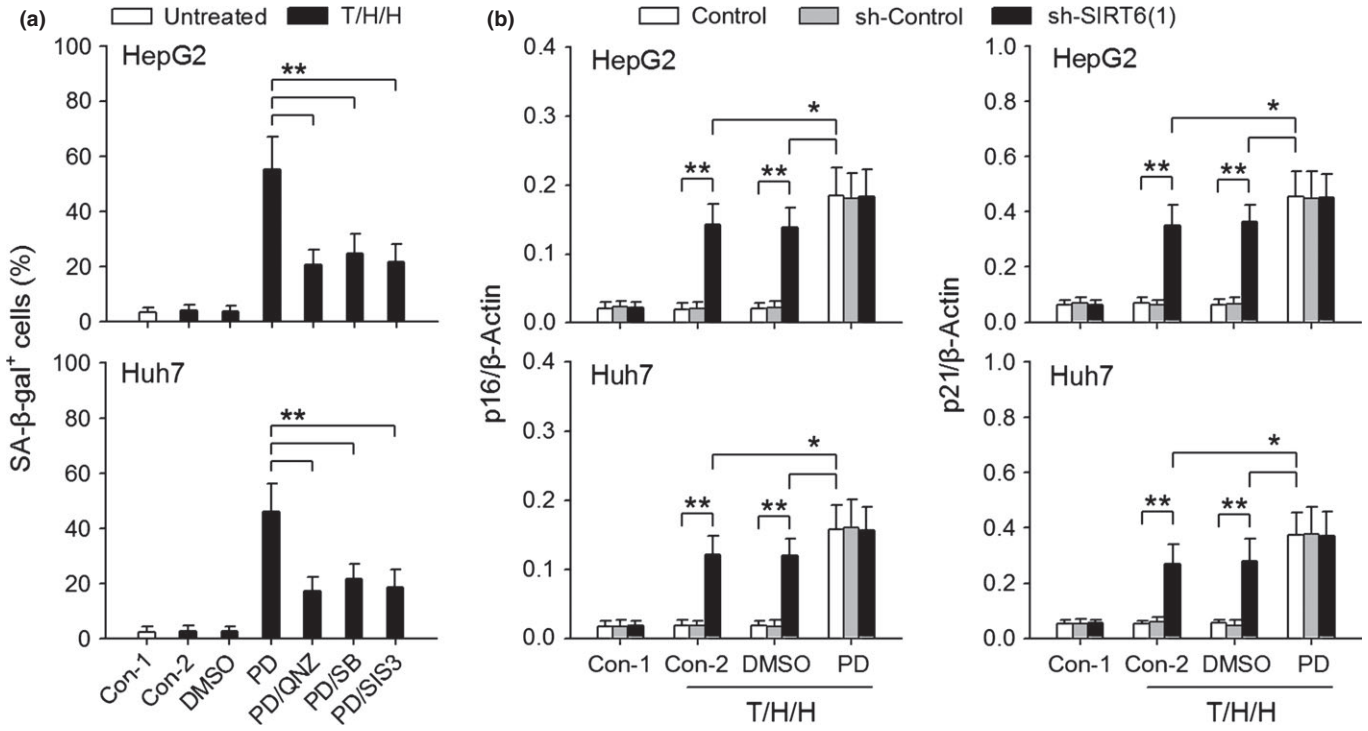

Fig. 5. Sirtuin 6 (SIRT6) contributes to the inhibitory effect of the ERK pathway on cellular senescence. HepG2 and Huh7 cells, non-transfected (a) or transfected with the indicated vectors (b), were untreated or treated for 10 days with transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H) in the absence or presence of PD98059 (PD; 10 μM), 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ; 40 nM), SB203580 (SB; 10 μM), and specific inhibitor of Smad3 (SIS3; 2 μM). (a) Cells were then stained for observation of senescence-associated (SA)-β-gal$^+$ cells. The percentage of SA-β-gal$^+$ cells was calculated. (b) Expression of p16 and p21 was analyzed by Western blot. The relative levels of p16 to β-actin and p21 to β-actin were calculated after densitometric analysis of Western blots. $*P < 0.05$, $**P < 0.01$. Con-, control.

as these factors could be produced by neutrophils (H_2O_2/HOCl) and other stromal cells (TGF-β1) 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-β1/H_2O_2/HOCl (Fig. S8). Taken together, these results suggest that TGF-β1 and H_2O_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,[13,15] our data in this study showed that TGF-β1/H_2O_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-β1/H_2O_2/HOCl on cellular senescence. Although SIRT6 could not abrogate the promoting effect of TGF-β1/H_2O_2/HOCl on the aerobic glycolysis and apoptosis resistance of HCC cells, TGF-β1/H_2O_2/HOCl failed to promote clonogenicity and tumorigenicity of HCC cells if the upregulation of SIRT6 expression was suppressed.

Transforming growth factor-β1/H_2O_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-β1/H_2O_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,[22] could induce the expression of SIRT6,[23] and that Smad3 could cooperate with c-Fos in modulating gene expression.[24,25] Nevertheless, TGF-β1 alone could not induce higher expression of SIRT6 in HCC cells. Our previous study showed that TGF-β1 could induce the transient activation of ERK and Smad pathways in HCC cells, but prolonged stimulation with TGF-β1/H_2O_2/HOCl was required for inducing the sustained and enhanced activation of these pathways.[7] 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-β1/H_2O_2/HOCl was required for inducing higher expression of SIRT6.

Cellular senescence could be induced by the activation of different signaling pathways, including NF-κB, Smad, and p38 MAPK. Transforming growth factor-β1 and H_2O_2/HOCl could activate these pathways,[7,26] 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-κB, Smad, and p38 MAPK on cellular senescence. Therefore, TGF-β1/H_2O_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,[4,6,27] whereas the ERK pathway suppresses cellular senescence by suppressing the expression of p16 and p21.[6,21] When HCC cells were

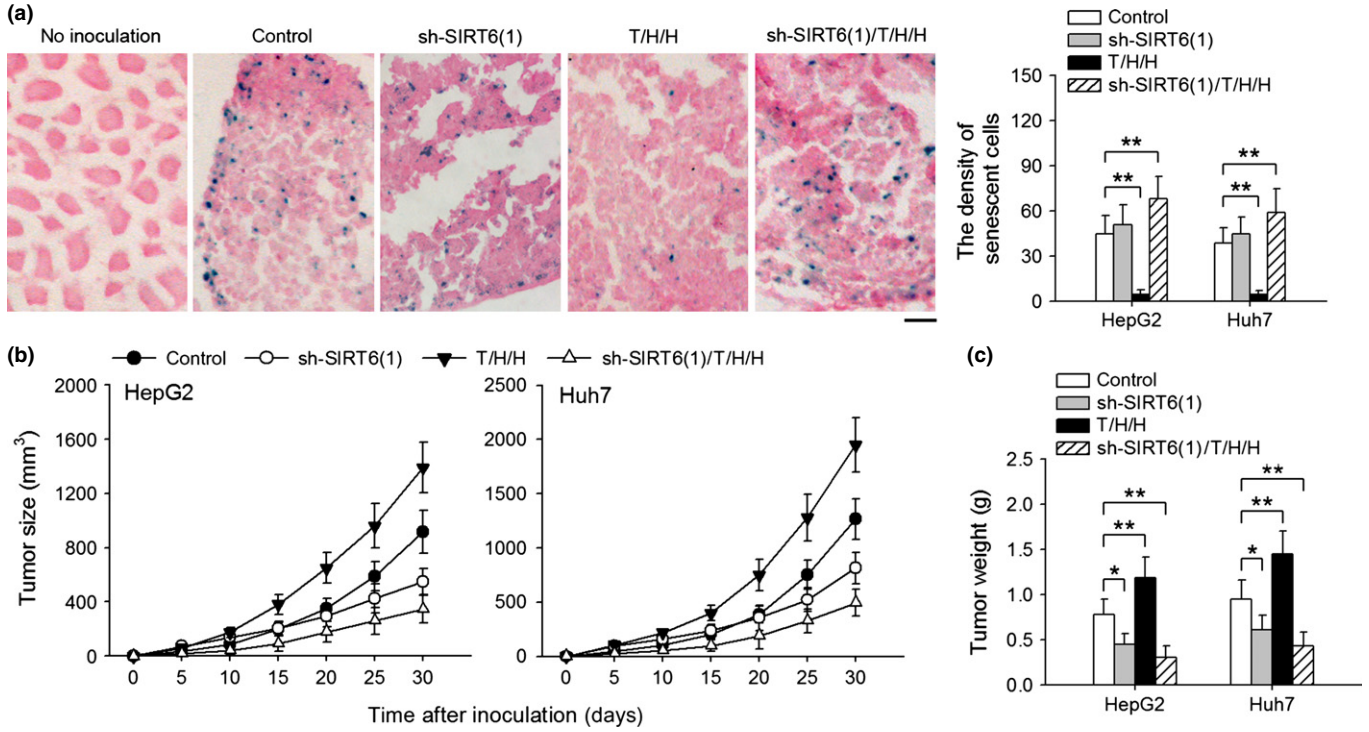

Fig. 6. Sirtuin 6 (SIRT6) is required for transforming growth factor-β1 (TGF-β1)/H_2O_2/HOCl (T/H/H) to promote the tumorigenicity of hepatocellular carcinoma cells. HepG2 and Huh7 cells, non-transfected or transfected with sh-SIRT6(1), were untreated or treated for 10 days with T/H/H. The cells were then used in the following experiments. (a) The cells were 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 cells. 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). $*P < 0.05$, $**P < 0.01$.

stimulated by TGF-β1/H_2O_2/HOCl, the ERK pathway was crucial in suppressing the cellular senescence of HCC cells. Increasing the activity of Myc is one of the mechanisms by which the ERK pathway inhibits cellular senescence.[6] Our data showed that upregulating SIRT6 expression might be another mechanism for the ERK pathway to suppress cellular senescence, as SIRT6 could downregulate the expression of p21 and p16. Moreover, SIRT6 could directly inhibit the expression of a subset of NF-κB target genes, especially those associated with senescence.[28] When HCC cells were simulated by TGF-β1/H_2O_2/HOCl, the activation of the ERK pathway was not sufficient to completely antagonize the effect of NF-κB, Smad, and p38 MAPK pathways on cellular senescence if SIRT6 expression was inhibited. Therefore, SIRT6 plays an important role in suppressing TGF-β1/H_2O_2/HOCl-mediated induction of cellular senescence.

Tumor cells secrete large amount of lactate due to increased aerobic glycolysis (Warburg effect), which can enhance tumorigenesis.[29,30] Both HIF-1α and AMPK can promote aerobic glycolysis by inducing the phosphorylation of PDH.[20] Sirtuin 6 has been found to suppress aerobic glycolysis by influencing the expression and function of HIF-1α.[18,19] However, H_2O_2/HOCl strongly activated AMPK, but only slightly increased the expression of HIF-1α, as shown by our data. This result is supported by recent reports that H_2O_2 might have the potential to increase HIF-1α expression,[31] but AMPK could negatively regulate the expression of HIF-1α in HCC cells.[20] Inhibiting SIRT6 expression only slightly influenced the phosphorylation of PDH and the production of lactate, as the activation of AMPK was not influenced by SIRT6. Accordingly, TGF-β1/H_2O_2/HOCl could augment the Warburg effect even if SIRT6 expression was upregulated. Moreover, TGF-β1/H_2O_2/HOCl could efficiently promote the anoikis resistance of HCC cells by inducing the sustained activation of the ERK and p38 MAPK pathways.[7] Sirtuin 6 could not abolish the promoting effect of TGF-β1/H_2O_2/HOCl on anoikis resistance. Therefore, SIRT6 cannot function as a tumor suppressor in the presence of TGF-β1/H_2O_2/HOCl.

In summary, in this study we showed that TGF-β1/H_2O_2/HOCl could upregulate SIRT6 expression in HCC cells, which disabled TGF-β1/H_2O_2/HOCl from inducing cellular senescence. As TGF-β1/H_2O_2/HOCl could induce the enhanced and sustained activation of the Smad and p38 MAPK pathways in HCC cells, TGF-β1/H_2O_2/HOCl should be able to reduce the tumorigenicity of HCC cells by inducing cellular senescence. However, the upregulation of SIRT6 expression by the ERK pathway abrogated the inducing effect of TGF-β1/H_2O_2/HOCl on cellular senescence of HCC cells. Therefore, TGF-β1/H_2O_2/HOCl promoted the tumorigenicity of HCC cells by promoting anoikis resistance and glycolysis. If the upregulation of SIRT6 is suppressed, the promoting effect of TGF-β1/H_2O_2/HOCl on the tumorigenicity of HCC cells could be abrogated, suggesting that the abolishment of TGF-β1/H_2O_2/HOCl-mediated senescence is in favor of tumorigenicity of HCC cells and the development of tumor. The ERK pathway is crucial for upregulating SIRT6 expression. Therefore, targeting the ERK pathway to suppress the upregulation of SIRT6 could abolish the promoting effect of TGF-β1/H_2O_2/HOCl on tumorigenicity of HCC cells. This might be a potential approach in a comprehensive strategy for HCC therapy.

Acknowledgments

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

Disclosure Statement

The authors have no conflict of interest.

References

1 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
2 Dasari A, Bartholomew JN, Volonte D, Galbiati F. Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements. *Cancer Res* 2006; **66**: 10805–14.
3 Volonte D, Liu Z, Musille PM *et al.* Inhibition of nuclear factor-erythroid 2-related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence. *Mol Biol Cell* 2013; **24**: 1852–62.
4 Supanji, Shimomachi M, Hasan MZ, Kawaichi M, Oka C. HtrA1 is induced by oxidative stress and enhances cell senescence through p38 MAPK pathway. *Exp Eye Res* 2013; **112**: 79–92.
5 Senturk S, Mumcuoglu M, Gursoy-Yuzugullu O, Cingoz B, Akcali KC, Ozturk M. Transforming growth factor-beta induces senescence in hepatocellular carcinoma cells and inhibits tumor growth. *Hepatology* 2010; **52**: 966–74.
6 Massagué J. G1 cell-cycle control and cancer. *Nature* 2004; **432**: 298–306.
7 Feng XX, Liu M, Yan W *et al.* β3 integrin promotes TGF-β1/H_2O_2/HOCl-mediated induction of metastatic phenotype of hepatocellular carcinoma cells by enhancing TGF-β1 signaling. *PLoS One* 2013; **8**: e79857.
8 Li YW, Qiu SJ, Fan J *et al.* Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection. *J Hepatol* 2011; **54**: 497–505.
9 Zhou SL, Dai Z, Zhou ZJ *et al.* Overexpression of CXCL5 mediates neutrophil infiltration and indicates poor prognosis for hepatocellular carcinoma. *Hepatology* 2012; **56**: 2242–54.
10 Xu Q, Ma P, Hu C *et al.* Overexpression of the DEC1 protein induces senescence in vitro and is related to better survival in esophageal squamous cell carcinoma. *PLoS One* 2012; **7**: e41862.
11 Ohta K, Haraguchi N, Kano Y *et al.* Depletion of JARID1B induces cellular senescence in human colorectal cancer. *Int J Oncol* 2013; **42**: 1212–8.
12 Minagawa S, Araya J, Numata T *et al.* Accelerated epithelial cell senescence in IPF and the inhibitory role of SIRT6 in TGF-β-induced senescence of human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2011; **300**: L391–401.
13 Marquardt JU, Fischer K, Baus K *et al.* SIRT6-dependent genetic and epigenetic alterations are associated with poor clinical outcome in hepatocellular carcinoma patients. *Hepatology* 2013; **58**: 1054–64.
14 Lefort K, Brooks Y, Ostano P *et al.* A miR-34a-SIRT6 axis in the squamous cell differentiation network. *EMBO J* 2013; **32**: 2248–63.
15 Sebastian C, Zwaans BMM, Silberman DM *et al.* The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. *Cell* 2012; **151**: 1185–99.
16 Dimri GP, Lee X, Basile G *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 1995; **92**: 9363–7.
17 Van Meter M, Mao Z, Gorbunova V, Seluanov A. SIRT6 overexpression induces massive apoptosis in cancer cells but not in normal cells. *Cell Cycle* 2011; **10**: 3153–8.
18 Zhong L, D'Urso A, Toiber D *et al.* The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 2010; **140**: 280–93.
19 Zhong L, Mostoslavsky R. SIRT6: a master epigenetic gatekeeper of glucose metabolism. *Transcription* 2010; **1**: 17–21.
20 Hsu CC, Wang CH, Wu LC *et al.* Mitochondrial dysfunction represses HIF-1α protein synthesis through AMPK activation in human hepatoma HepG2 cells. *Biochim Biophys Acta* 2013; **1830**: 4743–51.
21 Liu D, Liu J, Lin B *et al.* Lewis y regulate cell cycle related factors in ovarian carcinoma cell RMG-I in vitro via ERK and Akt signaling pathways. *Int J Mol Sci* 2012; **13**: 828–39.
22 Watanabe T, Hiasa Y, Tokumoto Y *et al.* Protein kinase R modulates c-Fos and c-Jun signaling to promote proliferation of hepatocellular carcinoma with hepatitis C virus infection. *PLoS One* 2013; **8**: e67750.
23 Min L, Ji Y, Bakiri L *et al.* Liver cancer initiation is controlled by AP-1 through SIRT6-dependent inhibition of survivin. *Nat Cell Biol* 2012; **14**: 1203–11.
24 Wu Y, Zhang X, Salmon M, Lin X, Zehner ZE. TGFβ1 regulation of vimentin gene expression during differentiation of the C2C12 skeletal myogenic cell line requires Smads, AP-1 and Sp1 family members. *Biochim Biophys Acta* 2007; **1773**: 427–39.
25 Funaba M, Ikeda T, Murakami M *et al.* Transcriptional regulation of mouse mast cell protease-7 by TGF-β. *Biochim Biophys Acta* 2006; **1759**: 166–70.
26 Zhou YH, Liao SJ, Li D *et al.* TLR4 ligand/H_2O_2 enhances TGF-β1 signaling to induce metastatic potential of non-invasive breast cancer cells by activating non-Smad pathways. *PLoS One* 2013; **8**: e65906.
27 Spallarossa P, Altieri P, Barisione C *et al.* p38 MAPK and JNK antagonistically control senescence and cytoplasmic p16INK4A expression in doxorubicin-treated endothelial progenitor cells. *PLoS One* 2010; **5**: e15583.
28 Kawahara TL, Michishita E, Adler AS *et al.* SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* 2009; **136**: 62–74.
29 Wu CA, Chao Y, Shiah SG, Lin WW. Nutrient deprivation induces the Warburg effect through ROS/AMPK-dependent activation of pyruvate dehydrogenase kinase. *Biochim Biophys Acta* 2013; **1833**: 1147–56.
30 Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**: 1029–33.
31 Kim MC, Cui FJ, Kim Y. Hydrogen peroxide promotes epithelial to mesenchymal transition and stemness in human malignant mesothelioma cells. *Asian Pac J Cancer Prev* 2013; **14**: 3625–30.

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 H_2O_2/HOCl but not transforming growth factor-β1 (TGF-β1)/H_2O_2/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)/H_2O_2/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)/H_2O_2/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.