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Down-regulation of iASPP in human hepatocellular carcinoma cells inhibits cell proliferation and tumor growth

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The oncoprotein inhibitory member of the ASPP family (iASPP) is a key inhibitor of the p53 tumor suppressor and is upregulated in patients with acute leukemia and breast carcinoma. To investigate the effect of iASPP inhibition on the proliferation of hepatocellular carcinoma cells, a recombinant lentivirus vector expressing a small interfering RNA (siRNA) against iASPP gene expression was constructed and used to infect human hepatocellular carcinoma cells (HepG2 and Hep3B). The results showed that iASPP mRNA and protein levels were significantly down-regulated in both cells infected with the siRNA against iASPP. siRNA-mediated down-regulation of iASPP repressed tumor cell proliferation and colony formation *in vitro* and induced a growth delay of the tumor *in vivo*, suggesting that iASPP plays an important role in the proliferation of hepatocellular carcinoma cells. iASPP may be a valuable candidate target gene in hepatocellular carcinoma therapy.

Key words: iASPP; hepatocellular carcinoma; proliferation

Hepatocellular carcinoma is one of the most common cancers in the world. As with most malignant solid tumors, hepatocarcinogenesis is considered to be a multi-step process involving uncontrolled cellular proliferation, detachment from the extracellular matrix and invasion into the surrounding tissue, along with modulation of both the immune system and the blood supply to promote tumor growth [1-3]. The mechanisms of hepatocellular carcinoma cell proliferation and apoptosis remain unknown, and the understanding of these mechanisms is important for disease treatment.

RNA interference (RNAi) involves sequence-specific post-transcriptional gene silencing by short small interfering RNA (siRNA), which is thought to be a powerful approach for studying gene function and gene therapy. There is no doubt that RNAi, as a tool for gene function analysis in mammals, has been widely promoted as a possible gene-targeted therapeutic strategy of exquisite specificity [4].

iASPP belongs to the ASPP family consisting of three proteins (ASPP1, ASPP2, and iASPP), which are unified by their protein structure. Each of the three proteins contains four ankyrin repeats, an SH3 domain, and a proline-rich region at the C terminus [5, 6]. The ASPP family of proteins specifically modulates the p53-dependent apoptotic response. ASPP1 and ASPP2 bind to and cooperate with p53 to induce apoptosis. In contrast, iASPP prevents p53 from triggering the cell death pathway [7-9]. Analysis in human tumor samples indicated that iASPP might play an important role in tumorigenesis. For example, the expression of iASPP is frequently up-regulated in human breast carcinomas and in patients with acute leukemia [10-15].

In the present study, HepG2 (wild-type p53) and Hep3B cells (p53 null) were selected as cell models to explore the potential role of iASPP in hepatocellular carcinoma, in which high expression levels of iASPP were observed (data not shown). The *in vitro* proliferation and colony-formation abilities of HepG2 and Hep3B cells infected with iASPP siRNA and the tumor growth in an *in vivo* nude mice model injected with Hep3B iASPP knockdown cells were investigated.

Materials and methods

Cell culture. The human hepatocellular carcinoma cell lines HepG2 and Hep3B were purchased from American Type Culture Collection (ATCC). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Construction and transfection of iASPP siRNA. iASPP siRNA (sequence: CACATGGATCTGAAGCAGA) and non-

sense siRNA (sequence: TTCTCCGAACGTGTCACGT) were inserted into the pGCSIL-GFP lentivirus RNAi expression system (Shanghai GeneChem Co., Ltd, Shanghai, China). The recombinant lentiviral siRNA expression vector targeting iASPP or the nonsense siRNA expression vector was transfected into HEK293T cells using the lentiviral helper plasmids pHelper1.0 and pHelper2.0 and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to generate the lentivirus. Viral stocks were made and used to infect HepG2 and Hep3B cells at an MOI of 10. At 72 h after infection, the cells were collected for measuring iASPP mRNA and protein expression levels.

Real-time quantitative PCR. Total RNA was extracted and reverse transcribed using M-MLV-RTase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed in triplicate using the SYBR Green Master PCR Mix (Applied Biosystem, Foster City, CA, USA). The primer sequences for the genes were as follows: iASPP forward primer: 5'-GGCGGTGAAGGAGATGAAC-3'; iASPP reverse primer: 5'-TGATGAGGAAATCCACGATAGAGA-3'. For the internal standard β-actin, the forward primer was 5'-GGCACCCAGCACAATGAAG-3', and the reverse primer was 5'-GGCCGGACTCGTCATACTC-3'. The expression of mRNA was assessed by evaluating the threshold cycle (C_T) values. The C_T values were normalized with the expression level of β-actin.

Western blot analysis. Cells were harvested 72 h after transfection and washed twice in PBS. Cell pellets were lysed in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 600 mM NP-40, 5 mM EDTA and 2% Triton X-100). The lysates were cleared by centrifugation and boiled with SDS sample buffer (240 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 20% mercaptoethanol and 0.4% bromophenol blue). The samples were subjected to SDS-PAGE, transferred onto PVDF membranes (Millipore, Bedford, MA, USA) and detected with a mouse anti-iASPP antibody (Abcam, Cambridge, UK; Cat. No. Ab49805; dilution 1/1000) followed by a horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz, CA, USA; Cat. No. sc-2005; dilution 1/5000). GAPDH was used as a loading control, and a mouse anti-GAPDH antibody was used (Santa Cruz, CA, USA; Cat. No. sc-32233; dilution 1/5000). The blotting signals were detected using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA).

MTT assay. Cells (2×10^3 cells/well) were plated on 96-well cell culture plates and cultured at 37°C in a 5% CO_2 atmosphere. MTT reagents (Shanghai Dingguo Biotechnology Co., Ltd., Shanghai, China) were added (10 μl/well) at different time points and incubated at 37°C for 4 h. DMSO (100 μl/well) was added to stop the reaction, and the optical density at 570 nm was determined using a multi-well plate reader.

BrdU assay. Cells were seeded on 96-well cell culture plates (1500 cells/well). At different time points, BrdU reagents (Chemicon, Temecula, CA, USA; Cat. No. 2750) were added (20 μl/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The cells were then fixed and washed three times with washing buffer. An anti-BrdU antibody was added (50 μl/well), and the cells were incubated at 37°C for 1 h. The cells were then washed three times, a second antibody was then added (50 μl/well), and the cells were incubated at 37°C for 30 min. Fifty microliters of tetramethylbenzidine (TMB) substrate was then added and incubated in the dark for 30 min. The optical density at 490 nm was determined using a multi-well plate reader.

Colony formation assay. Cells were seeded on 6-well cell culture plates (200 cells/well) in duplicate wells and cultured at 37°C in a 5% CO_2 atmosphere. After 2 weeks of incubation, the cells were fixed with paraformaldehyde for 30 min and then stained with Giemsa for 10 min. The cells were washed three times using ddH_2O to obtain a clean background, and then the number of colonies were counted and statistically analyzed.

***In vivo* model of tumor growth.** BALB/c nude mice (male, 4-6 weeks old, 20 ± 2 g, purchased from Shanghai SLAC Laboratory Animal Co., Ltd) were used for the *in vivo* tumor growth model. Animal experimentation was approved by the Animal Care and Ethics Committee of the Third Affiliated Hospital of Sun Yet-Sen University. The mice were divided into three groups, with six mice in each group. Hep3B cells (CON), Hep3B cells treated with nonsense siRNA (NC), and Hep3B cells treated with iASPP siRNA (KD) were resuspended at 2×10^7 cells/ml, and an aliquot of 0.25 ml cell suspension was injected subcutaneously into the athymic nude mice. Tumors were measured on the 10^{th}, 14^{th}, 18^{th}, and 22^{nd} days using calipers, and the volumes were calculated according to the equation $V = [L \times W^2] \times 0.52$ (V = volume, L = length, and W = width). The mice were sacrificed after 22 days, and the tumor weight was determined at this time.

Statistical analysis. Each experiment was conducted at least three times with consistent results. All statistical analyses were done using the Statistical Package for Social Science (SPSS) version 11.5 (SPSS Institute, Chicago, IL, USA). Data were expressed as the means ± SD. Statistical significance was determined by Student's *t*-test and one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered indicative of significance.

Results

Reduction of iASPP mRNA and protein expression by siRNA in hepatocellular carcinoma cells. A recombinant lentivirus expressing GFP and siRNA against iASPP or a nonsense control was infected into HepG2 and Hep3B cells. After a 72-h infection, noticeable green fluorescence was observed (Fig. 1 A). As expected, the cells transfected with iASPP siRNA resulted in a lower expression of iASPP mRNA and protein than those with the empty vector or the parental cells (Fig. 1 B and C).

iASPP down-regulation inhibits the proliferation of hepatocellular carcinoma cells. An MTT assay was performed to investigate whether the reduction of iASPP had an effect on cell growth and proliferation. As shown in Fig. 2 A and B, a striking variation in the growth pattern was observed

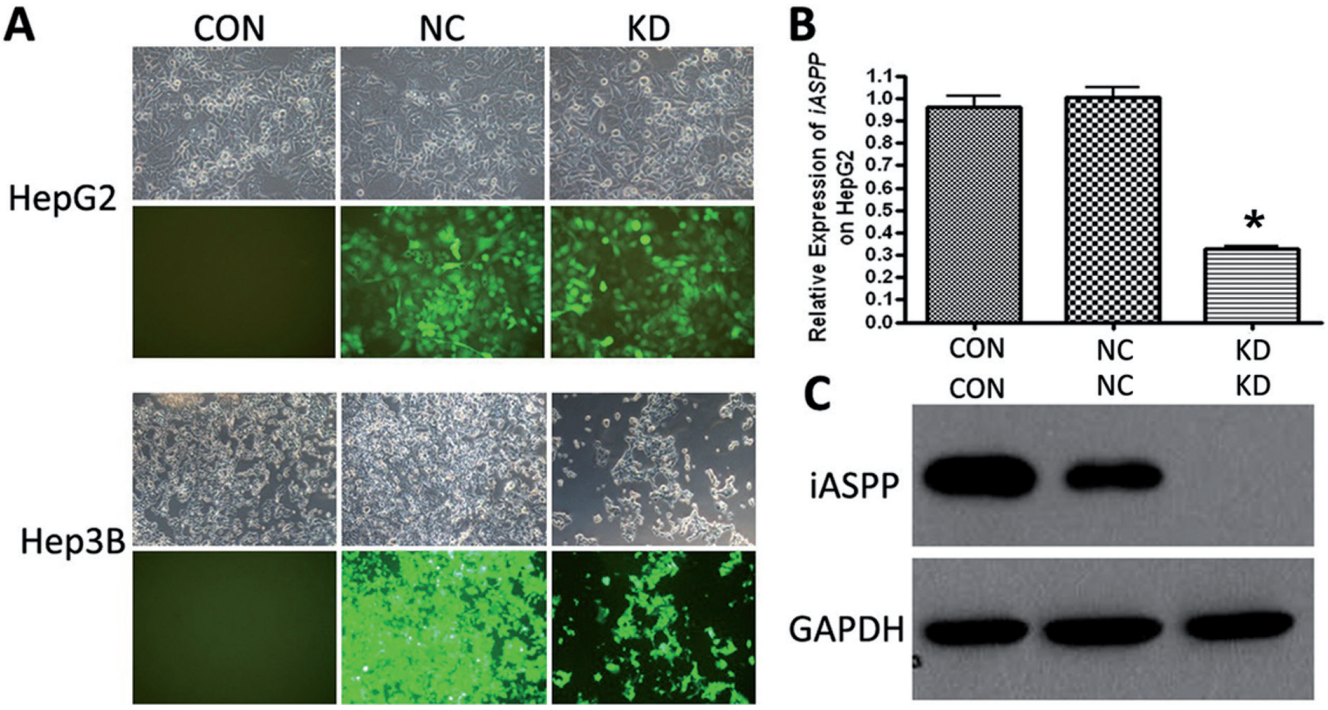

Figure 1. Lentivirus-mediated iASPP RNAi led to the down-regulation of iASPP in hepatocellular carcinoma cells. (A) HepG2 and Hep3B cells were infected with a lentivirus expressing both GFP and an siRNA targeting iASPP (KD) or a nonsense siRNA (NC). Cells not infected served as a blank control (CON). (B) The mRNA level of iASPP was down-regulated by iASPP siRNA in HepG2 cells. * $p < 0.05$ compared to the NC group. (C) The protein level of iASPP was down-regulated by iASPP siRNA in HepG2 cells. Indicated values are the means ± SD of three experiments.

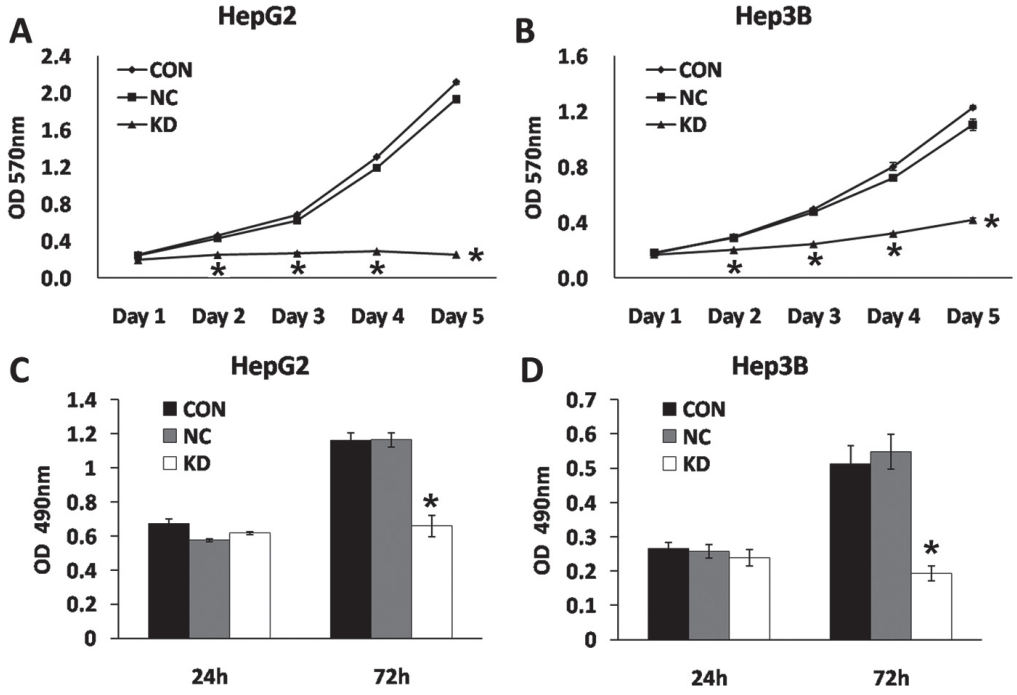

Figure 2. The proliferation in hepatocellular carcinoma cells was inhibited after iASPP siRNA treatment. Using an MTT assay, the proliferation in HepG2 cells (A) and Hep3B cells (B) was inhibited when the cells were treated with iASPP siRNA. A BrdU assay indicated that the proliferation of HepG2 cells (C) and Hep3B cells (D) was inhibited by iASPP siRNA. Indicated values are the means ± SD of three experiments. CON: blank control; NC: nonsense siRNA; KD: iASPP siRNA. * $p < 0.05$ compared to the NC group.

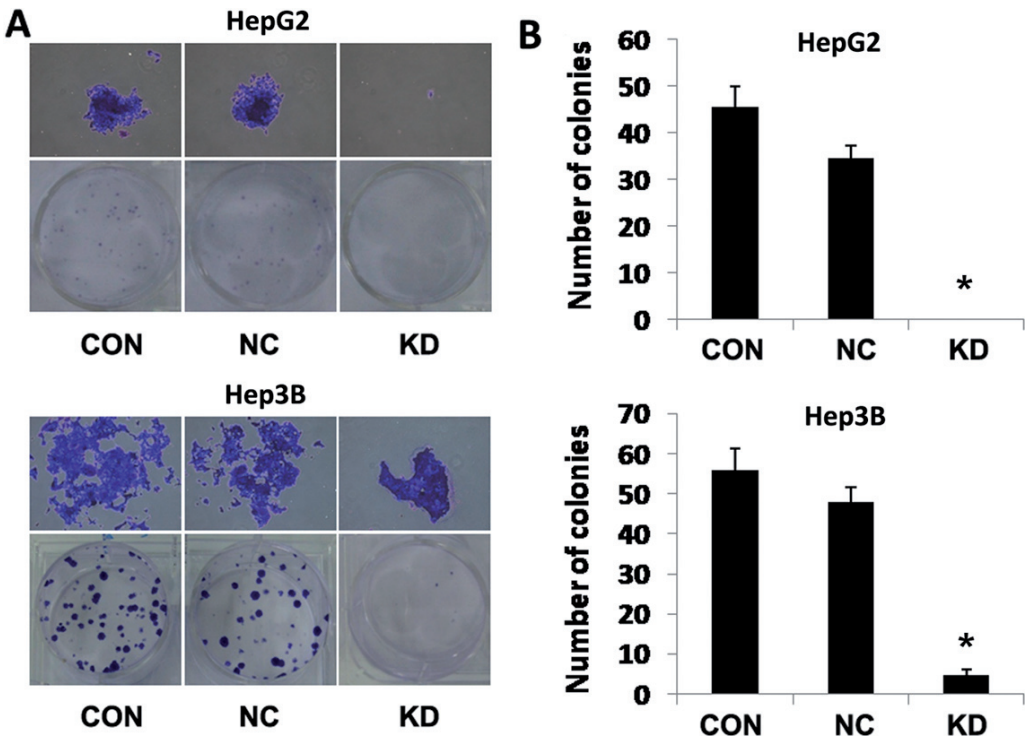

Figure 3. Colony formation of hepatocellular carcinoma cells was inhibited after iASPP siRNA treatment. (A) Representation of the 6-well colony formation assay is shown. Upper panel: Giemsa-stained light microscopy photographs showing cell numbers per colony; lower panel: photographs of plates showing the colony formation by HepG2 and Hep3B cells. (B) Decreased colony numbers were observed in HepG2 and Hep3B cells treated with iASPP siRNA, indicating that both cells had an impaired proliferative capacity. Indicated values are the means ± SD of three experiments. CON: blank control; NC: nonsense siRNA; KD: iASPP siRNA. * $p < 0.05$ compared to the NC group.

among transfectants from the second day to the fifth day in both HepG2 and Hep3B cells. The iASPP siRNA transfected cells showed a lower growth rate compared to the controls. To confirm this result, we then conducted another proliferation assay, the BrdU assay. Consistently, both HepG2 and Hep3B cells showed lower proliferation activation after the cells were treated with iASPP siRNA compared to the blank control or the nonsense siRNA at 72 h (Fig. 2 C and D).

iASPP down-regulation inhibits the colony formation of hepatocellular carcinoma cells. To further investigate tumor repression by downregulation of iASPP, we examined the colony formation of the lentivirus-infected cells. Compared with the controls, both HepG2 and Hep3B cells infected with iASPP siRNA formed substantially fewer colonies (Fig. 3).

iASPP down-regulation inhibits tumor growth ***in vivo.*** To show the effectiveness of the iASPP knockdown for the treatment of hepatocellular carcinoma, nude mice were subcutaneously injected with the established cell line Hep3B. When inoculated subcutaneously into athymic nude mice, down-regulation of iASPP by treatment of Hep3B cells with iASPP siRNA (KD) dramatically reduced tumor volumes and tumor weights compared with the Hep3B blank control cells (CON) and Hep3B cells treated with nonsense siRNA (NC) (Fig. 4). These *in vivo* results further demonstrate that down-regulation of iASPP potently inhibits tumor growth of hepatocellular carcinoma.

Discussion

The ASPP (apoptosis-stimulating protein of p53) family is the most effective p53 regulatory protein family found to date [16-18]. iASPP is the only inhibitory member of the ASPP family, which includes three p53-regulating proteins, ASPP1, ASPP2, and iASPP. p53 is one of the best known tumor suppressors to play a critical role in the regulation of cell proliferation through induction of growth arrest or apoptosis. It lies at the center of a large and intricate network of regulatory pathways. Damage to any of these regulatory mechanisms can potentially result in the improper function of p53 [19]. Human tumors can be classified into two groups: one is cancer caused by a p53 gene mutation, leading to a loss of the ability to induce apoptosis; the other group is tumors caused by the existence of p53 inhibitors in cells, which cause wild-type p53 to fail to play its normal cancer-suppressing role. iASPP was recently discovered to be an important inhibitor of p53. Some recent reports have indicated that iASPP is overexpressed in breast cancers and certain leukemias, and downregulation of iASPP could inhibit the proliferation of these cancer cells [10-15].

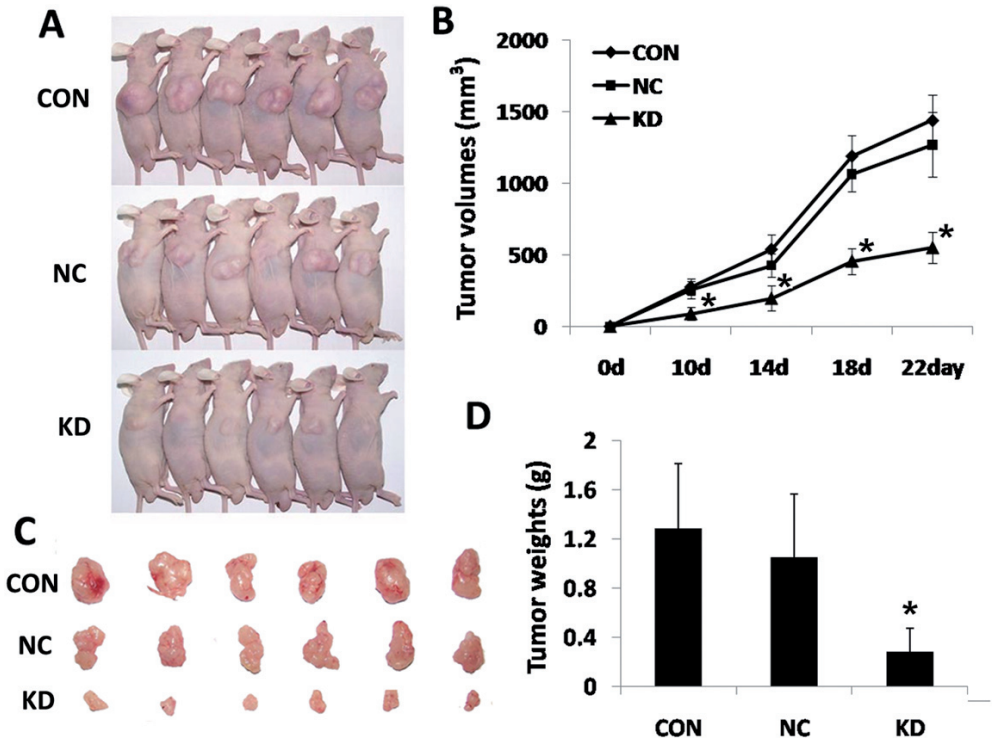

Figure 4. Tumor growth was inhibited after iASPP siRNA treatment. (A) Representative images of nude mice inoculated with Hep3B cells (CON), Hep3B cells treated with nonsense siRNA (NC), and Hep3B cells treated with iASPP siRNA (KD) on the 22nd day. (B) Tumor volumes were determined by external measurements and calculated. * $p < 0.05$ compared to the NC group. (C) On day 22 after inoculation, tumors were taken from mice that had been sacrificed moments before dissection. (D) Average tumor weights were determined. * $p < 0.05$ compared to the NC group. Indicated values are the means ± SD of six experiments.

This result may be due to the ability of p53 to resume apoptosis by reducing the inhibition of endogenous wild-type p53 by iASPP. It was suggested that inhibition of the overexpression of iASPP may be a new strategy to rescue the cancer-suppressive function of p53.

However, iASPP has also been reported to be a regulator of the NF-κB pathway. iASPP can bind to RELA/p65, which is a key mediator of genes involved in the control of the cellular proliferation and apoptosis, and inhibit its transcriptional activity [20]. Thus, the p53 and NF-κB pathways are two important pathways regulated by iASPP.

In the present study, two hepatocellular carcinoma cell lines, HepG2 (wild-type p53) and Hep3B cells (p53 null), were selected to investigate the effect of iASPP depletion on cell proliferation by downregulating the expression level of iASPP with siRNA. The cell proliferation and colony formation analyses were performed in cells with and without iASPP siRNA. It was shown that the proliferation and colony-formation abilities were effectively reduced in both HepG2 and Hep3B cells. Additionally, iASPP siRNA inhibited tumor growth *in vivo*. Our data showed that inhibition of iASPP hindered the cancer cell proliferation rate of both cell lines. Inhibition of growth and proliferation potency is an important strategy for tumor therapy. It was suggested that iASPP could regulate the biological function of hepatocellular carcinoma cells and that targeted inhibition of iASPP could be a promising strategy for the therapy of hepatocellular carcinoma. Here the proliferation of Hep3B cells (p53 null) decreased when treated with iASPP siRNA, which indicates that the effects shown were not p53-dependent. Thus, it seems that the effect of iASPP inhibition on proliferation was not relevant to the status of p53 in hepatocellular carcinoma. However, further investigation will be required to dissect the role of iASPP in the inhibition of hepatocellular carcinoma cell growth.

In conclusion, the proliferation and colony-formation abilities of both HepG2 and Hep3B cells were inhibited *in vitro* when the cells were infected with a lentivirus expressing an siRNA against iASPP. Tumor growth was also inhibited in an *in vivo* nude mouse model by incubating Hep3B cells with iASPP siRNA. These results strongly suggest that iASPP plays an important role in hepatocellular carcinoma cell proliferation, and it may be a potential target for anti-hepatocellular carcinoma therapy. However, further study is needed to obtain insight into the molecular mechanisms of induced anti-proliferative effects.

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