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Original Article

PIAS3/SOCS1-STAT3 axis responses to oxidative stress in hepatocellular cancer cells

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Abstract: The participation of STAT3 and its upstream inhibitors, PIAS3 and SOCS1, in the oxidative response of hepatocellular carcinoma (HCC) cells was uncertain. Here, the expression of PIAS3 and SOCS1 in HCC tissues and cell lines was explored, and we sought to determine whether oxidative stress epigenetically regulated PIAS3 and SOCS1 expression and STAT3 activation in HCC cells. The expression of PIAS3 and SOCS1 was markedly decreased in HCC cell lines and tissues compared to normal hepatic cells and tissues. In HCC patients, low PIAS3 and SOCS1 expression were associated with poor survival. Oxidative stress induced by H_2O_2 in HepG2 cells was indicated by low antioxidant levels and high protein carbonyl content. Moreover, oxidative stress in HepG2 cells contributed to reduced proliferation but increased apoptosis, migration, and invasion capacity, which might be counteracted by antioxidants, such as tocopheryl acetate (TA). PIAS3 and SOCS1 expression was markedly decreased, while STAT3 was activated in HepG2 cells in response to H_2O_2 exposure. Co-treatment with antioxidant TA effectively increased the expression of PIAS3 and SOCS1, but it dephosphorylated STAT3 in H_2O_2-treated cells. PIAS1 or SOCS1 overexpression in HepG2 cells after H_2O_2 treatment restored cell viability and anti-oxidative responses and decreased apoptosis, migration, and invasion ability, and dephosphorylated STAT3 levels. Co-administration of the STAT3 activator, colivelin, partially abolished the effect of PIAS3 and SOCS1 overexpression in these processes. Therefore, oxidative stress in HCC cells may improve their migration and reduce proliferation through STAT3 activation through the repression of PIAS3 and SOCS1 expression.

Keywords: HCC, oxidative stress, PIAS3, SOCS1, STAT3, colivelin

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver, representing 80% of all primary liver cancers worldwide. Other types of liver cancer include cholangiocarcinoma, which begins in the cells that line the bile duct; angiosarcoma (or hemangiosarcoma), which starts in the blood vessels of the liver; and hepatoblastoma, which is very rare and usually affects young children. HCC accounts for up to 75% to 85% of primary liver cancers globally [1-3].

Oxidative stress in the tumor microenvironment has been reported to participate in HCC development. Cancer cells generate more reactive oxygen species (ROS) than normal cells, leading to increased oxidative stress in the tumor microenvironment [4, 5]. ROS, including free oxygen radicals and non-radical ROS, are derived from the sequential reduction of oxygen through the addition of electrons [6]. It has been widely recognized that a low level of ROS is indispensable in several physiologic processes of the cell, including proliferation, apoptosis, cell cycle arrest, and cell senescence [7]. However, abundant ROS induces oxidative stress and a potentially toxic microenvironment, which facilitates cancer development primarily by increasing DNA damage and altering specific cellular processes [8]. Oxidative stress plays a significant role in cancer development mainly by enhancing DNA damage caused by hydroxyl radicals and modifying key cellular processes, such as cell proliferation, apoptosis, and motility cascades by superoxide radicals. Hydrogen peroxides play an important role in cancer

development [9]. However, excessive ROS generation is still fatal to cancer cells; therefore, nuclear factor erythroid 2-related factor 2 (Nrf2) is upregulated and activated to combat ROS during oxidative stress [10]. In HCC tissues, Nrf2 expression is increased and associated with tumor development and poor survival [11]. It has been suggested that oxidative stress is excessive in HCC tissues and patients, confirming that ROS can promote DNA methylation in cancer cells [12-14].

Several STAT members, especially STAT3, are involved in growth control [15, 16], and the aberrant activation of STAT3 is associated with tumorigenesis [17], including that of liver cancer [18]. STAT3 is transiently activated and then inactivated by a group of signaling proteins, such as protein inhibitors of activated STATs (PIAS), suppressors of cytokine signaling proteins (SOCS), and SH2-containing tyrosine phosphatase (SHP1 and SHP2) cascades [19]. PIAS proteins appear to regulate STAT activity [20]. Upon cytokine stimulation, PIAS3 binds to activated STAT3 and prevents binding to DNA [20]. PIAS3 expression is increased in human cancers, including cervical cancer [21], prostate cancer [22, 23], osteosarcoma [24], brain tumors [25], and lung cancer [26]. Moreover, several studies have reported that the PIAS3-STAT3 axis is involved in many types of cancers, including glioblastoma [27], breast cancer [28], and lung cancer [29]. SOCS1 blocks the cytokine signal transduction through direct interaction with JAK proteins. The SH2 domain of SOCS1 binds to the JH1 domain of JAK and inhibits its phosphorylation, and therefore downregulates the JAK/STAT pathway [30]. The *SOCS1* gene is frequently repressed in HCC, and SOCS1-deficient mice show high susceptibility to experimental HCC, developing larger and more numerous tumor nodules [31, 32]. Although previous studies have indicated a direct correlation between STAT3 and oxidative stress in HCC [33-35], the detailed mechanisms underlying PIAS3/SOCS1-STAT3 interactions in HCC in response to oxidative stress remain unclear.

Therefore, in the present study, we investigated the expression of PIAS3 and SOCS1 in HCC tissues and cell lines. The clinical relevance of these two signals and STAT3 was also evaluated. With oxidative stress induced by H_2O_2, the influence of PIAS3 and SOCS1 expression on HepG2 cell proliferation, migration, and invasion was experimentally investigated for the first time.

Materials and methods

Patients and paraffin-embedded tissues

A total of 62 pairs of resected HCC tissue samples and matched normal specimens from HCC patients were collected from September 2016 to May 2020 at Affiliated Hospital of Yanbian University. The HCC patients included 32 males and 30 females (aged 30-68 years old) with an average age of 46.5±11.6 years old. All patients were diagnosed and histopathologically confirmed as HCC, and had not received adjuvant chemotherapy, radiation therapy, or immunotherapy before surgical excision of tumors. Samples were pathologically confirmed by two experienced doctors and rapidly immersed in liquid nitrogen after surgical resection. Immunohistochemical staining for PIAS3 and SOCS1 was performed using three paraffin-embedded liver sections from HCC patients. The research protocol and procedures were approved by the Ethics Committee of Affiliated Hospital of Yanbian University (Approval number: 2021170). All study participants provided written informed consent before participating in the study.

Immunohistochemical staining

Immunohistochemical staining was performed according to the manufacturer's instructions with the following reagents and instruments: horse serum (RTU Vectastain Kit, PK-7200), 1:200 mouse anti-Nrf2 antibody (AF3925, R&D Systems), ABC reagent (LS-J1026-1, Vector labs), and IHC slide staining system (NanoMtrx 100, BioGenex). Staining intensity was scored visually by two experienced pathologists independently as follows: 0= no staining, 1= weak staining, 2= moderate staining, and 3= strong staining. Six visual fields with tumor cells were randomly selected and scored based on the percentage of positively stained cells (0-100%). The final IHC score was calculated by multiplying the intensity score by the percentage of positive cells [36].

Cell culture and transfection

HCC cell lines were purchased from The Cell Bank of Type Culture Collection of Chinese

Academy of Sciences, Shanghai, China, and maintained in DMEM containing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin at 37°C 5% CO_2, and 95% humidity. Cellular oxidative stress was induced by H_2O_2 (Merck Schuchardt OHG, Germany) and attenuated by tocopherol acetate (TA, 7695-91-2, Sigma, USA) at 300 μM [37].

PIAS3 and SOCS1 expression plasmids (pcDNA3.1-PIAS3 and pcDNA3.1-SOCS1) were designed and synthesized by GenePharma (Shanghai, China). All vectors were then transfected into HepG2 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Protein carbonyl determination

The procedure for protein carbonyl measurement was the same as that in a previous study [38] using Bradford assays kit (Merck Millipore, USA) and was repeated more than three times.

Total antioxidant capacity (TAC) determination

TAC was determined using the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (A1888, Sigma, USA) method [14]. TAC of each sample was expressed in terms of vitamin C equivalent antioxidant capacity (VCEAC; mM). Assays were repeated in triplicate.

Real-time PCR

Cells or specimens (100 mg) were mixed with TRIzol reagent (Invitrogen) to obtain total cellular RNA, and its concentration was calculated using a Nanodrop2000 instrument (OD260; Thermo Fisher Scientific). DNA was synthesized from an RNA template by reverse transcription using a HifairTM II 1st Strand cDNA Synthesis Kit (11119ES50, Yeasen Biotech, Shanghai, China). The primers used for real-time PCR were as follows: PIAS3 F: 5'-TGT CAC CAT GAA ACC ATT GC-3', PIAS3 R: 5'-AGG TAA AGT GCG CTT CCT CA-3'; SOCS1 F: 5'-GTC CCC CTG GTT GTT GTA G-3', SOCS1 R: 5'-AAG AGG TAG GAG GTG CGA G-3'; GAPDH F: 5'-GGG AAG GTG AAG GTC GGA G-3', and GAPDH R: 5'-GGG GTC ATT GAT GGC AAC A-3'. Real-time PCR was performed under the following conditions using a HifairTM II One Step RT-qPCR SYBR Green Kit (11125ES50, Yeasen Biotech, Shanghai, China): denaturation (10 min, 95°C), denaturation (15 s, 95°C for 40 cycles), annealing (15 s, 37°C), and extension (40 s, 60°C), with *GAPDH* as an internal control. Gene mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blotting (WB)

RIPA buffer supplemented with protease inhibitor cocktail (Cat. NO 03969-21 COSMOCORE, Japan) was used to lyse cells for extracting total protein, the concentration of which was determined using a Pierce BCA Protein Assay Kit (23225, Thermo Scientific, USA). Electrophoresis was performed by loading proteins onto sodium dodecyl sulfate-polyacrylamide gels and transferring them onto Durapore PVDF membrane filters (Sigma-Aldrich, USA) after electrophoresis. After incubation with primary and secondary antibodies, protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (46641, Thermo Scientific, USA). The details of antibodies obtained from Abcam are as follows: primary antibodies against Nrf2 (1:2000, ab31163), PIAS3 (1:2500, ab58406), SOCS1 (1:1000, ab9870), STAT3 (1:1000, ab31370), phosphor STAT3 (1:200, ab16431), and Tubulin (1:5000, ab6046), as well as horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibody (1:5000, ab205719 and ab205718).

Cell counting kit-8 (CCK-8) assay

Initially, cells were seeded into 96-well plates that were treated with CCK-8 reagent (0.01 mL, 96992, Sigma-Aldrich, USA) at multiple time points following transfection, and the plates were then incubated for 2 h at 37°C. Next, optical absorbance at 450 nm was measured using a Tecan Infinite M200 Pro plate reader (Life Science).

MTT assay

Cells were treated with 20 μL MTT (0.5 mg/mL, CT01-5, Sigma-Aldrich, USA). The supernatant was discarded, and the cells were treated with DMSO (150 μL). Optical absorbance at 540 nm was determined using a Tecan Infinite M200 Pro plate reader (Life Science).

Transwell invasion assay

Cells were trypsinized, and the Matrigel with a 8 μm pore size was inserted in 24-well plates.

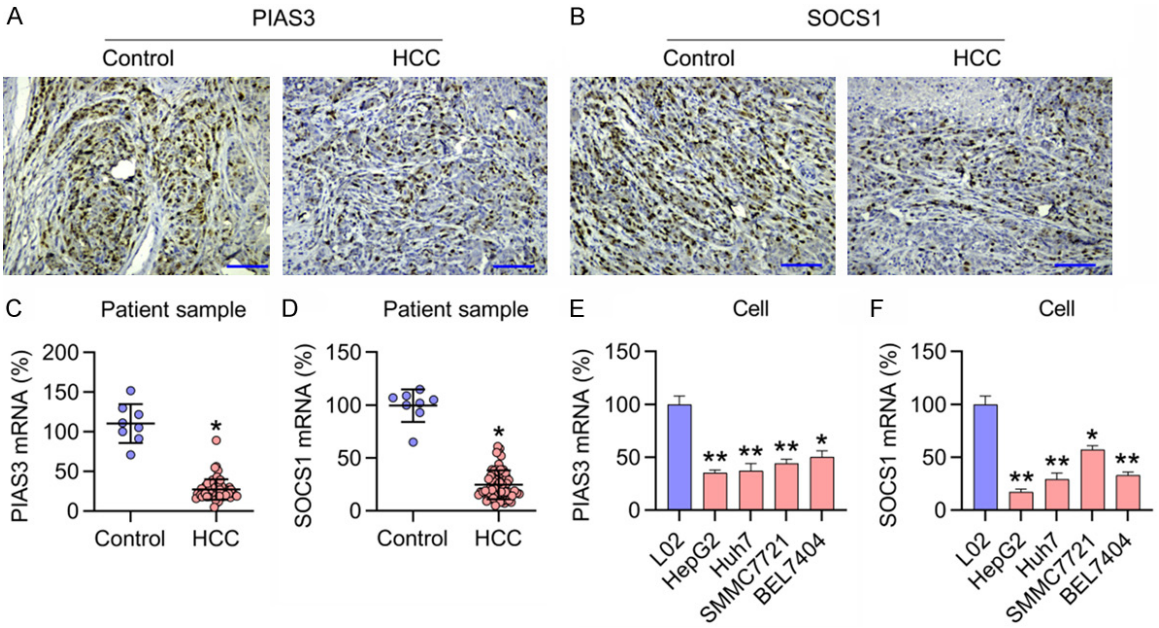

Figure 1. Expression of PIAS3 and SOCS1 in HCC tissue and cells. A. Representative micrographs of PIAS3 expression in noncancerous and HCC tissues (n=8). B. Representative micrographs of SOCS1 expression in noncancerous and HCC tissues (n=8). Scale bar, 200 μm. C, D. Expression of PIAS3 and SOCS1 in HCC specimens (n=62) and in healthy hepatic tissues (n=8). E, F. Expression levels of PIAS3 and SOCS1 in HCC cell lines (HepG2, Huh-7, SMMC7721, and BEL7404) and L02 cells (n=3). $^{*}P<0.05$, $^{**}P<0.01$.

Then, 0.4 mL F-12 hepatocyte growth factor (HGF; 0.02 μg/mL 10%) was added to the lower chamber. Next, approximately 1×10^5 cells were placed in the upper chamber. Then, cells were incubated for 20 h. Finally, the stained cells that migrated through pores were counted under a microscope.

Flow cytometry

Flow cytometry was performed using the combination of Annexin V-FITC (fluorescein isothiocyanate; Sigma, MA, USA) and propidium iodide (PI) method. First, the cell density was adjusted to about 1×10^6 cells/mL, the medium was removed, and the cells were washed twice with phosphate-buffered saline. Second, the cells were gently resuspended with 0.5 mL of pre-cooled 1× binding buffer, and 1.25 μL Annexin V-FITC and 10 μL of PI were added for incubating the cells at room temperature in the dark for 15 min. The sample was placed on ice, stored in the dark, and immediately analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA) and BD FACSDiva software.

Wound healing assay

A 10 μL pipette tip was used to scratch a wound. The cells were then placed under a microscope with a relative objective of 20×. Cell migration was evaluated as (width at 0 h-width at 36 h)/width at 0 h.

Data analysis

Data were expressed as the means ± standard deviation. Comparison among multiple groups and between two groups was evaluated using one-way analysis of variance (ANOVA) with Tukey's post hoc test and Student's *t*-test, respectively. Statistical significance was defined as *P*-value <0.05.

Results

Reduced expression of PIAS3 and SOCS1 in HCC tissues and cell lines

First, IHC staining showed that in noncancerous hepatic regions, PIAS3 and SOCS1 were positively expressed. IHC staining data also clearly indicated that PIAS3 and SOCS1 were downregulated in human HCC tissues (**Figure 1A**, **1B**). PIAS3 and SOCS1 were downregulated in HCC samples compared with paracancerous tissues (**Figure 1C**, **1D**). Furthermore, PIAS3 and SOCS1 expression was also lower in HCC cell lines (HepG2, Huh-7, SMMC7721, and

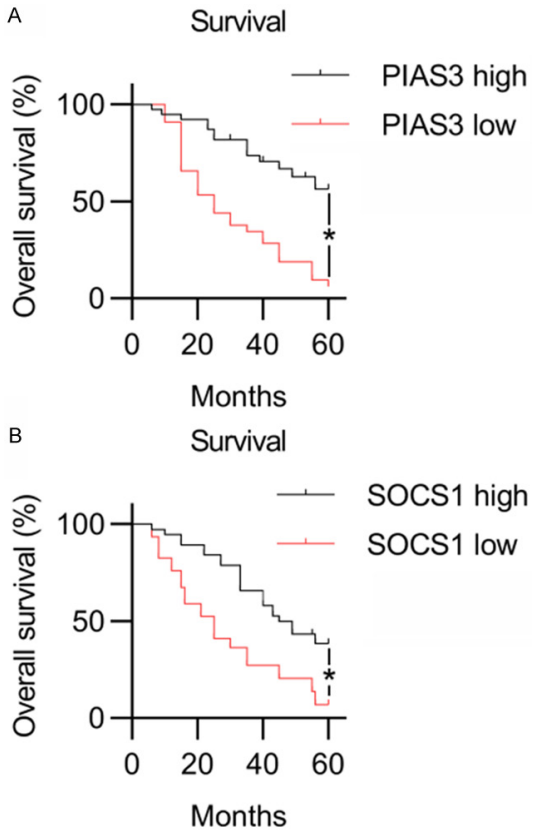

Figure 2. Kaplan-Meier curve analysis. A. PIAS3 levels and overall patient survival. B. PIAS3 levels and overall patient survival. n=62. *P<0.05.

BEL7404) than in normal hepatic cells (**Figure 1E**, **1F**).

Poor survival associated with low expression of PIAS3 and SOCS1

PIAS3 and SOCS1 expression was classified as high expression (IHC score ≥12) and low expression (IHC score <12). Through Kaplan-Meier curve analysis, we found that in HCC patients, low expression of PIAS3 and SOCS1 indicated shorter survival time than in patients with high expression (**Figure 2A**, **2B**).

Induction of oxidative stress and PIAS3 and SOCS1 expression in HepG2 cells stimulated by H_2O_2

H_2O_2 was used to induce oxidative stress in HepG2 cells, whereas TA (300 μM) was used as an antioxidant. Treatment with H_2O_2 (50 μM) for 24 h resulted in higher protein carbonyl content and lower TAC in HepG2 cells (**Figure 3A**, **3B**). The level of Nrf2, a well-recognized antioxidant protein, was reduced after H_2O_2 treatment (**Figure 3C**). However, co-treatment with TA significantly reversed the changes in protein carbonyl content, TAC, and Nrf2 protein levels, indicating that H_2O_2 successfully induced oxidative stress in HepG2 cells.

Next, we evaluated whether oxidative stress caused a change in the tumor-related processes of HCC cells. The CCK-8 and MTT assay results revealed decreased proliferation and growth of HepG2 cells after H_2O_2 induction (**Figure 3D**, **3E**). Flow cytometry data revealed that H_2O_2 treatment promoted apoptosis of HepG2 cells (**Figure 3F**). Furthermore, the wound healing and transwell experiments showed that H_2O_2 administration also increased the invasion and migration rate of HepG2 cells (**Figure 3G**, **3H**). These findings demonstrated that oxidative stress suppressed cell proliferation and growth but promoted apoptosis, invasion, and migration of HepG2 cells.

Reduction of PIAS3 and SOCS1 levels and STAT3 activation by H_2O_2 administration

Real-time PCR and WB data showed that TA treatment significantly elevated PIAS3 and SOCS1 levels in H_2O_2-treated cells at both the mRNA and protein levels (**Figure 4A-C**). The expression of downstream STAT3 in HepG2 cells did not change; however, the phosphorylation of STAT3 was further increased by H_2O_2 treatment, which could be counteracted by co-treatment with TA. Therefore, oxidative stress inhibited PIAS3 and SOCS1 expression in HCC cells, which subsequently increased the phosphorylation status of STAT3.

Effects of PIAS3 and SOCS1 on oxidative stress-induced tumor cell processes

To evaluate the influence of PIAS3 and SOCS1 expression on oxidative stress-induced tumor cell processes, these two proteins were respectively overexpressed in HepG2 cells following H_2O_2 stimulation. Real-time PCR and WB showed that either PIAS3 or SOCS1 were markedly upregulated in the cells after transfection of PIAS3 or SOCS1 overexpression vectors, respectively (**Figure 5A-C**). PIAS3 or SOCS1 overexpression contributed to the loss of STAT3 phosphorylation in H_2O_2-treated HepG2 cells (**Figure 5C**). To demonstrate whether the roles of PIAS3 and SOCS1 were STAT3-

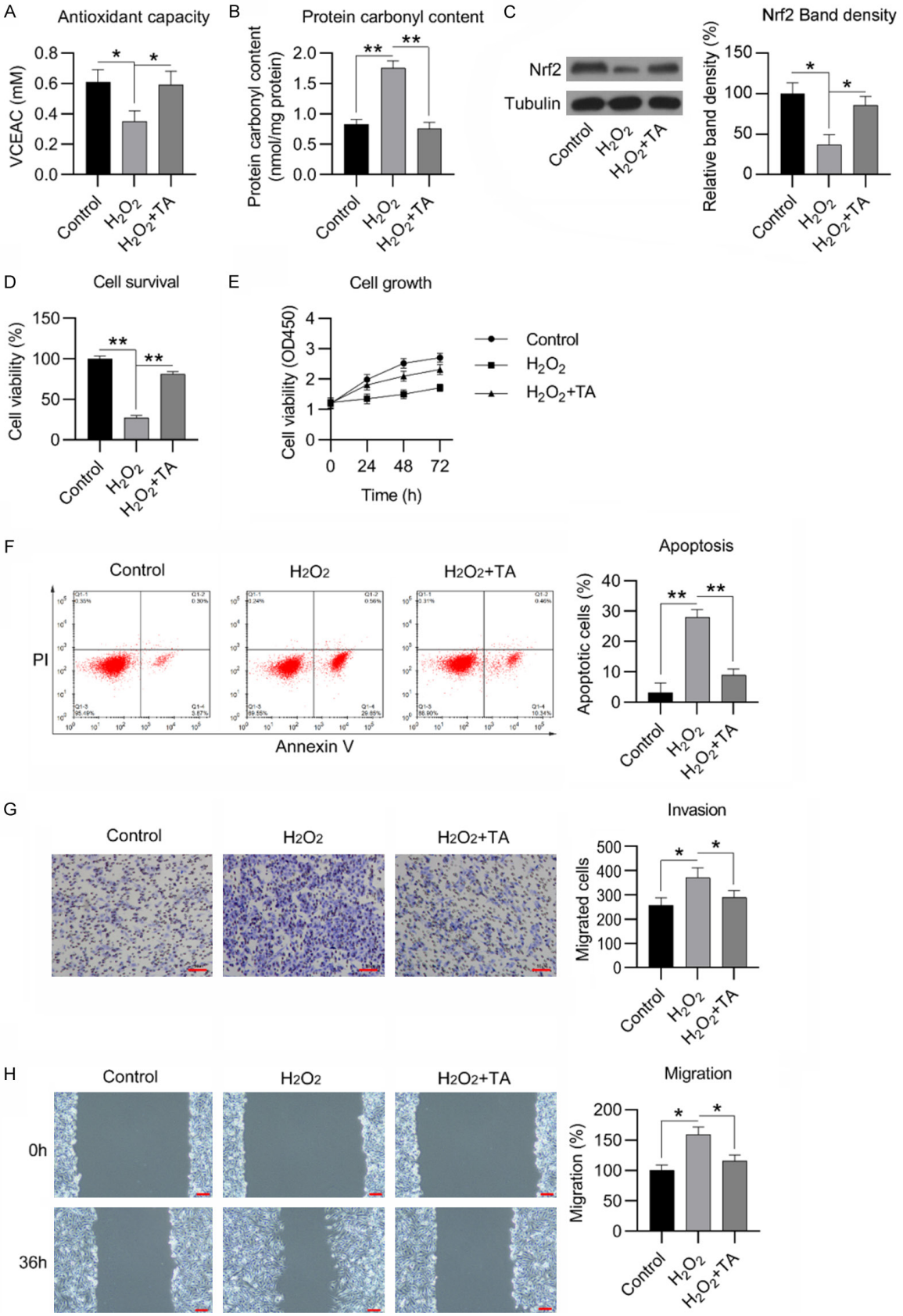

Figure 3. Effect of H_2O_2 treatment on oxidative stress induction and malignant processes in HCC cells. A. H_2O_2 (50 μM for 24 h) and/or antioxidant TA treatment. Total antioxidant capacity (TAC) in HepG2 cells was determined.

VCEAC: vitamin C equivalent antioxidant capacity (n=3). B. Protein carbonyl content in HepG2 cells was detected (n=3). C. Western blotting detected the expression of Nrf2 protein in cells (n=3). The band density for Nrf2 protein are shown (n=3). D. HepG2 cell proliferation 48 h after different treatments (n=3). E. Cell growth of HepG2 cells 24-72 h post treatment (n=3). F. Cell apoptosis in HepG2 cells. G. Invasion ability of HepG2 cells (n=3). Scale bar, 50 μm. H. Migration capacity of HepG2 cells (n=3). Scale bar, 50 μm. *P<0.05, **P<0.01.

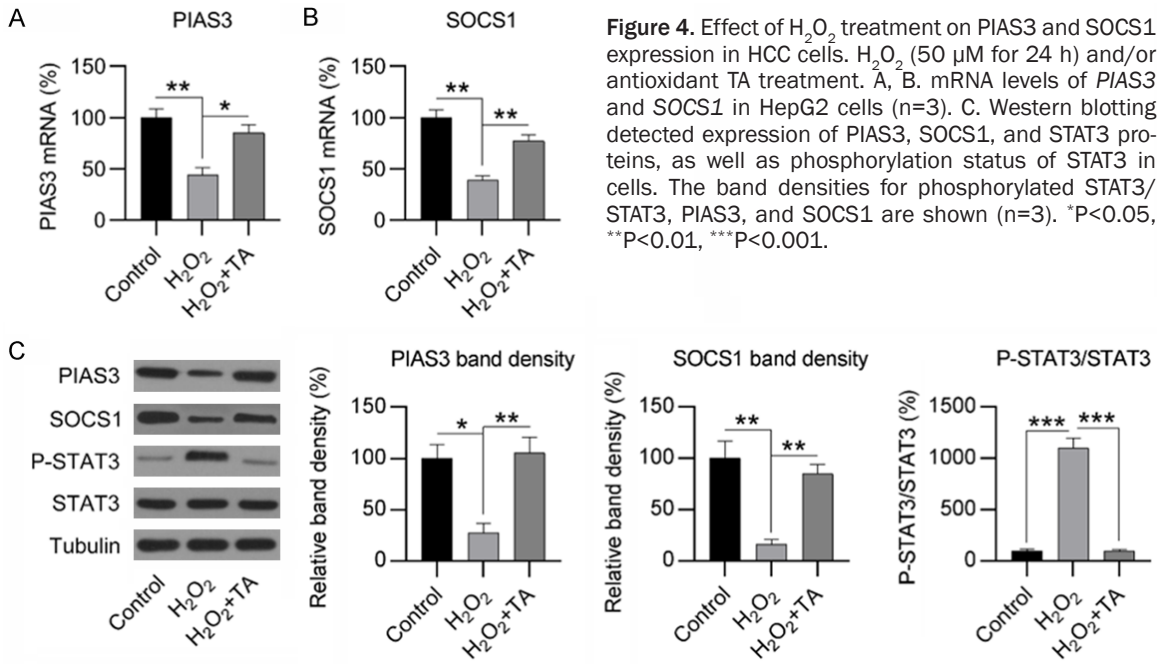

Figure 4. Effect of H_2O_2 treatment on PIAS3 and SOCS1 expression in HCC cells. H_2O_2 (50 μM for 24 h) and/or antioxidant TA treatment. A, B. mRNA levels of *PIAS3* and *SOCS1* in HepG2 cells (n=3). C. Western blotting detected expression of PIAS3, SOCS1, and STAT3 proteins, as well as phosphorylation status of STAT3 in cells. The band densities for phosphorylated STAT3/STAT3, PIAS3, and SOCS1 are shown (n=3). *P<0.05, **P<0.01, ***P<0.001.

dependent, HepG2 cells with H_2O_2 treatment and PIAS3/SOCS1 overexpression were co-administrated with colivelin (CO) to re-activate STAT3. WB data showed that CO treatment led to the upregulation of STAT3 phosphorylation (**Figure 5C**), indicating successful reactivation of STAT3 in HepG2 cells with H_2O_2 treatment and PIAS3/SOCS1 overexpression.

Next, we determined the TAC, protein carbonyl content, and Nrf2 expression, which might indicate oxidative stress in cells. The results showed that TAC and Nrf2 expression were promoted by PIAS3 and SOCS1 overexpression while protein carbonyl content was impaired. Furthermore, these alterations were abolished by co-administration of CO (**Figure 6A-C**).

Then, the effects of PIAS3 and SOCS1 overexpression and CO treatment on proliferation, apoptosis, migration, and invasion of HepG2 cells were investigated. The proliferation and growth of H_2O_2-treated HepG2 cells were increased (**Figure 6D**, **6E**), while apoptosis, migration, and invasion of H_2O_2-treated HepG2 cells decreased after PIAS3 or SOCS1 overexpression (**Figure 6F-H**). In addition, we observed that these changes in cellular processes could be counteracted by CO treatment (**Figure 6D-H**).

Discussion

HCC is a complex and heterogeneous tumor with multiple genetic aberrations. Many types of pathways participating in the modulation of proliferation, and cell death is implicated in hepatocarcinogenesis. The major etiological factors for HCC are hepatitis B virus and hepatitis C virus infection that induce continuous oxidative stress, which have recently been reported to be associated with hepatocarcinogenesis. Moreover, HCC is characterized by significant downregulation of certain oxidoreductive enzymes, including Nrf2 [6]. The present study considered the association of the PIAS3/SOCS1-STAT3 pathway and cellular responses to H_2O_2-induced oxidative stress in the human HCC cell line, HepG2. Expression of PIAS3 and SOCS1 was downregulated in HCC tissues compared to noncancerous tissues. Low expression of either PIAS3 or SOCS1 was correlated to poor survival of HCC patients. *In vitro* experi-

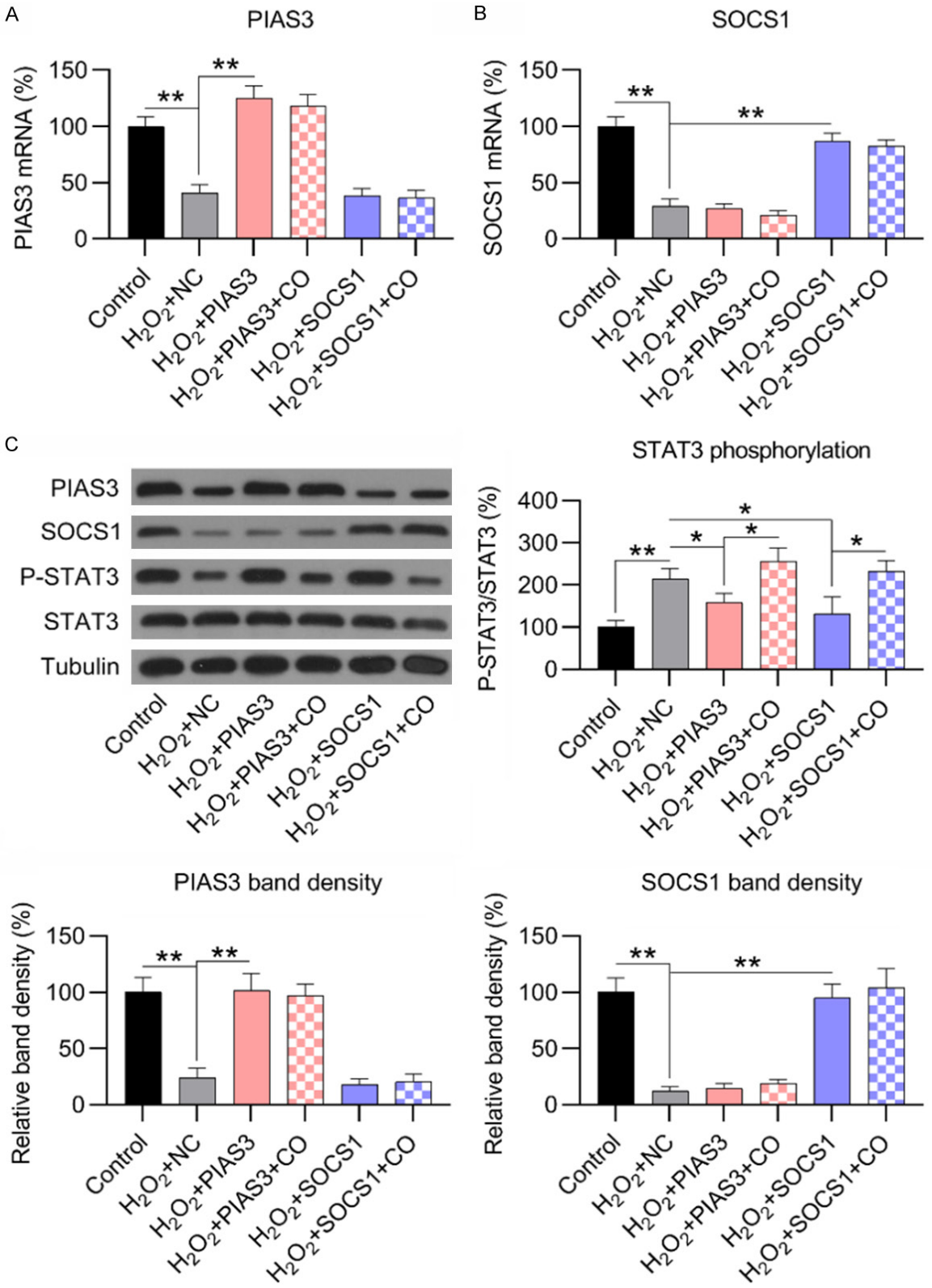

Figure 5. Overexpression of PIAS3 and SOCS1 and co-administration of colivelin in HCC cells. HepG2 cells were transfected with PIAS3 or SOCS1 overexpression vector for 36 h and then treated with H_2O_2 (50 μM) and/or 0.5 μM CO for 24 h. A, B. Real-time PCR was performed to examine mRNA levels of *PIAS3* and *SOCS1* in HepG2 cells (n=3). C. Western blotting detected the expression of PIAS3, SOCS1, and STAT3 protein and phosphorylation status of STAT3 in cells. The band density for phosphorylated STAT3/STAT3, PIAS3, and SOCS1 are shown (n=3). *P<0.05, **P<0.01.

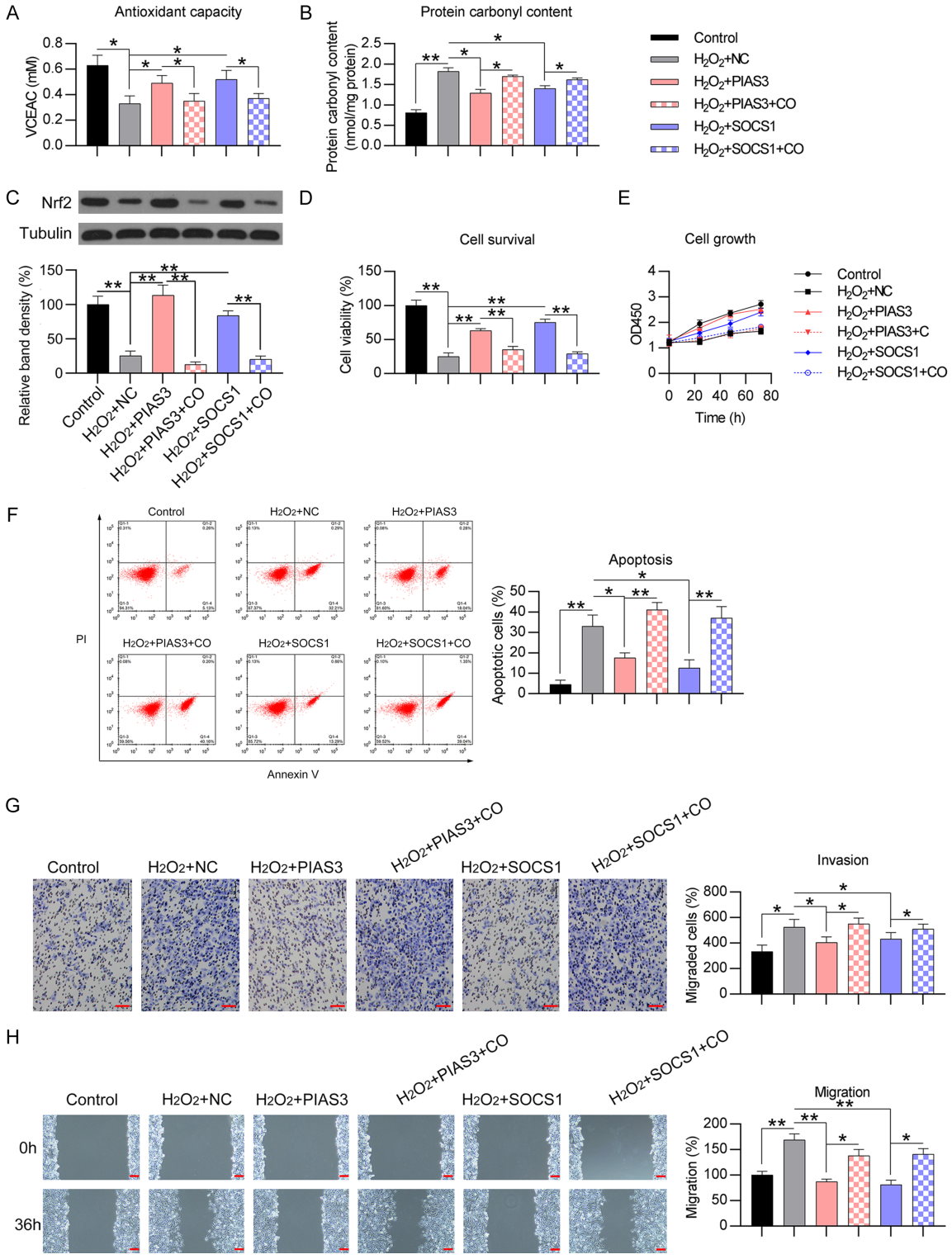

Figure 6. Effects of PIAS3 and SOCS1 overexpression and colivelin treatment on oxidative stress induction and malignant processes of HCC cells. HepG2 cells were transfected with PIAS3 or SOCS1 overexpression vector for 36 h and then treated with H_2O_2 (50 μM) and/or 0.5 μM CO for 24 h. A. Total antioxidant capacity (TAC) in HepG2 cells was determined. VCEAC: vitamin C equivalent antioxidant capacity (n=3). B. Protein carbonyl content in HepG2 cells was detected (n=3). C. Western blotting detected the expression of Nrf2 protein in cells (n=3). Quantification data from three independent repeats were showed below the blot. D. HepG2 cell proliferation 48 h after different treatments (n=3). E. MTT assays detected cell growth of HepG2 cells 24-72 h post treatment (n=3). F. Cell apoptosis of HepG2 cells. G. Invasion ability of HepG2 cells (n=3). Scale bar, 50 μm. H. Migration capacity of HepG2 cells (n=3). Scale bar, 50 μm. *P<0.05, **P<0.01.

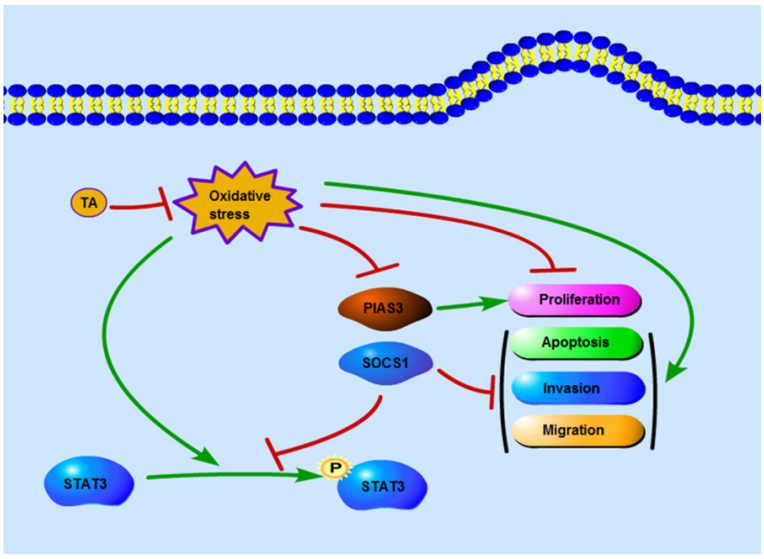

Figure 7. Schematic diagram of this study. Oxidative stress in HepG2 cells contributed to a significant decrease in PIAS3 and SOCS1 abundance, as well as phosphorylation of STAT3 in HCC cells. Oxidative stress represses cell proliferation and promotes apoptotic death, migration, and invasion, which can be counteracted by overexpression of PIAS3 and SOCS1.

ments using HepG2 cells showed that oxidative stress could be triggered by H_2O_2 incubation. H_2O_2 contributed to a significant decrease in PIAS3 and SOCS1 expression and further activation of STAT3 in HepG2 cells. Several key tumor-related processes, including cell proliferation, growth, apoptotic death, migration, and invasion, were associated with oxidative stress in HCC cells (**Figure 7**). However, these changes in cellular processes were counteracted by overexpression of PIAS3 or SOCS1, while the effects of PIAS3 or SOCS1 were subsequently partially abolished by STAT3 reactivation by CO administration. Our findings provide evidence that an oxidative microenvironment can be induced by H_2O_2 in HCC tumors, and it regulates tumor progression. The PIAS3/SOCS1-STAT3 signaling axis is involved in this H_2O_2-regulated HCC development.

The IHC and real-time PCR data revealed the downregulation of PIAS3 and SOCS1 expression in HCC tissue and cell lines compared with noncancerous tissue and normal hepatic cells. To the best of our knowledge, this is the first report showing low expression of PIAS3 in HCC tissues. Dysregulation of PIAS3 expression has been found in various types of human cancers, including cervical [21], prostate [22, 23], osteosarcoma [24], and lung cancer [26]. For SOCS1, our data agreed well with a previous study showing that SOCS1 expression is reduced in HCC tissue [39]. The inhibitory effect of PIAS3 and SOCS1 on STAT3 and continent activation of STAT3 in HCC [40] indicates that low PIAS3 and SOCS1 expression are required for HCC to maintain activation of STAT3 signaling.

In response to oxidative stress induced by H_2O_2 stimulation, TAC and Nrf2 expression in HepG2 cells decreased. A previous study reported that the Keap1-Nrf2 pathway promotes tumor development [41]. Upregulation and activation of Nrf2 are required to maintain the redox homeostasis in the cells to limit oxidative damage. An elevation of oxidative DNA lesion (8-OHdG) could be found in the HCC tissues, suggesting oxidative injury in the HCC tumor [42]. This raises the question of why Nrf2 was not upregulated to counterbalance the oxidative injury in HCC cells. Recent studies have demonstrated a correlation between Nrf2 and STAT3 activation in different cells and conditions [43-45]. This study found an inverse correlation between Nrf2 expression and STAT3 phosphorylation in H_2O_2-induced HepG2 cells. STAT3 deactivation, caused by either PIAS3 or SOCS1, resulted in Nrf2 upregulation, while reactivation by CO administration reduced cellular Nrf2 levels in HepG2 cells. These data suggest that oxidative stress causes further phosphorylation of STAT3 to evade the antioxidant activity of Nrf2.

A previous study has shown that Ascochlorin, an isoprenoid antibiotic, can induce PIAS3 to inhibit STAT3 activation in HCC cells and thereby suppress growth and invasion of HCC cells [46]. Negative regulators of the STAT3 signaling cascade, such as PIAS3, can effectively block the DNA binding activity of STAT3 [47]. Moreover, the lack of PIAS3 may activate STAT3 protein in various cancers, and PIAS3 is also found to be inactive in lymphoma [48] and gastric carcinoma [49]. Notably, it has also been reported that anticancer agents such as curcumin and

8-hydrocalamenene can also exhibit tumor inhibition through enhancing PIAS3 expression in tumor cells [50, 51]. Downregulation of SOCS1, a tumor suppressor, can affect human cancer progression [52]. SOCS1 acts as an inhibitor of STAT1 and STAT3 activity [53, 54]. The apoptosis-inhibitory function of SOCS1 in HCC and hematopoietic malignancies has also been described [32]. A previous study found that the antioxidant properties of SOCS1-mediated JAK/STAT inhibition through a coordinated cytoprotective response, restore the redox balance in diabetic vascular complications [55, 56]. Here, we found that H_2O_2 decreased the expression of PIAS3 and SOCS1 at the protein and mRNA levels, which correlated with its ability to negatively regulate STAT3 phosphorylation. Transfection with PIAS3 or SOCS1 overexpression vector reversed the STAT3 activation induced by H_2O_2. Meanwhile, increased oxidative stress was accompanied by regulation of tumor-associated processes: reduced proliferation and antioxidant capacity and increased apoptosis, migration, and invasion. Moreover, overexpression of PIAS3 or SOCS1 restored cell proliferation and antioxidant capacity but abolished the augmented migration and invasion of H_2O_2-treated HepG2 cells. These data demonstrated that targeted induction of PIAS3 and SOCS1 protein could form an important strategy to reduce dysregulated STAT3 activation in tumor cells. Oxidative stress damaged the cells, caused inhibition of proliferation, and increased apoptosis, but why were invasion and migration of cells enhanced? Such a bidirectional role of oxidative stress on proliferation and metastasis is counterintuitive. Actually, oxidative stress-promoted metastasis, migration, and invasion have been substantially documented in many previous reports [57-61]. Cancer cells depend on an increased antioxidant capacity, which keeps ROS levels higher than in normal cells, but below a critical threshold able to maintain their viability. It has been observed that the same stimuli that promote oxidative stress, such as detachment from the cell matrix, also increase the selective pressure on cells to adapt by building up a powerful antioxidant response [62]. Selection of such a phenotype associated with mitochondrial superoxide production directly promotes cell migration, invasion, and metastasis. One possible reason for this notion is that oxidative stress increases mutation rate and accelerates tumor progression: ROS cause strand breaks, alterations in guanine and thymine bases, and sister chromatid exchanges [63]. This may inactivate additional tumor suppressor genes within tumor cells, or further increase expression of proto-oncogenes. Genetic instability due to persistent carcinoma cell oxidative stress will therefore increase the malignant potential of the tumor [64]. Second, production of oxidative stress may link to activation of key signal pathways to promote cell migration and invasion. The p38 MAPK is activated by oxidative stress [65], and the phosphorylation of heat shock protein-27 (HSP27) by p38 MAPK has been shown to induce changes in actin dynamics [66, 67]. Phosphorylated HSP27 promotes the migration of breast cancer cells in vitro [68, 69]. Another signal Rac1 can activate the NADPH-oxidase in tumor cells, causing superoxide production. ROS have been shown to mediate the role of Rac1 in actin cytoskeleton reorganization [70, 71]. High levels of MMP-2 correlate with poor prognosis in cancer patients, and active MMP-2 is detected more frequently in malignancy [72-74]. Oxidative stress has been shown to activate MMP-2, possibly by the reaction of oxygen radicals with thiol groups within MMP-2 [75, 76]. Finally, ROS within the tumor microenvironment may promote metastasis by increasing vascular permeability [58].

In conclusion, we reported reduced expression of both *PIAS3* and *SOCS1* in human HCC tissues. Low expression of these two genes may indicate shorter survival time in HCC patients. Our data suggest that approaches targeting the PIAS3/SOCS1-STAT3 axis to minimize oxidative stress in the tumor microenvironment may clinically benefit HCC treatment. However, one of the limitations of this study is the lack of animal experiments to confirm our *in vitro* findings. To further elucidate the role of PIAS3 and SOCS1 on HCC development, HCC xenograft experiments based on mice with knockdown or overexpression of PIAS3 and SOCS1 would need to be carried out.

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Disclosure of conflict of interest

None.

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