Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth.

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Abstract

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a poor prognosis. Treatment of HCC is complicated by the fact that the disease is often diagnosed at an advanced stage when it is no longer amenable to curative surgery, and current systemic chemotherapeutics are mostly inefficacious. Sirtuin 1 (SIRT1) is a class III histone deacetylase that is implicated in gene regulations and stress resistance. In this study, we found that SIRT1 is essential for the tumorigenesis of HCC. We showed that while SIRT1 was expressed at very low levels in normal livers, it was over-expressed in HCC cell lines and in a subset of HCC. Tissue microarray analysis of HCC and adjacent non-tumoral liver tissues revealed a positive correlation between the expression levels of SIRT1 and advancement in tumor grades. Down-regulation of SIRT1 consistently suppressed the proliferation of HCC cells via the induction of cellular senescence or apoptosis. SIRT1-silencing also caused telomere dysfunction-induced foci and nuclear abnormality that were clearly associated with reduced expressions of telomerase reverse transcriptase (TERT), and PTOP, which is a member of the shelterin complex. Ectopic expression of either TERT or PTOP in SIRT1-depleted cells significantly restored cell proliferation. There was also a positive correlation between the level of induction of SIRT1 and PTOP in human HCC. Finally, SIRT1-silencing sensitized HCC cells to doxorubicin treatment. Together, our findings reveal a novel function for SIRT1 in telomere maintenance of HCC, and they rationalize the clinical exploration of SIRT1 inhibitors for HCC therapy.

Precis

Findings offer a preclinical proof of concept for the clinical exploration of SIRT1 inhibitors for liver cancer treatment.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide (1). Epidemiological evidence suggests a number of environmental factors associated with the development of HCC, including viral hepatitis type B (HBV) and C (HCV) infections, dietary aflatoxin, the male gender, and chronic liver disease (1, 2); however, the molecular mechanisms of HCC pathogenesis remains elusive. Although the cancer can be eradicated by curative surgery, most HCC patients are diagnosed at advanced stages when it is no longer amenable to curative therapies. The prognosis is very poor for patients who have unresectable tumors, with the median survival of around 6 months (3). HCC is highly refractory to chemotherapy. Even with the most effective forms of therapy, such as treatment with the anthracycline-based drug, doxorubicin, the response rate has been low (<20%) (4). Therefore there is an urgent need to understand HCC carcinogenesis at the molecular level, and to identify novel molecular targets for the development of more efficacious therapeutics.

Sirtuins are mammalian homologs of the yeast silent information regulator 2 (SIR2), which are histone deacetylases (HDAC) that utilizes NAD^+ as a cofactor for their functions (5). The yeast SIR2 plays a critical role in the extension of life span by repressing genome instability through establishing the transcriptional silencing of the mating-type loci, the ribosomal DNA locus, and the telomeres (6). In mammals, SIR2 is represented by seven homologs (SIRT1-7), of which SIRT1 is considered to be the human orthologue of SIR2 (7). SIRT1 is a key regulator of energy metabolism by regulating fuel utilization according to the energy status of the organism (8). At the cellular level, SIRT1 deacetylates both histones and non-histone targets including p53, FOXOs, E2F1, and Ku70 (9), and participates in stress response, DNA repair, and apoptosis (7, 10). The deacetylated form of these proteins enhances cell survival by directing the cell to growth arrest for undergoing DNA repair over apoptosis in response to DNA damage. In addition, recent studies showed that SIRT1 interacts with telomeric repeats (11), and plays a role in telomeric maintenance (11, 12) and genomic stability (13). Overexpression of SIRT1 attenuates telomeric shortening associated with aging and augments homologous recombination(11).

The clinical relevance of SIRT1 expression in HCC has not been examined previously. In this study, we found that SIRT1 is over-expressed in a subset of HCC.

Reduced SIRT1 expression inhibits proliferation of hepatoma cells. Furthermore, we discovered that SIRT1 plays a role in telomeric maintenance via regulating the expression of telomerase and members of the shelterin complex. Our data suggest that the inhibition of SIRT1 activity may be a strategy for the development of anti-HCC therapeutics.

Materials and Methods

Plasmids and antibodies

Lentivirus plasmid vectors pLKO.1-puro, and pLKO.1-puro vectors containing MISSION shRNA targeting SIRT1 (clone SH2421) or non-targeting shRNAs (clone SHC001) were from Sigma-Aldrich. Vectors expressing TERT (pLV102-TERT), PTOP (pLV102-TPP1) and GFP (pLV102-GFP) were from GeneCopoeia. SIRT1 (1104-1) and TERT (1531-1) antibodies were from Epitomics; POT1 (ab21382) and PTOP (ab57595) antibodies were from Abcam; p53 (2524), acetyl-p53 (K382) (2525), phospho-H2AX (Ser139) (2577), FOXO1 (2880) and PARP (9542) antibodies were from Cell Signaling Technology; p16 (C-20) (sc-468) and Ac-FOXO1(sc-49437) antibodies were from Santa Cruz Biotechnology; p27 (554228) antibody was from BD Biosciences. β-ACTIN antibodies (A5316) and α-tubulin antibodies (T5168) were from Sigma-Aldrich.

HCC specimens

Tumorous liver tissues and the corresponding adjacent non-tumoral liver tissues were collected from 40 patients who underwent curative surgery for HCC at The Prince of Wales Hospital, Hong Kong. For these patients, the surgery was conducted in the morning after eight hours of fasting. The patients were not subjected to any form of chemotherapy prior to the surgery. Informed consent was obtained from each patient recruited, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. For these patients, the surgery was conducted in the morning after eight hours of fasting. The patients were not subjected to neo-adjuvant chemotherapy prior to surgery. Total RNAs and proteins were extracted from these specimens.

Tissue microarrays and immunohistochemistry

The HCC tissue microarrays were generated from formalin-fixed, paraffin-embedded archive tissues of 150 paired HCC. The samples were collected at The Prince of Wales Hospital from 1995 to 2002. Tissue slides were deparaffinized, followed by quenching of endogenous peroxidase activity by hydrogen peroxide. SIRT1 antibody was applied at a dilution of 1:400. Chromogen development was performed using the

universal HRP multimer ultraview kit (Ventana Medical System). The scoring of SIRT1 was carried out by two independent pathologists according to the proportion of tumor cells with positive nuclear staining (negative, none; weak, <=10%; moderate, 10 to <=50%; strong, >50%).

Cell culture

HepG2, SK-Hep-1, PLC5, Hep3B, SNU-449, SNU-423, and Huh-7 cells were obtained from American Type Culture Collection recommendations (ATCC, Rockville, MD). Huh7 cell line was acquired from the Health Science Research Resources Bank (Osaka, Japan). HKCI-4 and HKCI-2 cells were established in Prof. Nathalie Wong's laboratory (14). Huh7, HepG2, Hep3B and PLC5 were cultured according to recommendations in DMEM medium containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY). SNU-449, and SNU-423, were maintained in complete RPMI medium (Gibco BRL, Grand Island, NY). HKCI-4 and HKCI-2 cell lines were maintained in RPMI 1640 glutamax with HEPES buffer supplemented with 10% fetal bovine serum, 10 µg/ml selenium, 10 µg/ml transferrin and 10 µg/ml insulin. All cells were authenticated by short tandem repeat profiling analysis.

Lentivirus production

Lentivirus expressing shSIRT1-1, shSIRT1-2, or shCont was produced in HEK-293FT cells using the corresponding pLKO.1-puro vector with the aid of packaging plasmids pLP1, pLP2, and pLP/VSVG from BLOCK-iT Lentiviral RNAi Expression System (Invitrogen). The viruses were concentrated using PEG-it™ virus precipitation solution (System Biosciences) and stored at -80^{o}C.

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA preparation and cDNA synthesis were carried out as described (15). Quantitative PCR experiments were performed using the SYBR Green PCR core reagent kit (Applied Biosystems). Detailed information of the qPCR reaction and the primer used are listed in the Supplementary Information, S1.

Western blotting analysis

Protein lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and

immunoblotted with antibodies as indicated. Blots were developed with ECL™ Western blotting reagents. Band intensities were quantified using ImageJ (N.I.H.).

Cell proliferation and BrdU assay

Cell proliferation in response to SIRT1-silencing was determined by trypan blue exclusion assay. DNA synthesis was determined by the bromodeoxyuridine (BrdU) assay according to manufacturer's instructions (Roche Diagnostics Basel, Switzerland). The result was expressed as a percentage of the maximum absorbance at 450 nm based on three independent experiments. The effect of doxorubicin treatment on cell proliferation was measured by the MTT assay as described previously (16).

Colony formation assay and soft agar assay

The colony formation assay was carried out as described (17). Crystal violet-stained colonies were scored and results from duplicate assays were expressed as the mean from four independent experiments. For the soft agar assay, the base layer of each well consisted of 1.5 ml solidified media containing DEME with 10% FBS and 0.5% low melting point agarose. The top agar layer consisted of 5,000 SK-Hep-1 cells suspended in DEME with 0.35% low melting point agarose. Cells were allowed to grow for 3 weeks and the number of total colonies was counted under a microscope. Each assay was done in triplicate.

Analysis of cell cycle distribution and apoptosis

Cell cycle distribution and apoptosis were determined by fluorescence-activated cell sorting (FACS) analysis as described (18). Flow cytometry was carried out using a FACSCalibur flow cytometer (BD Bioscience). Data acquisition and analysis were done with CellQuest (BD Bioscience).

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining of cells was performed according to the method of Dimeri et al. (1995).

Quantification of telomere dysfunction induced foci (TIF)

TIF was monitored by TRF2-γH2AX co-localization using immunofluorescence (IF). Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, ice-cold

methanol:acetone (1:1) for 30 minutes and with methanol for another 30 minutes. Cells were then permeabilized using PBST (PBS, 0.2 % Triton X-100) and blocked with 10% normal goat serum. IF was carried out using the rabbit monoclonal anti-γH2AX (ser-139) antibody (1:50) and mouse anti-TRF2 antibody (1:100) overnight at 4^{o}C. The cells were then rinsed with PBS and incubated with goat anti-mouse fluorescein isothiocyanate (FITC) and goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) antibodies. Cells were counterstained with DAPI (4,6-diamidino-2-phenylindole). Digital images were captured with a Zeiss Axiovert 200 M fluorescence microscope with a CoolsnapTMES2 (Photometrics®) camera.

Statistical Analysis

SIRT1 expression in HCC and non-tumoral liver tissues were compared by the paired Student's *t*-test. Correlations between SIRT1 and individual clinicopathological parameters were evaluated by the non-parametric Chi-square test and Spearmans rho rank test. The Kaplan-Meier method was used to estimate the survival rates for SIRT1 expression. The equivalences of the survival curves were tested by log-rank statistics. All statistical analyses were carried out by the statistical program SPSS version 16.0.

Results

SIRT1 expression in HCC

We first determined the expression of SIRT1 in a panel of nine HCC cell lines and three normal liver biopsies. While the level of SIRT1 was almost undetectable in the normal livers, it was significantly over-expressed in all of the HCC cell lines (9/9) examined (Figure 1A). To establish the relevance of SIRT1 expression in HCC, we further examined its protein expression in primary HCC and in adjacent non-tumoral liver, which is often considered the premalignant lesion of HCC. Western blotting analysis indicated that a significant portion of patients showed elevated SIRT1 level in tumor tissues (28 of 40 cases) (Figure 1B). Furthermore, the average level of SIRT1 was found to be significantly higher in the tumor (median 0.25, quartiles 0.95-0.08) relative to the non-tumoral liver (median 0.05, quartiles 0.07 – 0.03) (paired t-test $p < 0.001$) (Figure 1C). On the other hand, the analysis of SIRT1 mRNA level from these patients by real-time quantitative PCR revealed that the average SIRT1 mRNA levels in the tumor and non-tumoral liver did not differ significantly (Figure 1D), suggesting that tumor-specific over-expression of SIRT1 was regulated in a transcription-independent manner.

Next, large-scale immunohistochemical analysis of SIRT1 expression was performed on tissue microarrays that contained 150 early resectable HCC and paired adjacent non-tumoral livers. The clinicopathological parameters of these HCC cases are summarized in Supplementary Table 1. Distinct SIRT1 staining was found in certain tumors (46/150 cases), whereas the staining was negative in all adjacent non-tumoral livers. In SIRT1 positive HCC, immunostaining could be identified in the tumor hepatocytes, and the staining was predominantly localized in the nucleus (Figure 1E). SIRT1 positive tumors were further classified into weak, moderate and strong categories according to the score obtained by determining the percentage of positively stained cells on the section. Among the 46 positive cases, 21 cases showed strong staining of SIRT1, whereas 8 and 17 cases exhibited moderate and weak staining respectively. SIRT1 expression in immunohistochemistry correlated positively with the tumor grade (Spearman rho test, $P<0.001$) but did not show association with any other clinicopathological parameter (Table 1). No prognostic significance of SIRT1 expression was demonstrated ($P=0.161$).

Effect of SIRT1 silencing on HCC cell proliferation, senescence and apoptosis

To further elucidate the functional role of SIRT1 in HCC, we transduced HCC cells SK-Hep-1 (p53 wild-type), HepG2 (p53 wild-type), Hep3B (p53 deleted), and PLC5 (p53 inactivated) with lentiviruses containing short hairpin RNAs (shRNA). Two independent shRNAs (shSIRT1-1 and shSIRT1-2) showed efficient SIRT1 knockdown in these cell lines (> 90%) compared to the untreated or scramble shRNA (shCont) transduced cells (Figure 2A).

Down-regulation of SIRT1 resulted in a marked decrease in cell number of all cells tested over a course of six days, independent of their p53 status (Figure 2B and Supplementary Figure 1). Furthermore, knockdown of SIRT1 reduced the number and size of SK-Hep-1 cell colonies as determined by colony formation assays (Figure 2C). It also inhibited anchorage-independent growth of the cells as determined by soft agar assays (Figure 2D). Concordantly, SIRT1 knockdown reduced DNA synthesis as measured by BrdU incorporation (Figure 2E). Cell cycle analysis showed that significant G1 arrest was observed in SK-Hep-1 and HepG2 cells, whereas G2 arrest was observed in PLC5 and Hep3B cells (Figure 2F). Taken together, these data suggest that while reduced levels of SIRT1 repressed cell proliferation in general, it has disparate effects on cell cycle distribution in different HCC cells.

An earlier study showed that inhibition of SIRT1 leads to senescence-like growth arrest in breast and lung cancer cells (19). We also observed that gene silencing of SIRT1 in SK-Hep-1 and HepG2 (p53 wild-type) resulted in cells that were enlarged in size, flattened in shape, and highly positive for senescence-associated β-galatosidase (SA-β-gal) staining, whereas negative staining was observed in Hep3B and PLC5 (p53 null or mutated) cells (Figure 3A). SIRT1 silencing was associated with increased acetylation of FOXO1 (Supplementary Figure 2), a well-known SIRT1 substrate, suggesting that there is a concordant reduction of SIRT1 expression and activity. Cellular senescence in SK-Hep-1 and HepG2 cells was associated with enhanced p53 acetylation on lysine 382 and induction of p21 that are known for their role in senescence induction (20). Enhanced p53 acetylation, but not p21 induction, was observed in PLC5 cells, consistent with its mutated p53 status. On the other hand, the expression of two other proteins implicated in senescence, namely p27 and p16, was not changed significantly upon SIRT1 knockdown (Figure 3B). Increased propensity to apoptotic cell death was evidenced by the enhanced PARP

cleavage in Hep3B and PLC5 cells, compared with HepG2 and SK-Hep-1 cells (Figure 3C). Collectively, these data suggest that SIRT1 knockdown is associated with G1 arrest and cell senescence in p53 wild-type HCC cells, but leads to G2 arrest and apoptosis in p53 mutated HCC cells.

The role of SIRT1 in telomeric maintenance

Telomeres are nucleoprotein structures that protect the ends of chromosomes. Mammalian telomeres are protected from being recognized as sites of DNA damage by the shelterin complex, which is composed of TRF1, TRF2, POT1, TIN2, PTOP and RAP1 respectively (21). Either critically shortened telomeres, or the inhibition of shelterin causes telomeric dysfunction that is characterized by the activation of DNA damage response and the formation of telomere dysfunction-induced foci (TIF), leading to cellular senescence (22, 23). To determine whether the observed cellular senescence or cell death is associated with telomeric dysfunction, we evaluated the expression of telomerase (TERT), members of the shelterin complex, and other telomere-associated proteins (PINX1, TANK1, and KU-70) respectively by quantitative real-time PCR.

Compared to untreated or shCont-expressing cells, SK-Hep-1 cells expressing SIRT1 shRNAs resulted in a significant reduction of TERT, POT1 and PTOP mRNA respectively (Figure 4A). Concordantly, a marked reduction of TERT and PTOP protein expression upon SIRT1 knockdown were observed in all hepatoma cells examined (SK-Hep-1, HepG2, Hep3B and PLC5), suggesting that SIRT1 may play an important role in PTOP and TERT expression in liver tumors (Figure 4B). A reduction of POT1 expression upon SIRT1 knockdown was observed only in SK-Hep-1 and Hep3B cells but not in HepG2 and PLC-5 cells, suggesting that the POT1 gene may be subjected to differential regulation in different cells (Figure 4B). Importantly, when PTOP or TERT, but not GFP, was ectopically expressed in cells transduced with lentiviruses expressing shSIRT1-1 and shSIRT1-2 respectively (Figure 4C), a significant enhancement in cell growth (Figure 4D) and a reduction in cell senescence (Supplementary Figure 3) were observed in SK-Hep-1 cells. Similarly, ectopic expression of PTOP or TERT also promoted cell growth in SIRT1-depleted Hep3B cells (Supplementary Figure 4). Together, these data supported the notion that PTOP and TERT depletion are responsible, at least in part, for cell growth suppression by SIRT1 knockdown in HCC cells.

PTOP plays an essential organizing function in shelterin and protects telomeres from TIF via recruiting POT1 (24). It also recruits TERT to the telomere and regulates its activity (25, 26). Gene knockdown of PTOP results in the activation of DNA damage signaling pathway, leading to telomeric fusion detectable in metaphase spreads (24). Consistent with these known functions of PTOP, we observed that SIRT1 silencing significantly induced the formation of TIF in SK-Hep-1 cells as evidenced by co-localization of phosphorylated H2AX (γ-H2AX) and TRF2 (Figure 4E). However, we could not analyze telomeric fusion in cells (SK-Hep-1, PLC5, HepG2 and Hep3B) expressing SIRT1 shRNA, because these cells failed to undergo mitotic arrest in response to colchicine treatment (data not shown). We further showed that SIRT1 depletion probably disrupted mitotic checkpoint signaling because either one or more genes involved in mitotic arrest, including *AURK-A, AURK-B, CENP-A*, and *BUB1,* were repressed upon SIRT1 knockdown (Supplementary figure 5). In line with this observation, cells expressing SIRT1 shRNA exhibited increased nuclear abnormality characterized by multinuclei and micronuclei formation (Figure 4F). Collectively, these data suggested that telomeric dysfunction and genetic instability are the major contributing factors to cell growth suppression induced by SIRT1 reduction.

Correlation between SIRT1, TERT and PTOP expression in human HCC.

To determine the relevance of the above SIRT1-regulated pathways in human subjects, we probed for the expression of TERT and PTOP in HCC by Western blotting using the same 40 paired HCC that have been used (Figure 1B) to determine SIRT1 expression. Overall, TERT was expressed at a higher levels in HCC compared to adjacent non-tumoral liver tissues, whereas PTOP was expressed at a comparable level in these tissues (Figure 5A). Correlative analysis further revealed a significant association between tumoral induction of TERT and SIRT1 (Spearman's rank = 0.40, P=0.01) (Figure 5B), whereas the association between tumoral induction of SIRT1 and PTOP was not apparent (Spearman's rank = 0.08, P=0.21) (Figure 5C). These data suggest that a SIRT1-TERT regulatory axis may exist *in vivo*.

SIRT1 knockdown enhanced cytotoxicity of doxorubicin in HCC cells.

To further demonstrate the potential of targeting SIRT1 for HCC therapy, we determined whether SIRT1 inhibition enhances the anti-tumor effect of the DNA-damaging agent doxorubicin, the only clinically efficacious chemotherapeutic agent for HCC treatment. Cells were first transduced with lentiviruses expressing shSIRT1-1 and shSIRT1-2 for 2 days respectively, before doxorubicin was added to the medium at a final concentration of 0 – 12 μM and incubated for 48 hours. Remarkably, reduced SIRT1 expression increased the chemosensitivity of SK-Hep-1 and PLC5 cells to doxorubicin treatment by 8 fold and 4 fold respectively (Figure 6A). Concordantly, reduced SIRT1 expression also promoted doxorubicin-induced apoptosis of these cells, as evidenced by enhanced PARP cleavage (Figure 6B). Together these data suggest the therapeutic potential of combining a SIRT1 inhibitor and doxorubicin in the treatment of a subset of HCC in which SIRT1 is up-regulated.

Discussion

The role of SIRT1 in tumorigenesis is controversial. Increased SIRT1 expression has been found to reduce tumor formation in a mouse model of colon tumor (27), whereas SIRT1 mutant mice exhibited increased DNA instability and are more susceptible to tumor development (28). Reduced levels of SIRT1 mRNA and proteins are observed in breast tumors compared to normal tissue (28). Intriguingly, however, SIRT1 over-expression is found in acute myeloid leukemia (29), prostate (30), skin (31), gastric (32) and colorectal cancers (33), suggesting a tumorigenic role. Therefore, the function of SIRT1 may be tumor-type specific and may also depend on the stage of tumorigenesis being assessed.

With regard to HCC, Wang *et al* found that SIRT1 mRNA is expressed at a comparable level in both tumor and non-tumoral tissues, by analyzing pooled microarray data from HCC samples. They further concluded that SIRT1 protein expression is reduced in HCC based on the analysis of one paired HCC specimen (28). In agreement with their results, our study confirmed that SIRT1 mRNA levels do not differ between HCC and non-tumoral tissues. However, by analyzing SIRT1 protein expression using our large collection of paired frozen HCC tissues and histological sections, we have convincingly demonstrated that SIRT1 protein was indeed over-expressed in a subset of HCC by a post-transcriptional mechanism. We have also demonstrated that SIRT1 expression is low in normal and pre-malignant livers, and its positivity is closely associated with poorly differentiated histology. Furthermore, reduced levels of SIRT1 suppressed cell proliferation and anchorage-independent growth of HCC cells. Together our data support the notion that SIRT1 over-expression may play a role in HCC tumorigenesis.

Recent studies also suggested that inhibition of SIRT1 may have anti-cancer potential. RNAi-mediated silencing of SIRT1 genes resulted in growth arrest or apoptosis in some epithelial tumor cells (34), and reactivated tumor suppressor genes (35). The SIRT1 inhibitor, sirtinol, induced senescence-like growth arrest in tumor cells (19), whereas another inhibitor, cambinol, induced apoptosis of Burkitt lymphoma cells and suppressed the growth of tumor xenografts *in vivo* (36). Our study revealed that the knockdown of SIRT1 in HCC cells resulted in the apoptosis or the characteristic senescence-like growth arrest phenotype closely resembling that of breast and lung cells treated with sirtinol (19), suggesting that reduced SIRT1 activity may perturb pathways essential for cell proliferation in these cancer cells.

We further identified telomeric dysfunction as one of the major phenotypes of reduced SIRT1 levels in HCC tumor cells. Telomeres are shortened on every DNA replication cycle due to the requirement of short RNA to prime DNA synthesis by DNA polymerases (37). Progressive telomere shortening is also found in chronic liver injury and liver cirrhosis (38). Telomere shortening triggers the DNA damage response, leading to cell cycle arrest, senescence, or apoptosis (39), and it also serves as a tumor suppressor mechanism to limit the proliferation of transformed cells. Nevertheless, TERT is activated in most tumors including HCC to overcome the telomere barrier by adding back telomere repeats to chromosome ends (38). Inhibition of TERT shortens telomeres and causes cancer cell death (40). TERT antagonists also inhibit tumor growth in a xenograft animal model of HCC (41).

Although TERT plays a pivotal role in tumorigenesis, and we have showed that the proliferation of SIRT1-depleted HCC cells was rescued by TERT expression, we believe that telomere phenotypes in SIRT1-depleted cells did not result from telomeric attrition. This is because telomere shortening is a gradual process that requires a substantial number of cell division cycles for its effect on cell growth to become apparent. Therefore, the acute cell growth suppression effect as a result of SIRT1 knockdown argues against telomere attrition as the major mechanism. Indeed, telomeric length did not differ significantly between the control and SIRT1-depleted cells, as measured at 7 days after lentiviral transduction (Supplementary figure 6). Instead, our evidence suggests that TERT may contribute to cell survival via telomere-independent mechanisms (42) (43), and therefore, reduced expression of TERT in SIRT1 knockdown cells suppresses cell proliferation that can be rescued by its re-expression. Apparently, the TIF and nuclear abnormalities observed in SIRT1 knockdown cells are more likely due to the depletion of PTOP, or both PTOP and POT1. PTOP binds to and recruits POT1 to the telomere which is essential for telomere protection. The PTOP-POT1 complex also recruits TERT and is essential for its activity (25). Over-expression of telomere-binding proteins is also found in many cancers (44) (45). Presumably these proteins are required to cope with the increased levels of cell division in tumors. Consistent with the above notion, the depletion of telomeric-binding proteins, including PTOP and POT1, induces the DNA-damage response, leading to cell growth inhibition (46) (47).

The current understanding of the pro-survival function of SIRT1 is its role to direct the cells towards DNA repair via modulating the activity of MRE11-RAD50-

NBS1, p53, Ku70, and FOXO proteins, etc (48). Our findings extend the current understanding of the function of SIRT1 to include telomeric maintenance in HCC cells, and that its acute depletion could suppress tumor cell growth. It should be noted that SIRT1 depletion also impinges on other cellular processes including the repression of genes involved in mitotic checkpoint signaling. The underlying mechanistic basics remain to be determined. With regard to cancer therapy, the disruption of telomeric maintenance via targeting telomerase is one of the emerging anti-tumor strategies. However, one major limitation of using telomerase inhibitors for tumor therapy is the long lag time for its action, during which the tumor burden may have already increased substantially (40) (41). Therefore targeting SIRT1 may be a more efficient strategy to induce telomeric dysfunction and tumor cell death. The response rate of HCC to various chemotherapies has been low. Our work suggests that SIRT1 may serve as a potential target for the development of anti-HCC therapeutics, and inhibition of SIRT1 may be a novel strategy to target a subset of HCC patients in whom this protein is over-expressed.

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Reference

1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132:2557-76.
2. But D-Y-K, Lai C-L, Yuen M-F. Natural history of hepatitis-related hepatocellular carcinoma. World J Gastroenterol. 2008;14:1652-6.
3. Greten TF, Papendorf F, Bleck JS, Kirchhoff T, Wohlberedt T, Kubicka S, et al. Survival rate in patients with hepatocellular carcinoma: a retrospective analysis of 389 patients. British Journal of Cancer. 2005;92:1862-8.
4. Yeo W, Mok TS, Zee B, Leung TWT, Lai PBS, Lau WY, et al. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. J Natl Cancer Inst. 2005;97:1532-8.
5. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 2000;403:795-800.
6. Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem. 2004;73:417-35.
7. Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J. 2007;404:1-13.
8. Guarente L. Sirtuins as potential targets for metabolic syndrome. Nature. 2006;444:868-74.
9. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol. 2010;5:253-95.
10. Oberdoerffer P, Michan S, Mcvay M, Mostoslavsky R, Vann J, Park S-K, et al. SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. Cell. 2008;135:907-18.
11. Palacios JA, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco MA. SIRT1 contributes to telomere maintenance and augments global homologous recombination. The Journal of Cell Biology. 2010;191:1299-313.
12. El Ramy R, Magroun N, Messadecq N, Gauthier LR, Boussin FD, Kolthur-Seetharam U, et al. Functional interplay between Parp-1 and SirT1 in genome integrity and chromatin-based processes. Cell Mol Life Sci. 2009;66:3219-34.
13. Yuan J, Pu M, Zhang Z, Lou Z. Histone H3-K56 acetylation is important for genomic stability in mammals. Cell Cycle. 2009;8:1747-53.
14. Wong N, Yeo W, Wong W-L, Wong NL-Y, Chan KY-Y, Mo FK-F, et al. TOP2A overexpression in hepatocellular carcinoma correlates with early age onset, shorter patients survival and chemoresistance. Int J Cancer. 2009;124:644-52.
15. Tong EHY, Guo J-J, Xu S-X, Mak K, Chung SK, Chung SSM, et al. Inducible nucleosome depletion at OREBP-binding-sites by hypertonic stress. PLoS ONE. 2009;4:e8435.
16. Wong VKW, Chiu P, Chung SSM, Chow LMC, Zhao Y-Z, Yang BB, et al. Pseudolaric acid B, a novel microtubule-destabilizing agent that circumvents multidrug resistance phenotype and exhibits antitumor activity in vivo. Clin Cancer Res. 2005;11:6002-11.
17. Cheng IK-C, Ching AK-K, Chan T-C, Chan AW-H, Wong C-K, Choy K-W, et al. Reduced CRYL1 expression in hepatocellular carcinoma confers cell growth advantages and correlates with adverse patient prognosis. J Pathol. 2010;220:348-60.

18. Law BYK, Wang M, Ma D-L, Al-Mousa F, Michelangeli F, Cheng S-H, et al. Alisol B, a novel inhibitor of the sarcoplasmic/endoplasmic reticulum Ca(2+) ATPase pump, induces autophagy, endoplasmic reticulum stress, and apoptosis. Mol Cancer Ther. 2010;9:718-30.
19. Ota H, Tokunaga E, Chang K, Hikasa M, Iijima K, Eto M, et al. Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. Oncogene. 2006;25:176-85.
20. Pedeux R, Sengupta S, Shen JC, Demidov ON, Saito Si, Onogi H, et al. ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. Mol Cell Biol. 2005;25:6639-48.
21. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes & Development. 2005;19:2100-10.
22. Palm W, de Lange T. How shelterin protects mammalian telomeres. Annu Rev Genet. 2008;42:301-34.
23. Deng Y, Chan SS, Chang S. Telomere dysfunction and tumour suppression: the senescence connection. Nat Rev Cancer. 2008;8:450-8.
24. Kibe T, Osawa GA, Keegan CE, de Lange T. Telomere protection by TPP1 is mediated by POT1a and POT1b. Mol Cell Biol. 2010;30:1059-66.
25. Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature. 2007;445:506-10.
26. Xin H, Liu D, Wan M, Safari A, Kim H, Sun W, et al. TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature. 2007;445:559-62.
27. Firestein R, Blander G, Michan S, Oberdoerffer P, Ogino S, Campbell J, et al. The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth. PLoS ONE. 2008;3:e2020.
28. Wang R-H, Sengupta K, Li C, Kim H-S, Cao L, Xiao C, et al. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell. 2008;14:312-23.
29. Bradbury CA, Khanim FL, Hayden R, Bunce CM, White DA, Drayson MT, et al. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. Leukemia. 2005;19:1751-9.
30. Huffman DM, Grizzle WE, Bamman MM, Kim J-s, Eltoum IA, Elgavish A, et al. SIRT1 is significantly elevated in mouse and human prostate cancer. Cancer Res. 2007;67:6612-8.
31. Hida Y, Kubo Y, Murao K, Arase S. Strong expression of a longevity-related protein, SIRT1, in Bowen's disease. Arch Dermatol Res. 2007;299:103-6.
32. Cha EJ, Noh SJ, Kwon KS, Kim CY, Park B-H, Park HS, et al. Expression of DBC1 and SIRT1 is associated with poor prognosis of gastric carcinoma. Clin Cancer Res. 2009;15:4453-9.
33. Stünkel W, Peh BK, Tan YC, Nayagam VM, Wang X, Salto-Tellez M, et al. Function of the SIRT1 protein deacetylase in cancer. Biotechnol J. 2007;2:1360-8.
34. Ford J, Jiang M, Milner J. Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. Cancer Res. 2005;65:10457-63.
35. Pruitt K, Zinn RL, Ohm JE, Mcgarvey KM, Kang S-HL, Watkins DN, et al. Inhibition of SIRT1 reactivates silenced cancer genes without loss of promoter DNA hypermethylation. PLoS Genet. 2006;2:e40.

36. Heltweg B, Gatbonton T, Schuler AD, Posakony J, Li H, Goehle S, et al. Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res. 2006;66:4368-77.
37. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol. 1973;41:181-90.
38. Ozturk M, Arslan-Ergul A, Bagislar S, Senturk S, Yuzugullu H. Senescence and immortality in hepatocellular carcinoma. Cancer letters. 2008.
39. Stewart SA, Weinberg RA. Telomeres: cancer to human aging. Annu Rev Cell Dev Biol. 2006;22:531-57.
40. Shay JW, Keith WN. Targeting telomerase for cancer therapeutics. Br J Cancer. 2008;98:677-83.
41. Djojosubroto MW, Chin AC, Go N, Schaetzlein S, Manns MP, Gryaznov S, et al. Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. Hepatology. 2005;42:1127-36.
42. Smith LL, Coller HA, Roberts JM. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol. 2003;5:474-9.
43. Ahmed S, Passos JF, Birket MJ, Beckmann T, Brings S, Peters H, et al. Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. Journal of Cell Science. 2008;121:1046-53.
44. Poncet D, Belleville A, t'kint de Roodenbeke C, Roborel de Climens A, Ben Simon E, Merle-Beral H, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. Blood. 2008;111:2388-91.
45. Cookson JC, Laughton CA. The levels of telomere-binding proteins in human tumours and therapeutic implications. Eur J Cancer. 2009;45:536-50.
46. Veldman T, Etheridge KT, Counter CM. Loss of hPot1 function leads to telomere instability and a cut-like phenotype. Curr Biol. 2004;14:2264-70.
47. Barrientos KS, Kendellen MF, Freibaum BD, Armbruster BN, Etheridge KT, Counter CM. Distinct functions of POT1 at telomeres. Mol Cell Biol. 2008;28:5251-64.
48. Liu T, Liu PY, Marshall GM. The critical role of the class III histone deacetylase SIRT1 in cancer. Cancer Res. 2009;69:1702-5.

Figure Legend

Table 1. Correlation of SIRT1 expressions with clinicopathologic features. Strong SIRT1 protein score was associated with progressive advancement of histological differentiation.

Figure 1. Expression of SIRT1 in HCC. (A) Expression of SIRT1 protein was analyzed by Western blot analysis using SIRT1 antibodies. β-ACTIN was used as a loading control. (B) Level of SIRT1 in 40 paired HCC by Western blot analysis using SIRT1 antibodies. β-ACTIN was used as a loading control. For (A) and (B), total protein extracts were used for Western blotting analysis. (C) Quantification of SIRT1 protein expression in HCC and their adjacent non-tumoral livers. *, $p < 0.001$ (D) Real-time quantitative PCR analysis of SIRT1 mRNA expression in HCC and their adjacent non-tumoral livers. β-ACTIN mRNA expression was used as an internal control. (E) Immunohistochemical examination of SIRT1 expression in primary and paired adjacent non-tumoral HCC. Magnification X400. Insert, magnification X100.

Figure 2. Effect of SIRT1 gene silencing on the growth of HCC cells. (A) Western blot analysis of SIRT1 protein expression in liver tumor cells transduced with lentiviruses expressing scramble (shCont), or SIRT1-targeting (shSIRT1-1, and shSIRT1-2) shRNA respectively. Cells were harvested 4 days after viral transduction. β-ACTIN was used as a loading control. (B) Proliferation of SK-Hep-1 cells was suppressed by SIRT1 gene silencing. Cells were infected with the respective lentivirus and cell number was determined by trypan blue exclusion assay at the indicated number of days thereafter. Plots are cumulative cell numbers versus days in culture. *, $P<0.001$. (C) Colony formation assay of SK-Hep-1 cells transduced with lentiviruses expressing the indicated shRNA. Cells were grow for 14 days under the selection of puromycin (0.25 μg/ml) and stained with crystal violet. (D) Soft agar assay of SK-Hep-1 cells transduced with lentiviruses expressing the indicated shRNA. Cells were growth in soft agar for 3 weeks. Quantification of colonies were obtained from macroscopically visible colonies in each well, and expressed as percentage relative to the group expressing shCont. *, $P<0.001$. (E) BrdU incorporation of SIRT1 knockdown SK-Hep-1 cells. 3 days after lentiviral transduction, cells were pulsed

with BrdU (10 μM) for 4 h. Then cells were stained with anti-BrdU antibody and signal incorporation was measured at 450 nm. The O.D. values were expressed as percentage relative to the group expressing shCont. *, P<0.001. (F) Cell cycle analysis of SIRT1 knockdown cells using propidium iodide and flow cytometric analysis. *, P<0.01 when compared to shCont. For B, D, E and F, three independent experiments were performed, each in triplicate. Data obtained from representative experiment was shown. Bar represents the mean ± SD of three experiments.

Figure 3. Cellular senescence and cell death in liver tumor cells by SIRT1 knockdown. (A) SA-β-gal staining was performed for the analysis of cellular senescence of SK-Hep-1, HepG2, Hep3B and PLC5 cells transduced with lentiviruses expressing the indicated SIRT1 shRNAs for 6 days. Quantification of SA-β-gal–positive cells relative to total cells was obtained by counting 200 cells in three randomly chosen fields per dish. *, P<0.001. Three independent experiments were performed. Data obtained from representative experiment was shown. Bar represents the mean ± SD of three experiments. (B) Western blot analysis using SIRT1, Ac-p53(K382), p53, p21, p27, p16 and β-ACTIN antibodies. Cells were transduced with lentiviruses expressing the indicated shRNA for 4 days before the analysis. (C) Western blot analysis of Poly ADP ribose polymerase (PARP) cleavage of cells transduced with lentiviruses expressing the indicated shRNA for 4 days. β-ACTIN was used as a loading control.

Figure 4. SIRT1 knockdown induce telomeric dysfunction. (A) Real-time quantitative PCR analysis of gene expressions related to telomeric maintenance. SK-Hep-1 cells were transduced with lentiviruses expressing the indicated shRNA. β-actin mRNA expression was used as an internal control. Experiment was performed in triplicate. Values represent the mean ± SD of three independent experiments. *, P<0.001. (B) Western blot analysis of SIRT1, TERT, PTOP, POT1 and β-ACTIN expressions of SK-Hep-1 cells. Cells were transduced with lentiviruses expressing the indicated shRNA for 5 days before analysis. (C-E) Effects of TERT or PTOP over-expression on cells depleted for SIRT1. SK-Hep-1 cells were transfected with TERT, PTOP or GFP expressing vector. Cells were grow for 1 week under the selection of puromycin (0.25 μg/ml). Subsequently these cells were transduced with lentivirus expressing

shCont, shSIRT1-1 and shSIRT1-2 respectively. Cells were harvested for Western blot analysis (C), or subjected to cell proliferation assay (D) respectively after 6 days. For (D), values were expressed relative to the cell count in the shCont group, which was set to 100%. Bars represent the mean ± SD of three independent experiments. (E) Left, induction of TIF by knockdown of SIRT1. SK-Hep-1 cells at 4 days after lentiviral transduction were analyzed using TRF2 antibodies for telomeres (TRITC, red) and γ-H2AX antibodies for DNA damage (FITC, green). Arrow indicates telomeric γ-H2AX foci. Right, quantification of the TIF response. Cells shown were scored for 5 or more telomeric γ-H2AX foci. Bars represent the mean ± SD of three independent experiments. (F) Left, SK-Hep-1 cells at 4 days after lentiviral transduction were analyzed using α-tubulin antibodies (TRITC, red) and DAPI (blue). Arrow denotes multinuclei, arrow head denotes micronuclei. Right, quantification of nuclear abnormalities. 100 cells were randomly picked from 5 fields and scored. Bars represent the mean ± SD of three independent experiments.

Figure 5. (A) Expression of SIRT1, TERT, PTOP and β-ACTIN expression in HCC and adjacent non-tumoral liver tissues. (B-C) SIRT1, TERT and POTP expression level was first normalized by the expression level of β-ACTIN, and the fold induction of each of these protein in HCC over non-tumoral liver in each patient was calculated. Fold induction of SIRT1 was plotted against TERT (C), and PTOP (D) respectively, and was analyzed by Spearmans rho rank test.

Figure 6. SIRT1 knockdown sensitizes HCC cells to doxorubicin treatment. (A) SK-Hep-1 and PLC5 cells transduced with lentivirus expressing the indicated shRNA were treated with doxorubicin at various concentration for 2 days, and then processed for MTT assay. (B) SK-Hep-1 and PLC5 cells transduced with lentivirus were treated with 0.75 μM of doxorubicin for 2 days. Cells were harvested for PARP cleavage analysis using PARP antibodies. β-ACTIN was used as a loading control.

Table 1

Correlation of clinicopathological parameters with SIRT1 expression

Clinicopathological Parameters		SIRT1 Expression				P-value
		Negative	Weak	Moderate	Strong	
Sex	Male	90	12	5	17	0.162
	Female	14	5	3	4	
Age (median+/-SD)		57+/-13.4	46+/-16.3	56+/-13.8	53+/-11.5	0.544
Grade	1	30	4	1	0	<0.001*
	2	70	12	6	15	
	3	4	1	1	6	
Stage (T)	1	61	13	6	10	0.349
	2	27	2	2	7	
	3	16	2	0	4	
Multiple Tumor	No	83	14	8	16	0.519
	Yes	21	3	0	5	
Macroscopic Vascular Invasion	No	95	16	8	18	0.623
	Yes	9	1	0	3	
Microscopic Vascular Invasion	No	71	13	6	11	0.386
	Yes	33	4	2	10	
HBV	No	8	1	0	3	0.640
	Yes	82	12	5	15	
HCV	No	29	3	1	4	0.965
	Yes	1	0	0	0	
Steatosis	0%	73	14	6	14	0.099
	>0-33%	20	0	2	4	
	>33-66%	9	1	0	1	
	>66%	1	0	0	2	
Cirrhosis	No	33	7	1	4	0.317
	Yes	71	10	7	17	

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Figure 1

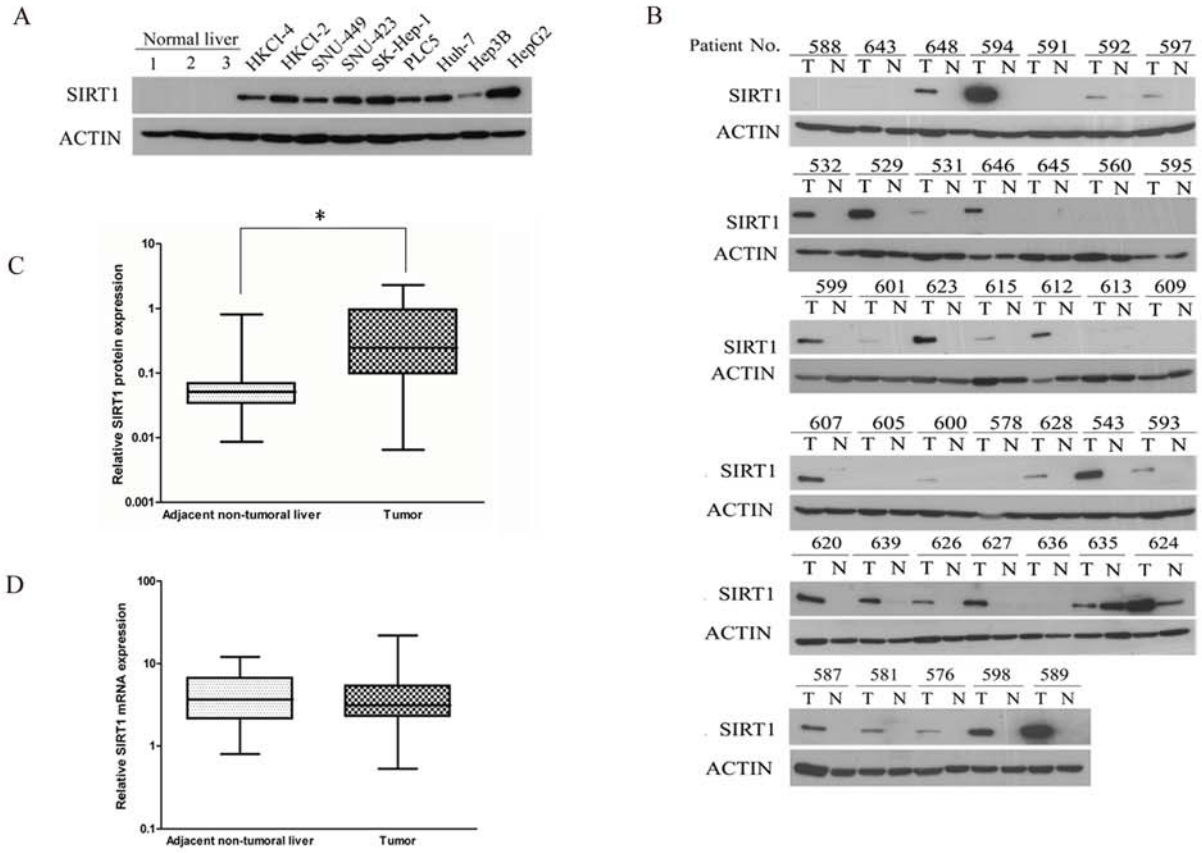
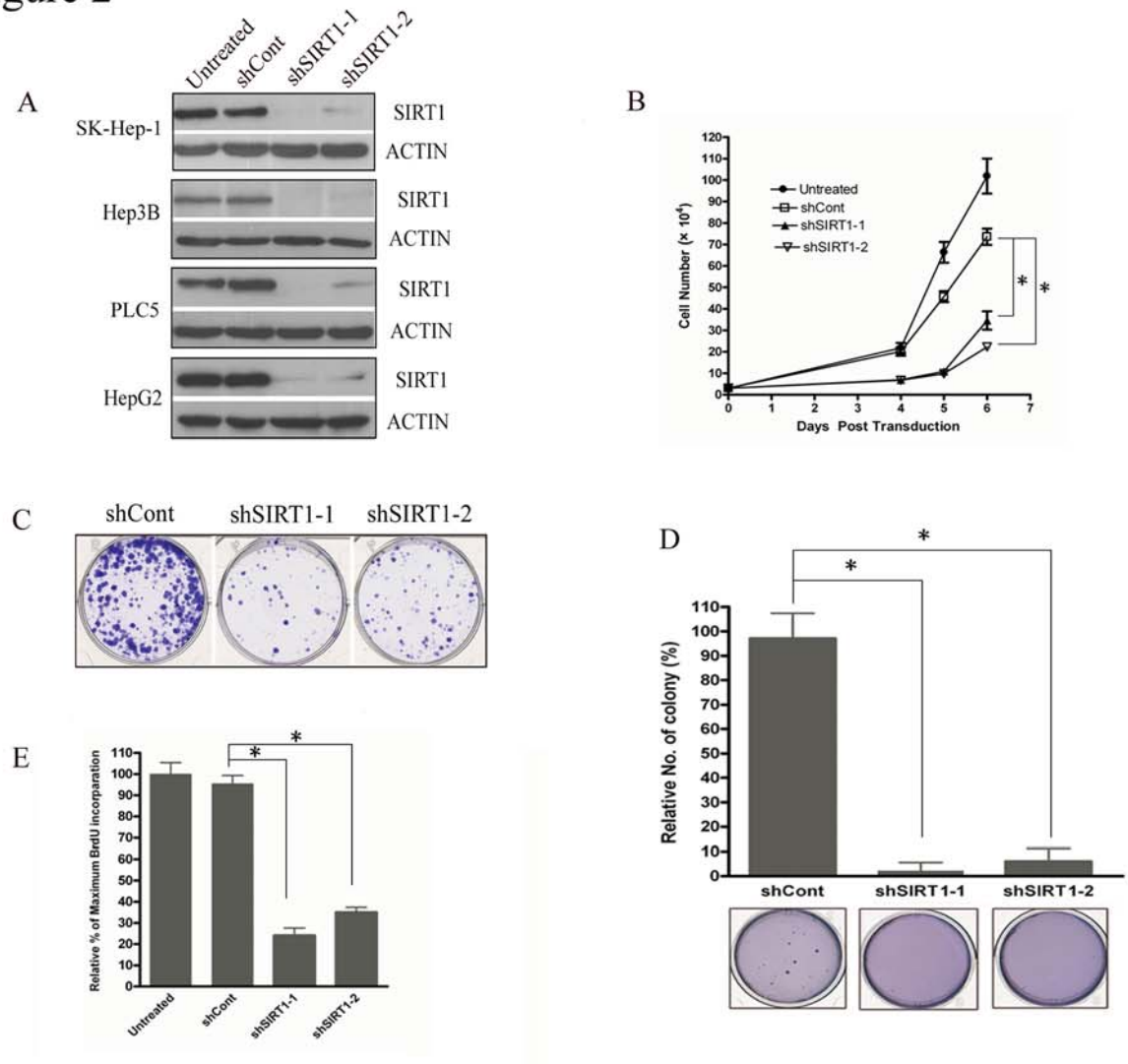


Figure 2



F

		G1	S	G2
SK-Hep-1	shCont	44.29 ±0.36	35.07±1.58	20.64 ±1.58
	shSIRT1-1	69.20±1.24 *	13.40±3.25 *	17.40 ±2.90
	shSIRT1-2	65.86 ±1.75 *	14.57 ±0.72 *	19.57 ±1.90
HepG2	shCont	49.71 ±1.50	27.63 ±0.81	22.66 ±0.35
	shSIRT1-1	68.52 ±0.90 *	20.29±2.50	11.19 ±1.56 *
	shSIRT1-2	65.92 ±2.32 *	20.84±2.56	13.24 ±1.76 *
Hep3B	shCont	57.65 ±0.91	27.84 ±1.21	14.51 ±0.94
	shSIRT1-1	26.14±1.21 *	26.61 ±1.43	47.25 ±1.95 *
	shSIRT1-2	34.21 ±2.41 *	27.73 ±2.76	38.06±0.75 *
PLC5	shCont	66.58 ±1.70	24.40 ±1.31	9.02 ±0.55
	shSIRT1-1	53.59 ±1.35 *	28.62±1.42	17.85 ±0.78 *
	shSIRT1-2	51.84 ±1.38 *	29.07 ±1.22	19.09 ±0.90 *

Figure 3

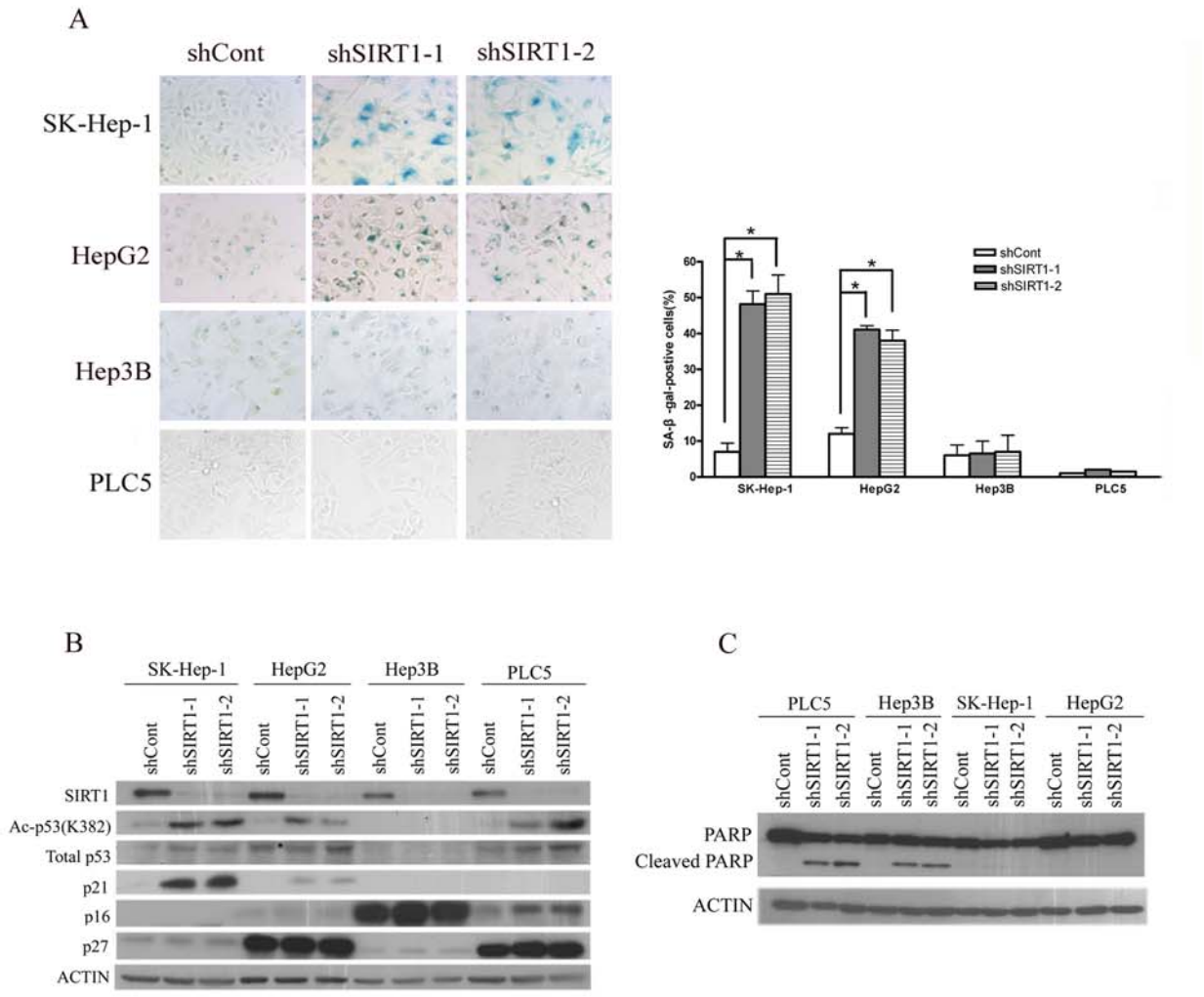


Figure 4

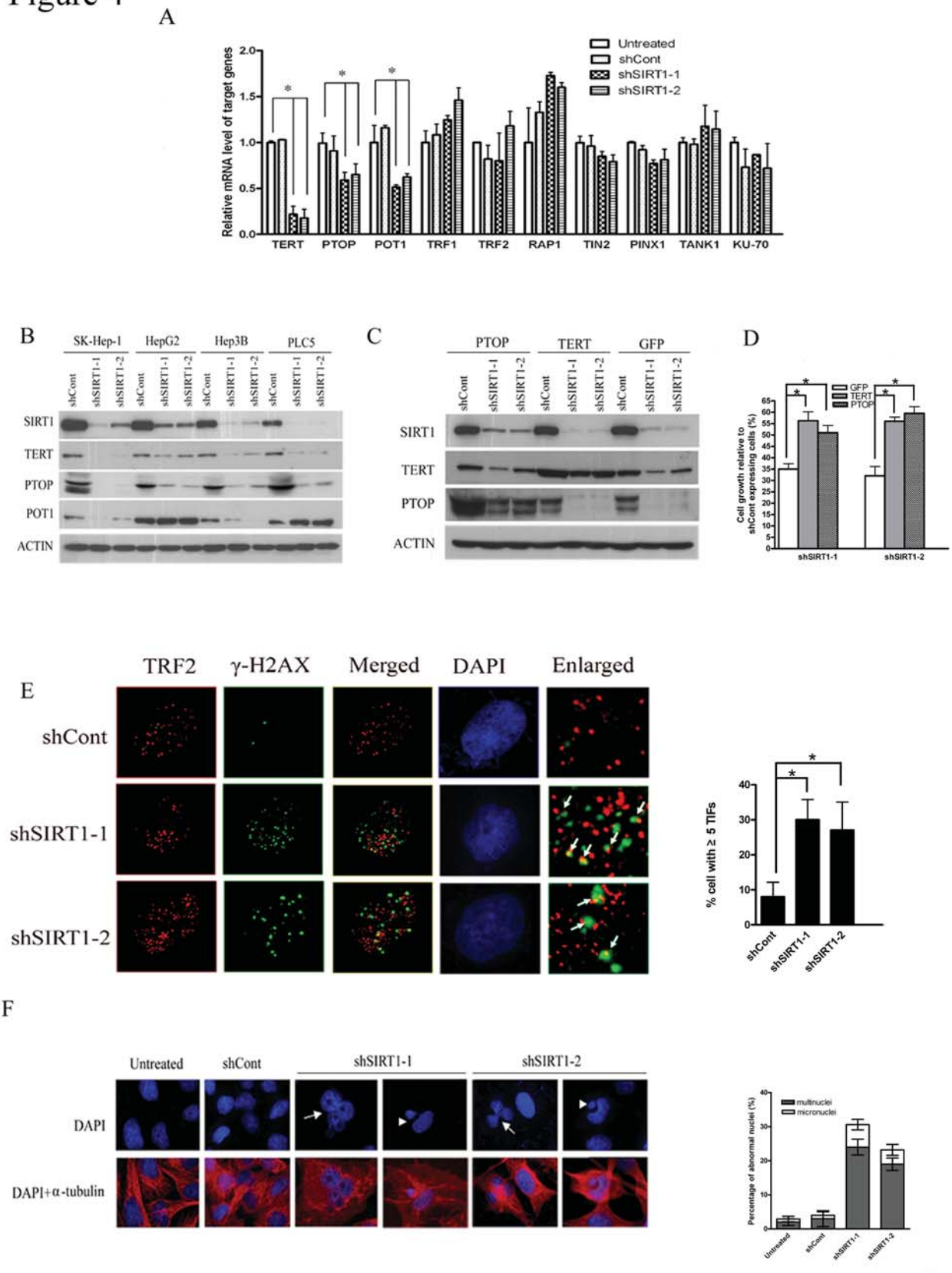


Figure 5

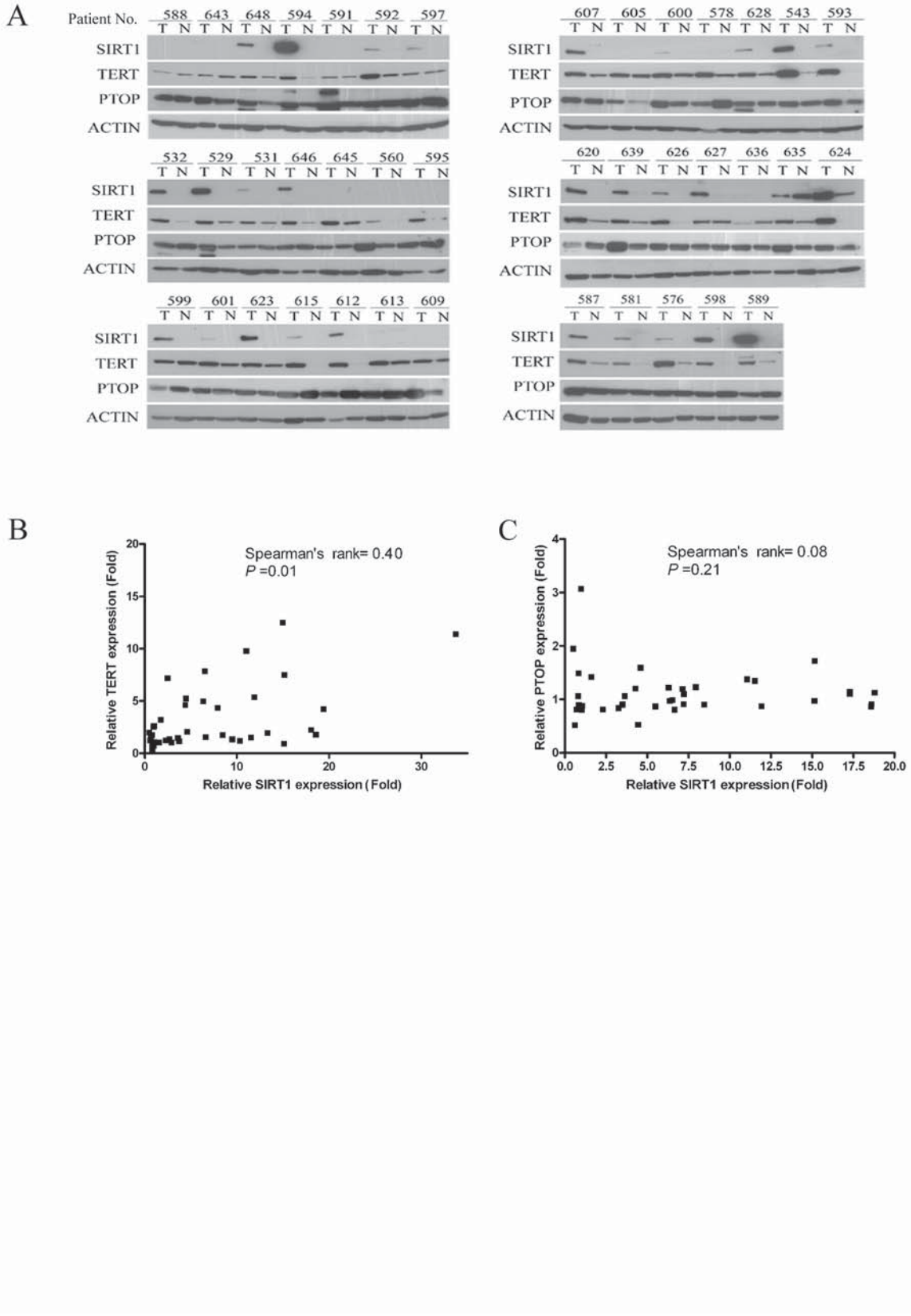
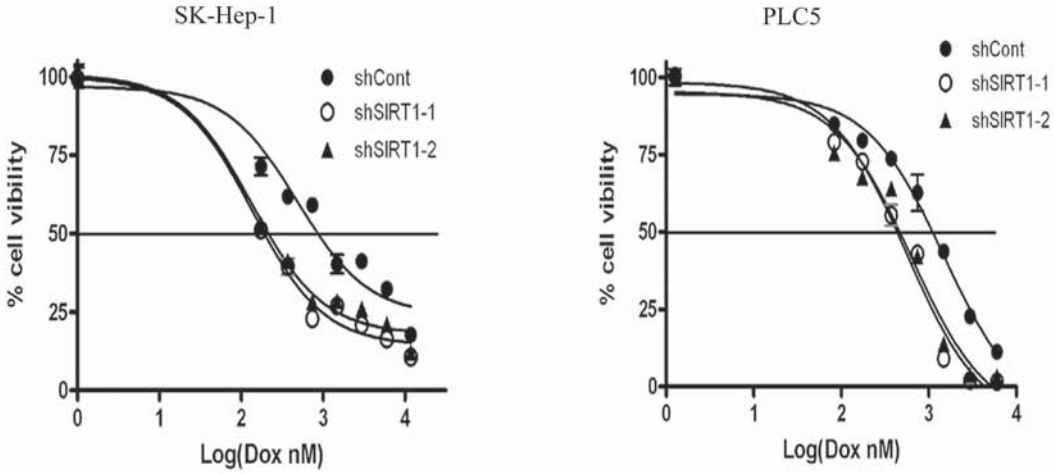


Figure 6

A



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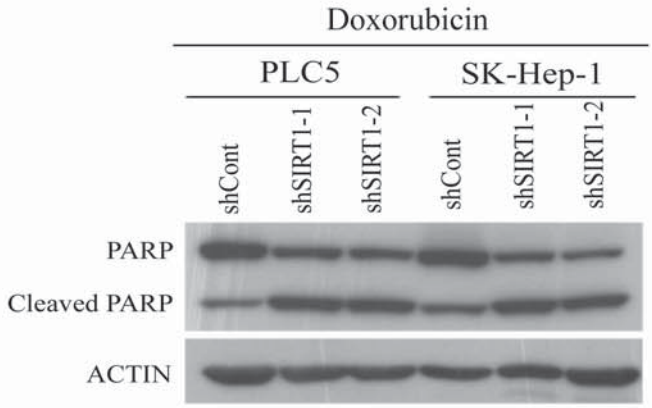

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Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth.

Juan Chen, Bin Zhang, Nathalie Wong, et al.

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