

STC2 is upregulated in hepatocellular carcinoma and promotes cell proliferation and migration *in vitro*

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The human glycoprotein, stanniocalcin 2 (STC2) plays multiple roles in several tumor types, however, its function and clinical significance in hepatocellular carcinoma (HCC) remain unclear. In this study, we detected STC2 expression by quantitative real-time PCR and found STC2 was upregulated in HCC tissues, correlated with tumor size and multiplicity of HCC. Ectopic expression of STC2 markedly promoted HCC cell proliferation and colony formation, while silencing of endogenous STC2 resulted in a reduced cell growth by cell cycle delay in G0/G1 phase. Western blot analysis demonstrated that STC2 could regulate the expression of cyclin D1 and activate extracellular signal-regulated kinase 1/2 (ERK1/2) in a dominant-positive manner. Transwell chamber assay also indicated altered patterns of STC2 expression had an important effect on cell migration. Our findings suggest that STC2 functions as a potential oncoprotein in the development and progression of HCC as well as a promising molecular target for HCC therapy. [BMB Reports 2012; 45(11): 629-634]

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly virulent malignancy and its incidence has increased rapidly during the past few decades worldwide, with only 9% of patients surviving more than 5 years (1, 2). About half of the cases and deaths are estimated to occur in China. Hepatitis B or C viral infection, alcohol abuse

and aflatoxin intake are the most common etiologies for the development of HCC. In recent years, advances in therapeutic modalities and strategies have improved the chance of curative treatment for HCC. Liver transplantation and surgical resection are regarded as the main therapeutic treatment after considering tumor extent and hepatic functional reserve (3). And molecular targeted chemotherapy such as sorafenib and monoclonal antibodies can also significantly improve the long-term survival of HCC patients (4). Because the genetic heterogeneity of different patients affect their response to chemotherapy and the limits of other therapies exist, the HCC patients' long-term prognosis is still poor for the metastasis and the frequent recurrence (4, 5).

During HCC tumorigenesis and progression, specific alterations in oncogenes and tumor suppressor-genes are required (6, 7). Hence identifying novel oncogenes as HCC therapeutic agents is a focal point of anti-cancer research. Stanniocalcin (STC) is a family of secreted glycoprotein hormones that originally discovered in the corpuscles of Stannius, an endocrine gland of fish (8, 9). STC1 and STC2, two homologues of STC family, are reported to involve in calcium and phosphate homeostasis (10, 11). Both two proteins differ at C-terminal half but share similar hydropathy profiles at the N-terminal half (12). STC1 is the first discovered Stanniocalcin family member and has been implicated to participate in different physiological functions (13, 14). STC2 is widely expressed in human tissues with high transcripts levels in skeletal muscle, heart and pancreas (12, 15). Several reports have revealed that STC2 overexpression could promote tumor cell proliferation, invasion and metastasis in prostate cancer, ovarian cancer or neuroblastoma (16-18). STC2 is also vital for cytoprotective properties when exposed to ER stress and hypoxia (19, 20). Although STC2 is well-known in other tumor types, the relationship between STC2 and HCC has not yet been reported so far.

In this current study, we examined the expression pattern of STC2 in HCC specimens and cell lines, and analyzed the potential correlation between STC2 and HCC. In addition, we also tested its effect on cell proliferation and migration, and investigated the potential role of STC2 on HCC cell cycle transition.

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[#]Haixiao Wang and Kuangjie Wu contributed equally to this work. <http://dx.doi.org/10.5483/BMBRep.2012.45.11.086>

Received 26 April 2012, Revised 14 May 2012, Accepted 6 June 2012

Keywords: Cell migration, Cell proliferation, Hepatocellular carcinoma, Stanniocalcin 2

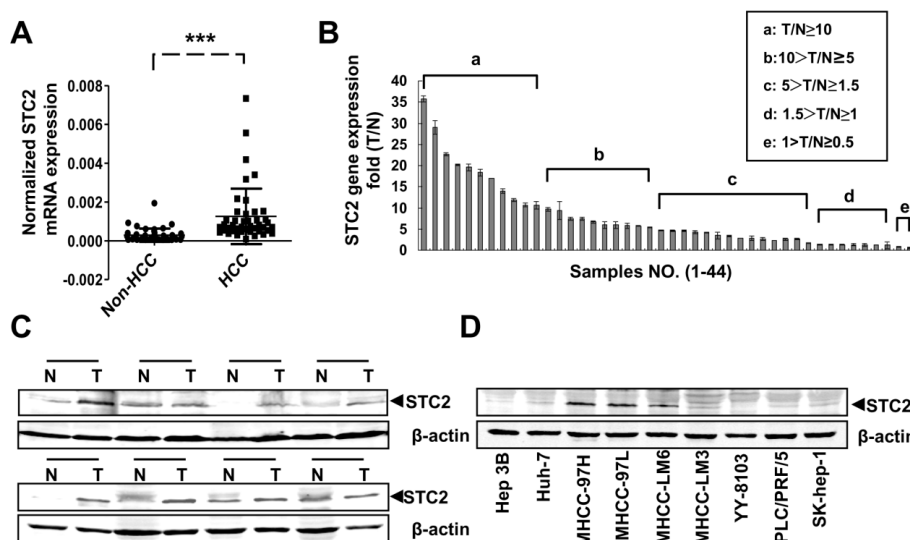


Fig. 1. Expression pattern of STC2 in HCC specimens and cell lines. (A, B) The mRNA expression levels of STC2 in 44 paired HCCs and non-HCCs were determined by quantitative real-time PCR and normalized by $2^{-\Delta\Delta Ct}$ method, where β -actin was used as internal control. Compared with noncancerous tissues, the mRNA level of STC2 was significantly higher in 79.5% HCC specimens. The mean ratios of STC2 mRNA levels in HCC tissues to those in paired paratumor tissues (T/N) in each case were calculated and showed by a column. (C) The protein levels of STC2 in 8 matched HCC specimens were analyzed by western blotting. β -actin was used as loading reference. (D) The relative expression levels of STC2 in HCC cell lines were evaluated by Western blotting. *** $P < 0.0001$.

Finally, we explored the underlying mechanism of STC2 functions in HCC. Therefore, the data strongly indicated that STC2 might represent as a promising novel therapeutic target for HCC.

RESULTS

STC2 is frequently upregulated in HCC

To evaluate the expression pattern of STC2 in HCC tissues, we performed quantitative real-time PCR approach in 44 pairs of HCCs and non-HCCs. The results indicated that STC2 was significantly upregulated in HCCs compared with the paired non-HCC tissues (Fig. 1A, $P < 0.0001$). Among the 44 pairs of clinical samples, 35/44 (79.5%) showed STC2 upregulation ($T/N \geq 1.5$), 21/44 (47.7%) ($T/N \geq 5$) had severe STC2 increase. 7/44 (15.9%) had no difference ($1.5 > T/N \geq 1$), and 2/44 (4.5%) was downregulated ($1 > T/N \geq 0.5$) (Fig. 1B). Moreover, total proteins were extracted from random 8 paired HCCs and paratumor tissues. The protein expression of STC2 was determined by western blot using a polyclonal antibody to STC2. 7/8 (87.5%) were significantly elevated in HCCs than paired non-HCC tissues in line with the quantitative real-time PCR results (Fig. 1C).

Subsequently, we accessed the correlation between STC2 expression and clinicopathological characteristics in HCC. The results demonstrated STC2 mRNA levels were higher in samples with diameter of tumor ≥ 5 cm than those < 5 cm, and in samples with multiple tumors than those with a single tumor (Supplemental Table 1, $P < 0.05$). Furthermore, it was trend to be associated with the high TNM staging (I/II vs III/IV) of HCC (Supplemental Table 1, $P=0.063$). Other clinicopathologic parameters, such as sex, patient age (≤ 50 years vs > 50 years), HBsAg, liver cirrhosis, Child-Pugh score, serum AFP, tumor differentiation, tumor encapsulation and microvascular invasion are

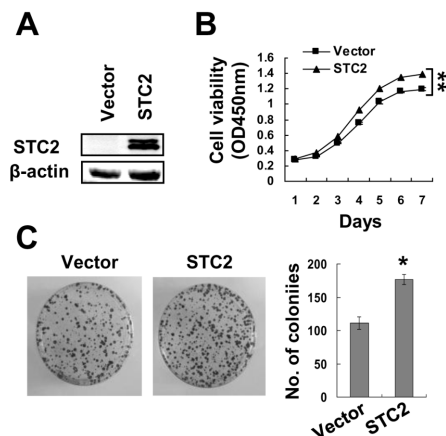


Fig. 2. STC2 overexpression promotes HCC cell proliferation and colony formation. (A) Exogenous STC2 was expressed in YY-8103 cells transfected with the pcDNA3.1-STC2. Parallel cells transfected with empty vector were used as a control. (B) STC2 overexpression promoted cell proliferation of YY-8103 cells. (C) Ectopic STC2 expression enhanced the colony formation ability of YY-8103 cells. The histogram represents the mean colony number of 3 independent experiments (mean \pm SD). * $P < 0.05$ and ** $P < 0.01$.

not significantly related to the upregulation of STC2 (Supplemental Table 1, $P > 0.05$). In addition, we measured the protein expression level of STC2 in available 8 HCC cell lines by western blot analysis. The results showed that STC2 level was higher in MHCC-97H, MHCC-97L and MHCC-LM6 cells, which all possess high metastatic potential (Fig. 1D). Taken together, the expression pattern of STC2 in clinical samples and cell lines suggested it may play an important role in the pathogenesis of HCC.

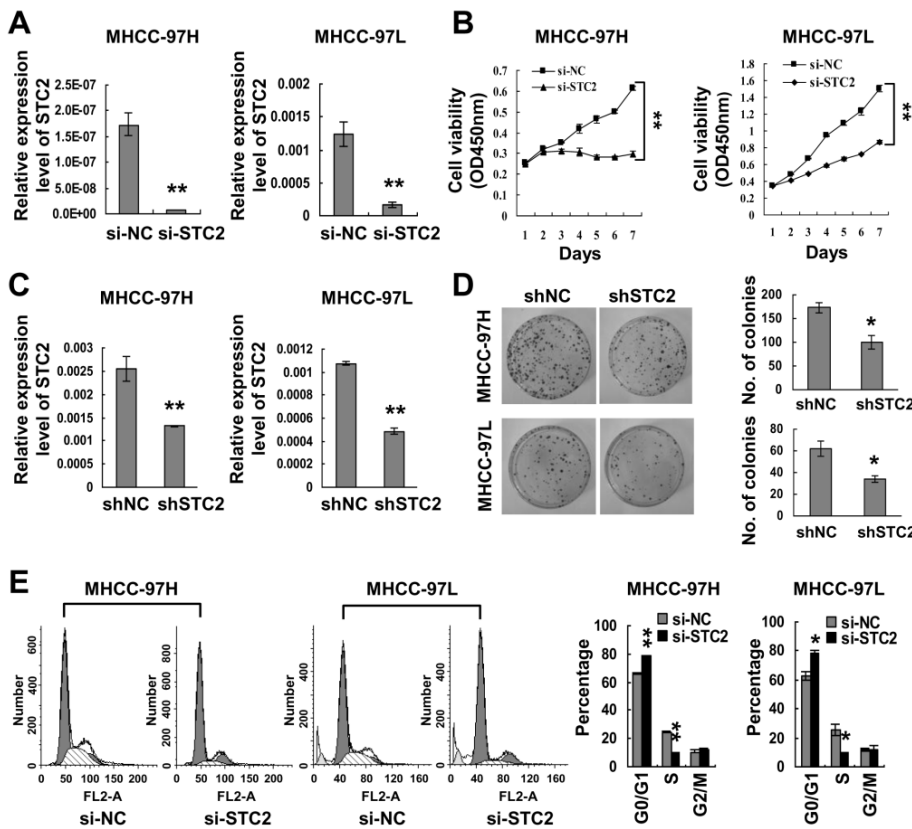


Fig. 3. STC2 silencing suppresses cell growth and induces cell cycle delay in HCC cells. (A) The specific siRNA for STC2 and control siRNA were transiently transfected into MHCC-97H and MHCC-97L cells. The RNAi efficiency was demonstrated by quantitative real-time PCR, where si-NC was used as control. (B) STC2 depletion by siRNA dramatically inhibited cell proliferation in both MHCC-97H and MHCC-97L cells using cell viability assay. (C) The pSUPER-shRNA against STC2 significantly knocked down the endogenous STC2 mRNA expression. (D) STC2 knockdown by shRNA reduced the colony formation, as shown by representative dishes of colony formation of those cells transfected with pSUPER-shSTC2 (right) and vector used as control (left). The mean colony number of three independent experiments (mean \pm SD) was indicated by the histograms (right). (E) STC2 silencing by siRNA resulted in cell cycle significantly arrest at G₀/G₁ phase both in MHCC-97H and MHCC-97L cells by flow cytometry, where si-NC was used as a control. The histograms (bottom) showed the mean and standard deviation of three independent experiments. *P < 0.05 and **P < 0.01.

STC2 overexpression promotes cell proliferation and colony formation

Since a high STC2 mRNA expression is associated with larger tumor size (Supplemental Table 1), we first evaluated the effect of STC2 overexpression on HCC cell growth. Compared to the empty vector control, STC2 overexpression markedly promoted cell proliferation of YY-8103 cells (Fig. 2B, P < 0.01). To further confirm the cell growth promotion induced by STC2, we performed colony formation assays and the result illustrated that ectopic expression of STC2 dramatically increased the number of colonies relative to controls (Fig. 2C, P < 0.05). These data indicated that STC2 is essential to maintain HCC cell proliferation.

Silencing of endogenous STC2 suppresses cell growth and colony formation of HCC cells

To investigate the effect of STC2 depletion on cellular growth and colony formation of HCC cells, cell proliferation and colony formation assays were employed. As shown in Fig. 3A and C, the siRNA-mediated or pSUPER-shRNA-mediated RNA interference dramatically reduced the STC2 mRNA expression level of MHCC-97H cells compared with the control si-NC or pSUPER-shNC. STC2 knockdown could significantly suppress cell growth and colony formation of MHCC-97H relative to the controls (Fig. 3B and D, P < 0.05). The same impacts of STC2 silencing on cell proliferation and colony formation were also observed in

MHCC-97L cells (Fig. 3).

To elucidate the mechanism by which STC2 affects cell growth, we investigated the cell cycle profile of MHCC-97H and MHCC-97L cells by flow cytometry. As shown in Fig. 3E, the populations of cells in G₀/G₁ phase increased remarkably while this G₀/G₁ arrest correlated with a decrease in the S phase population in both cell lines at 72 hours after STC2 knockdown by si-STC2 (Fig. 3E, P < 0.05). This result suggested that STC2 affects cell growth through involving in G₁ to S transition of cell cycle progression.

STC2 positively regulates cyclin D1 and pERK1/2 expression

Cyclin D1 is required for cell cycle progression from G₁ to S phase (21). To explore the mechanism by which STC2 induces G₀/G₁ phase arrest, the expression level of cyclin D1 was monitored by western blot. The results showed that the protein level of cyclin D1 was significantly reduced in MHCC-97H and MHCC-97L cells when treated with si-STC2 (Fig. 4). In contrast, STC2 overexpression increased the protein level of cyclin D1 in YY-8103 cells. As known, sustained ERK1/2 activity is required for the continued expression of cyclin D1 in G₁ phase. We next assessed the phosphorylation level of ERK1/2 and western blot results demonstrated that phosphorylated ERK1/2 was increased in YY-8103 cells transfected with STC2 plasmid and decreased in MHCC-97H and MHCC-97L cells when treated with si-STC2 (Fig. 4). Cyclin D1 ex-

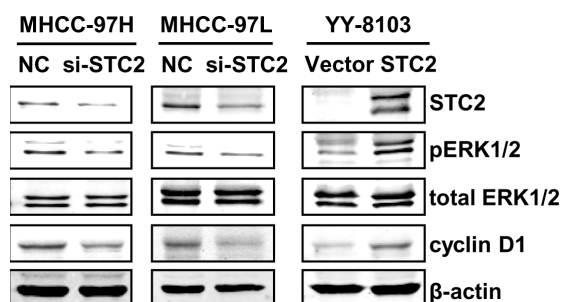


Fig. 4. STC2 regulates cyclin D1 expression and ERK1/2 phosphorylation level in a dominant-positive manner. STC2 knockdown by siRNA down-regulated cyclin D1 expression and phosphorylated ERK1/2 level in MHCC-97H and MHCC-L cells by western blot analysis. β -actin was used as internal control. STC2 overexpression in turn significantly up-regulated the protein level of cyclin D1 and phosphorylated ERK1/2 in YY-8103 cells, where pcDNA3.1 vector was used as a negative control. The experiment was done in triplicates.

pression and ERK1/2 activation by STC2 supported from molecular level that STC2 is involved in the regulation of cell cycle.

STC2 promotes HCC cell migration *in vitro*

Given that STC2 expression is highly correlated with the metastatic potential of HCC cells (Supplemental Table 1), pcDNA3.1-STC2 was transiently transfected into PLC/PRF/5 and SK-hep-1 cells in which STC2 expression are relatively lower. Cell migration assay indicated that larger number of cells migrated to the underlayer of membrane in overexpression group compared with vector group (Fig. S1A and B, $P < 0.0001$). And STC2 silencing in MHCC-LM6 cells dramatically suppressed cell migration ability compared to the control cells (Fig. S1C). Notably, we also detected the expression of some migration related gene, such as E-cadherin and its negative regulator Zeb1 and Snail. As shown in Fig. S1D, the expression of E-cadherin was decreased and Zeb1 and Snail were increased when STC2 was overexpressed in PLC/PRF/5 and Sk-hep-1 cells, supporting the phenotypic evidence that STC2 promotes cell migration from molecular level. To exclude that increased or decreased ability of cell migration were the consequence of changed growth, cell viability assay was employed. Etoposide expression of STC2 showed no significant impacts on PLC/PRF/5 and SK-hep-1 cellular proliferation between the treated cells and controls within 72 hours after transfection of pcDNA3.1-STC2 (Fig. S2A). Meantime STC2 knockdown did not affect cell growth in MHCC-LM6 cells within 72 hours (Fig. S2B). Taken together, these data suggested that STC2 can dramatically enhance HCC cell migration independent of cell proliferation.

DISCUSSION

To the best of our knowledge, the present study is the first report to validate mRNA and protein levels of STC2 in HCC and highlights the oncogenic role of STC2 in HCC. Among the 44 pairs of

HCC tissues detected in this study by quantitative real-time PCR, STC2 was frequently upregulated in HCC tumor tissues relative to matched nontumorous samples. Furthermore, we also showed that STC2 protein level was increased in 8 representative pairs of HCC samples. Thus, the expression pattern of STC2 in HCC is consistent with that in other tumors, implying that STC2 may play a similar role in various different tumor types. The analysis of clinical correlation illustrated that STC2 overexpression in HCC was associated with tumor size and tumor multiplicity, meanwhile, HCC cell lines with high STC2 expression are abundant with migration property. These data indicated that STC2 might boost HCC carcinogenesis and metastasis. As expected, cell biology experiments performed in this study revealed that STC2 overexpression promoted HCC cell proliferation and migration, and STC2 knockdown led to a suppression of cell growth and migration *in vitro*. Therefore, in this report, we have added new information about STC2 in tumor development.

Recent evidences suggested that STC2 is a poor prognostic marker for patients with renal cell carcinoma, gastric cancer and *et al.* (22, 23), while it is correlated with longer disease-free survival in breast cancer (24). Intriguingly, Schmitz KJ reported that activation of ERK1/2 in HCC was strongly correlated with a reduced overall survival and served as a significant predictor of prognosis (25). Importantly, large tumor size is a detrimental prognostic factor and tumor multiplicity is also an independent risk factor for survival and tumor recurrence in HCC (26). As a secreted protein, STC2 may be identified as a potential promising biomarker to monitor the recurrence and prognostic after treatment of HCC, which is required for further investigation.

As reported by many studies, the regulation of cell cycle G_1/S check-point was usually abnormal in tumor cells. To address the biological function of STC2 in HCC cells, flow cytometric analysis of MHCC-97H and MHCC-97L cells revealed that knockdown of STC2 arrested the cells in G_0/G_1 or at the G_1/S border. Concomitant with cell cycle arrest, STC2 knockdown decreased the phosphorylated ERK1/2 level and induced a reduction of cyclin D1. Since both ERK1/2 and cyclin D1 are responsible for G_1/S transition, our data indicated at molecular level that STC2 knockdown blocked cell cycle progression in experimental HCC cells. In support of this conclusion, when STC2 was overexpressed in YY-8103 cells, we also observed an increase in phosphorylated ERK1/2 and cyclin D1 level by western blot.

In summary, we present data that STC2 affects HCC cells viability, colony formation and migration ability in a dominant-positive manner. Furthermore, STC2 regulates cell cycle transition from G_1 to S phase. Finally, the protein level of cyclin D1 and pERK1/2 was decreased in STC2-downregulated cells (MHCC-H and MHCC-L) and increased in STC2-upregulated cells (YY-8103). All the data suggest that the upregulation of STC2 contributes to HCC progression and metastasis. The results of the present study not only help to understand the role of STC2 in HCC tumorigenesis and progression, but also strongly imply that STC2 might represent as a novel biomarker and a promising therapeutic target for the intervention of HCC in the Stanniocalcin family.

MATERIALS AND METHODS

Tissue specimens and cell culture

In this study, paired HCCs and adjacent paratumor liver tissues from 44 patients incorporating HCCs were obtained with informed consent. The experiments were approved by the Ethics Committee of the Wuxi People's Hospital. Clinical and pathological information was extracted from medical charts and pathology reports of these patients. The clinicopathological characteristics of the patients and their cancers are detailed in Supplemental Table 1. HCC cell lines were from American Type Culture Collection (Manassas). Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) (Gibco) at 37°C in 5% CO₂.

RNA extraction and quantitative real-time PCR

Total RNA was extracted by TRIZOL reagent (Invitrogen) and reverse-transcribed into cDNA using M-MLV reverse transcriptase kit (Promega). The following primers were used to specially amplify the STC2 gene and β-actin (monitored as a control). The primer sequences were: STC2, forward primer, 5'-ATGCTACCTC AAGCAGACC-3'; reverse primer, 5'-TCTGCTCACTGAACC TGC-3'. β-actin, forward primer, 5'-AGAGCCTCGCCTTTGCC GATCC-3'; reverse primer, 5'-CTGGGCCTCGTCCACATA-3'. The copy number of each sample was calculated and all the data were normalized to β-actin prior to comparative analysis using 2^{-ΔΔCt} method (27).

Plasmid construction

The open reading frame (ORF) of STC2 (human, NM_003714) was amplified by PCR from the cDNA of MHCC-97H cells. STC2 ORF was inserted into the c-myc-tagged pcDNA3.1(B) expression vector (Invitrogen) to construct pcDNA3.1-STC2 plasmid according to the manufacturer's protocol.

Cell transfection

Cell transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA interference

For siRNAs transfection, STC2-specific small interference RNA (siRNA) and the non-specific control siRNA were chemically synthesized by GenePharma. The special siRNA sequences used were as follows: siRNA_STC2 (sense 5'-GUGGAGAUGAUCCA UUUCATT-3' and antisense 5'-UGAAAUGGAUCAUCUCCAC TT -3'). The non-specific siRNA not targeting any annotated human genes was used as negative control. Moreover, for the construction of the RNAi plasmid, the special oligonucleotide fragment encoding short hairpin RNA (shRNA) for STC2 silencing was cloned into the pSUPER vector (OligoEngine). The oligonucleotide sequences for shRNA were: shRNA_STC2, forward, GAT CCCCCTGGAGATGATCCATTTTCATTC AAGAGATGAAATGGA TCATCTCCACTTTTTGGAA; reverse, AGCTTTTCCAAAAGTG

GAGATGATCCATTTTCATCTCTTGAATGAAATGGATCATCTCC ACGGG. The pSUPER vector containing irrelevant nucleotide was used as negative control.

Cell proliferation and colony formation assay

HCC cells were seeded into 96-well plates at a density of 3 × 10³ cells per well in 100 µl medium. The absorbance values were determined at a wavelength of 450 nm using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's manual. Cells transfected with vector or pcDNA3.1-STC2 were plated at a density of 8 × 10⁴ per 10 cm plate and selected in appropriate medium of 0.8 mg/ml G418 (Life Technologies) for colony formation at 37°C in 5% CO₂. After three weeks, the cells were rinsed with PBS, fixed with paraformaldehyde and stained with coomassie brilliant blue. The colonies were photographed and statistically analyzed.

Cell cycle analysis

The treated cells were harvested as single cell suspensions, washed with PBS and incubated with propidium iodide (10 µg/ml) and RNase A (10 mg/ml) for 30 min at 4°C in the dark. Cell cycle distribution was analyzed by a FACSCalibur flow cytometer (Becton-Dickinson).

Western blot analysis

The treated cells were washed twice with cold PBS and scraped off from the plates. Cells were resuspended in cold lysis buffer [25 mmol/L Tris (pH 6.8), 1% SDS, 5 mmol/L EDTA, protease inhibitor cocktail (Sigma)]. Tissue specimens were ground in liquid nitrogen and lysed in RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS]. The blotted membrane was incubated overnight with the primary antibody at 4°C. The membrane was incubated with the secondary antibody for 1 h at room temperature. Mouse antibody to cyclin D1 (Santa Cruz Biotechnology), and rabbit antibodies to STC2 (Abcam), pERK1/2 and total ERK1/2 (cell signaling technology) were used in this study.

Cell migration assay

The migration ability of HCC cells was assessed using the Transwell chambers. 3 × 10⁴ cells were resuspended in 500 µl DMEM without FBS. Then cells were plated in the upper chamber of 24-well transwells without matrigel and 750 µl DMEM containing 10% FBS were added to the lower chamber. After incubated for 36 h at 37°C in 5% CO₂, cells located upon the upper membranes were wiped with cotton swabs. The cells migrated to the lower surface of membrane were fixed in ethanol and stained with crystal violet. The migrated cells in five random fields (magnification, ×100) were photographed and counted for each group. The migration capability of cell was determined by the average number of migrated cells in the five fields.

Statistical analysis

All experiments were repeated at least three times and each ex-

periment was performed at least in duplicate. All results are presented as mean \pm standard deviations. Statistic analysis was performed using Student's t test analysis from GraphPad Prism-5.0. P value < 0.05 was considered statistically significant.

Acknowledgements

This work was supported by grants from the Academician Workstation of Wuxi People's Hospital (CYE00917), and the Chinese National Key Program on Basic Research (2010CB-529204), and the China National Key Projects for Infectious Disease (2008ZX10002-021).

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