Negative regulation of hepatocellular carcinoma cell growth by signal regulatory protein ?1

ARTICLE *in* HEPATOLOGY · SEPTEMBER 2004

Impact Factor: 11.06 · DOI: 10.1002/hep.20360 · Source: PubMed

Negative Regulation of Hepatocellular Carcinoma Cell Growth by Signal Regulatory Protein α1

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Signal regulatory protein (SIRP) α1 is a member of the SIRP family that undergoes tyrosine phosphorylation and binds SHP-2 tyrosine phosphatase in response to various mitogens. The expression levels of SIRPα1 were decreased in HCC tissues, compared with the matched normal tissues. Exogenous expression of wild type SIRPα1, but not of a mutant SIRPα1 lacking the tyrosine phosphorylation sites, in SIRPα1-negative Huh7 human HCC cells resulted in suppression of tumor cell growth both *in vitro* and *in vivo*. Treatment of Huh7 transfectants with EGF or HGF induced tyrosine phosphorylation of SIRPα1 and its association with SHP-2, which were accompanied by reduced ERK1 activation. Expression of SIRPα1 significantly suppressed activation of NF-κB and also sensitized Huh7 cells to TNFα or cisplatin-induced cell death. In addition, SIRPα1-transfected Huh7 cells displayed reduced cell migration and cell spreading in a fashion that was dependent on SIRPα1/SHP-2 complex formation. In conclusion, a negative regulatory effect of SIRPα1 on hepatocarcinogenesis is exerted, at least in part, through inhibition of ERK and NF-κB pathways. (HEPATOLOGY **2004;40:618–628.**)

Signal regulatory protein (SIRP) α1, also known as Src homology-containing phosphotyrosine phosphatase substrate (SHPS) 1, is a member of the immunoglobulin-like receptor superfamily proteins. The putative extracellular region of SIRPα1 possesses 3 immunoglobulin-like domains with multiple *N*-linked glycosylation sites. The cytoplasmic region of SIRPα1 contains 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) with 4 tyrosine residues that are phosphorylated in response to a variety of growth factors and integrin-mediated cell adhesion.[1–3] This phosphorylation enables recruitment and activation of Src homology-containing phosphotyrosine phosphatase 2 (SHP-2) that in turn dephosphorylates specific protein substrates involved in mediating various physiological effects.

SHP-2, a widely expressed cytoplasmic tyrosine phosphatase with 2 Src homology-2 domains, has been implicated in growth factor-induced cell proliferation probably through activation of the Ras–mitogen-activated protein kinase (MAPK) cascade.[4] SIRPα1/SHP-2 complex negatively or positively regulates intracellular signaling initiated either by tyrosine kinase-coupled receptors for growth factors or by cell adhesion to extracellular matrix proteins. For example, SIRPα1 overexpression in NIH3T3 cells inhibited DNA synthesis and MAPK phosphorylation following endothelial growth factor (EGF) or insulin stimulation.[1] In contrast, overexpression of wild-type SHPS-1 (the murine homolog of SIRPα1) was reported to increase MAPK activity in response to insulin or integrin stimulation.[5] SHPS-1 overexpression suppressed anchorage-independent cell growth and cancer dissemination; however, phosphorylation states of MAPK and c-jun NH2-terminal kinase (JNK) were almost similar.[6] In fibroblasts expressing an SHPS-1 mutant lacking most of the cytoplasmic region, growth factor–induced JNK

Abbreviations: SIRP, signal regulatory protein; SHPS, Src homology-containing phosphotyrosine phosphatase substrate; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP-2, Src homology-containing phosphotyrosine phosphatase 2; MAPK, mitogen-activated protein kinase; EGF, endothelial growth factor; JNK, c-jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; HCC, hepatocellular carcinoma; TNF, tumor necrosis factor; HGF, hepatocyte growth factor; cDNA, complementary DNA; PARP, poly(ADP-ribose) polymerase; IκB, inhibitor of κB; IKK, IκB kinase; kb, kilobase; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum.

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Received November 11, 2003; accepted May 21, 2004.

Supported by grants from High-Tech Research and Development Program of China (2001AA221021); China Key Basic Research Program (2002BA711A02-3); and National Natural Science Foundation of China (30270686 and 30370740).

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20360

activation was enhanced, whereas extracellular signal-regulated kinase (ERK) activation was dependent on the kind of growth factor.[7] Moreover, overexpression of SIRPα1 was reported to negatively regulate EGF-induced PI3-K activation and modulate nuclear factor (NF)-κB signaling.[8,9] Thus, the precise mechanism by which SIRPα1/SHP-2 complex regulates MAPK and other signaling pathways remains unclear.

Cell migration is crucial for multiple biological functions including embryonic development and tumor metastasis. Formation of SIRPα1/SHP-2 complex was implicated in regulation of cell migration. However, the effects of SIRPα1 on cell motility were also controversially reported. Overexpression of wild type SHPS-1 promoted CHO cell migration, whereas expression of SHPS-4Y mutant, which lacks the phosphorylation sites required for SHP-2 binding, had no effect.[10] Likewise, fibroblasts homozygous for expression of an SHPS-1 mutant lacking most of the cytoplasmic region of this protein exhibited defective migration associated with increased formation of actin stress fibers and focal adhesions.[7] In contrast, ectopically expressed SHPS-1/SIRPα1 in v-Src–transformed BALB/c3T3 cells or U87MG cells led to marked impairment of cell migration and cancer dissemination.[6,8] These observations therefore suggest that the functional consequences of tyrosine phosphorylation of SIRPα1 and its association with SHP-2 in cell migration have not been established.

CD47 (or integrin-associated protein [IAP]), an important component of the signaling pathway triggered by integrins or cell-cell adhesions, has been implicated as a ligand for SIRPα1.[11] The CD47/SHPS-1 system was recently shown to inhibit cell migration by cell-cell contact[10] and to play novel regulatory roles on immune cells.[3] SIRPα1 has also been shown to bind to various adaptor proteins such as FyB/SLAP130, SKAP55 and Grb2.[12] Thus, SIRPα1 appears to function in a variety of cellular signaling systems.

Recently it has been reported that the expression level of SIRPα1 was decreased in breast cancer tissues and leukemia myeloid cells, indicating a role of SIRPα1 in oncology.[6,13] In this study, we observed that SIRPα1 expression in hepatocellular carcinoma (HCC) tissues frequently seemed lower than that of paired normal tissues. Exogenous expression of SIRPα1 in the HCC cell line led to inhibition of cell cycle progression and cell growth, which is associated with reduced ERK1 activation after EGF or hepatocyte growth factor (HGF) stimulation. Furthermore, SIRPα1 conferred enhanced apoptosis following tumor necrosis factor (TNF) α or cisplatin treatment in Huh7 cells by negatively regulating NF-κB signaling. In addition, SIRPα1 expression also led to reduced cell spreading and migration. In brief, we present evidence that SIRPα1 functioned as a tumor suppressor in human HCC cells.

Patients and Methods

Patients and Samples. A total of 36 HCC specimens and adjacent nontumorous liver counterparts were studied. All samples were collected at the Eastern Hepatobiliary Surgery Hospital, Shanghai, China.

Complementary DNA (cDNA) Constructs, Antibodies, and Reagents. The human SIRPα1 and SIRPα1-4Y cDNAs contained in pLXSN[1] (kindly provided by A. Ullrich, Max –Planck Institute, Martinsried, Germany) were digested with *Eco*RI and *Bam*HI and ligated into expression vector pcDNA3.0 (Invitrogen, Carlsbad, CA). The plasmids pcDNA3-SHP-2 (WT) and pcDNA3-mIκBα were kindly provided by G. S. Feng (The Burnham Institute, La Jolla, CA) and C. Scheidereit (Mark –Delbruck Center, Berlin, Germany), respectively. Antibodies specific for poly(ADP-ribose) polymerase (PARP), total MAPKs and the phosphorylated and active ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), p38 (Thr180/Tyr182), Akt (S473), and phospho-inhibitor of κB (IκB) kinase (IKK) α (Ser180)/IKKβ (Ser181) were purchased from Cell Signaling Technology (Beverly, MA) and used as previously described.[14] Anti–IκBα1 was from IMGENEX (San Diego, CA) and anti–Bcl-xL from Oncogene (Bayer, Cambridge, MA). A polyclonal antibody reactive with SIRPα1 was raised against a GST fusion protein containing the C-terminal part of SIRPα1. The monoclonal antibody against phosphotyrosine (4G10) was obtained from Transduction Laboratories (BD Biosciences, San Diego, CA). HGF and TNF-α were obtained from PeproTech (Rocky Hill, NJ) and cisplatin was purchased from Sigma (St. Louis, MO). The PI3K inhibitor wortmannin (Calbiochem, Boston, MA) was used at a 100nM concentration.

Cell Culture and Transfection. All cell lines were grown in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Stable transfection was performed as previously described.[15] Established clones were isolated and screened by Western blot analysis with anti-SIRPα1 antibody. Positive clones expressing similar levels of SIRPα1 or mutant protein were pooled and cultured in the presence of 200 μg/mL G418 for maintenance of the transgene expression.

Northern Blotting. Total RNA was prepared using TRIzol (Invitrogen), RNA (40 μg) was separated and transferred to a nitrocellulose membrane and probed using SIRPα1 cDNA labeled by random oligonucleotide priming (Promega, Madison, WI).

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting analysis with whole-cell extracts were performed essentially as previously described.[16]

Luciferase Assay. NF-κB transcriptional activity was determined using NF-κB–driven luciferase plasmid. pRL-TK (Promega) was used as internal control. A dual luciferase reporter assay was carried out according to the manufacturer's suggestions.

Cell Cycle Analysis. Huh7 cells were synchronized in G_0 by serum starvation for 3 days (in DMEM without serum), followed by stimulation in DMEM supplemented with 10% FBS. Progression through the cell cycle was monitored by detection of the DNA content as previously described.[17]

Cell Spreading and Migration Assay. Cell Spreading and migration assays were performed essentially as previously described.[16,18]

Growth Curves, Viability, and Focus Formation Assay. Huh7 cells (10^5) were plated per well of 6-well plates. At each time point, the cells were trypsinized and counted. Each data point was performed in triplicate. The measurement of viable cell mass was performed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) to count living cells by WST-8. To visualize the nuclei of Huh7 cells, DNA was stained with DAPI (1 μg/mL) and determined using fluorescence microscopy. The luciferase-based *in vitro* cell viability assay was performed as described.[19] For assays of focus formation of stably transfected cells, previously described methods were employed.[8]

In Vivo *Tumorigenicity Assay.* Huh7 cells were released from tissue culture dishes and washed in serum-free medium. Tumor cells were diluted with phosphate-buffered solution and injected into the mid-dorsum of BALB/c nude mice (4–6 weeks old) in a total volume of 0.1 mL (5×10^6). Animals were inspected weekly for tumor development. Growing tumors were measured using vernier calipers, and tumor volume was calculated by the formula length × width^2 × 0.52, which approximates the volume of an elliptical solid. Statistical analysis was performed by Student *t* test (2-tailed). The criterion for statistical significance was taken as $P < .05$.

Results

Expression of SIRPα1 Was Decreased in HCC. SIRPα1 has been shown to negatively regulate cellular responses induced by growth factors. To search for the involvement of SIRPα1 in tumorigenesis, we evaluated SIRPα1 expression in HCC tissues from 36 patients. Northern blot analysis revealed 2 major RNA transcripts of 2.5 and 3.9 kilobase (kb) in the normal tissues. Quantified data of tumors relative to paired normal tissues demonstrate that the expression of SIRPα1, especially the 2.5 kb transcript, was frequently down-regulated in cancerous tissues (Fig. 1A). Of 36 HCC tissues, 19 appeared to have a decreased expression of SIRPα1. These results were consistent with the 2 previous reports concerning SIRPα1 expression in human HCC tissue.[20,21] Immunohistochemical analysis showed that SIRPα1 was down-regulated in HCC tissues and a loss of SIRPα1 expression was observed in some tumor cells and areas (Fig. 1B). Of 4 established HCC cell lines detected (HepG2, Huh7, Sk-Hep1, and Changliver), the expression of SIRPα1 was

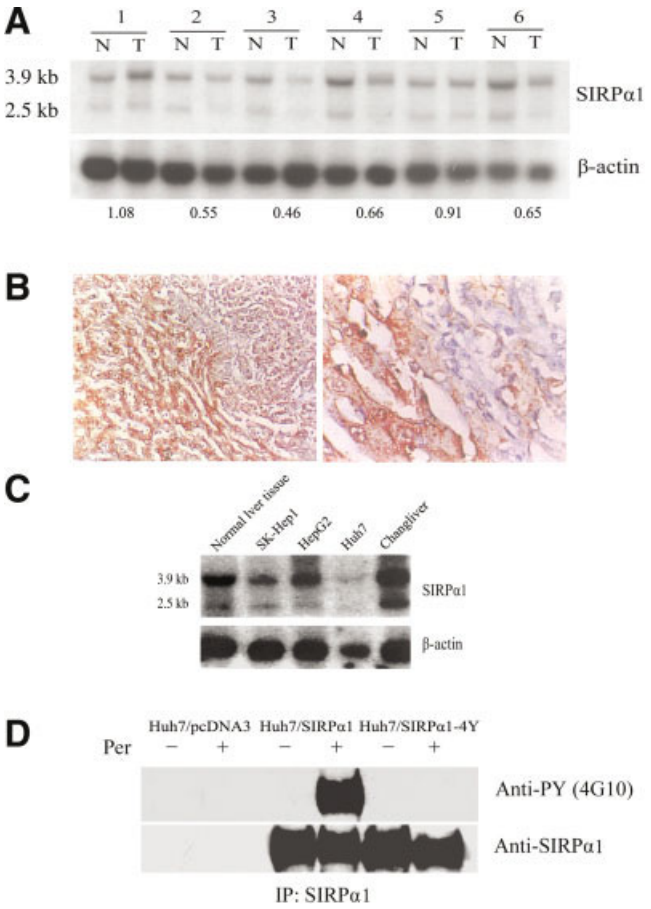

Fig. 1. Expression levels of SIRPα1 were decreased in HCC tissues. (A) Representative expression of SIRPα1 in paired normal (N) and tumor (T) tissues was analyzed by Northern blot (top panel). For quantification, band intensities were first normalized to the respective actin signal and then calculated as fold change of tumor tissues relative to paired normal tissues. (B) Immunohistochemical staining for SIRPα1 in paired normal (left) and tumor tissues (right). (Original magnification, (left) ×100, (right) ×400.) (C) Northern blot analysis of total RNA from various human HCC cell lines. A normal liver tissue served as a control for the comparison. (D) Western blots containing SIRPα1 immunoprecipitates from quiescent or pervanadate-treated Huh7/pcDNA3, Huh7/SIRPα1, and Huh7/SIRPα1-4Y cells were labeled with antiphosphotyrosine (anti-PY [4G10]; top) and anti-SIRPα1 antibodies (bottom). IP, immunoprecipitation.

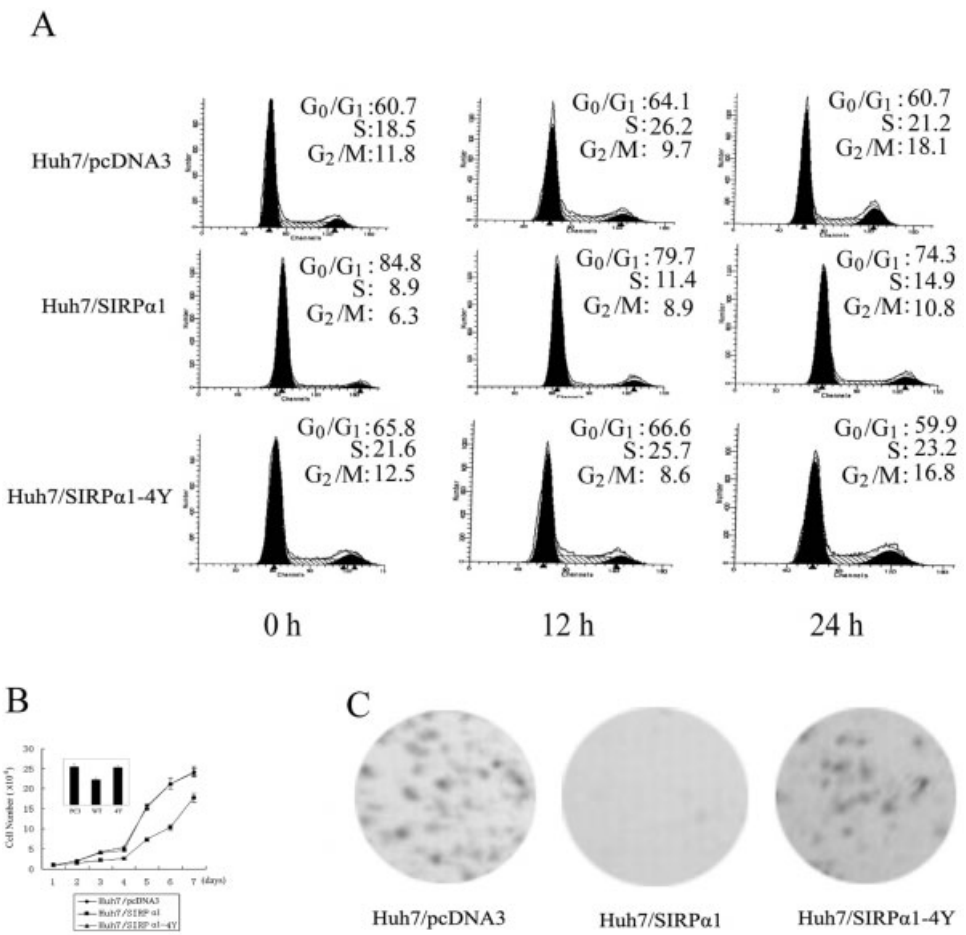

Fig. 2. Exogenous SIRPα1 expression altered cell cycle and proliferation. (A) FACS analysis of progression into S phase of 3 cell pools at 0, 12, or 24 hours after readdition of serum G, G phase; M, M phase. (B) Growth curves for stable Huh7 transfectants in complete medium containing 10% serum. Each measurement represents the mean of cell counts from at least 3 wells. (Inset) Saturation density of the 3 cell pools. (C) Focus formation of Huh7-derived cell lines: 10^4 cells from each of the cell pools were plated in a 6-well plate and grown in 10% FBS for 16 days. Foci were then stained and counted based on triplicate wells for each cell line. These data were confirmed by 3 independent experiments.

significantly decreased in Huh7 and SK-Hep1 cells (Fig. 1C).These results suggest that SIRPα1 expression might be involved in some steps of tumor development.

To elucidate the effects of SIRPα1 on liver cell functions, Huh7 HCC-derived cell line was stably transfected with either wild type SIRPα1, or a carboxyl terminal SIRPα1 mutant (SIRPα1-4Y) in which 4 carboxyl terminal tyrosines have been mutated to phenylalanine, making this mutant incapable of binding to SHP-2. Positive clones expressing similar levels of SIRPα1 or mutant protein were pooled to rule out clone-specific results. As shown in Fig. 1D, SIRPα1 became tyrosine-phosphorylated upon pervanadate treatment, but SIRPα1-4Y did not, even though their expression was comparable.

Expression of SIRPα1 Reduced Cell Proliferation in Human HCC Cells. To characterize the distribution of Huh7/pcDNA3, Huh7/SIRPα1 and Huh7/SIRPα1-4Y cells in the cell cycle, the progression of synchronized cells through the cell cycle was determined by DNA/flow cytometry analysis. As shown in Fig. 2A, SIRPα1 expression strongly delayed cell cycle progression. Growth curves also demonstrated a significant difference between the Huh7/SIRPα1 cells and Huh7/pcDNA3 or Huh7/SIRPα1-4Y cells (Fig. 2B), indicating that the alterations in cell cycle distribution resulted in decreased growth and reduced saturation densities. Furthermore, SIRPα1 expression nearly abolished the colony formation ability of Huh7 cells (Fig. 2C). These data suggest that SIRPα1 exerts an inhibitory effect on cell proliferation that was dependent on its carboxyl terminal sites mutated in the SIRPα1-4Y.

SIRPα1 Sensitized Huh7 Cells to TNF-α and Chemotherapeutic Agent-Induced Apoptosis. To examine the effect of SIRPα1 on cell survival, an *in vitro* cell viability assay was performed using a luciferase assay.[19] Following transfection with the luciferase reporter gene (pCMV-luc) and treatment with TNF-α, the luciferase activity in the Huh7/SIRPα1 cells was strongly reduced compared with that in Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells (Fig. 3A). This suggested that SIRPα1 sensitized Huh7 cells to TNF-α–induced cell death. Furthermore, DAPI staining showed clear evidence of condensed and fragmented nuclei in Huh7/SIRPα1 cells, confirming the SIRPα1-mediated sensitization of TNF-α–induced apoptosis (Fig. 3B).

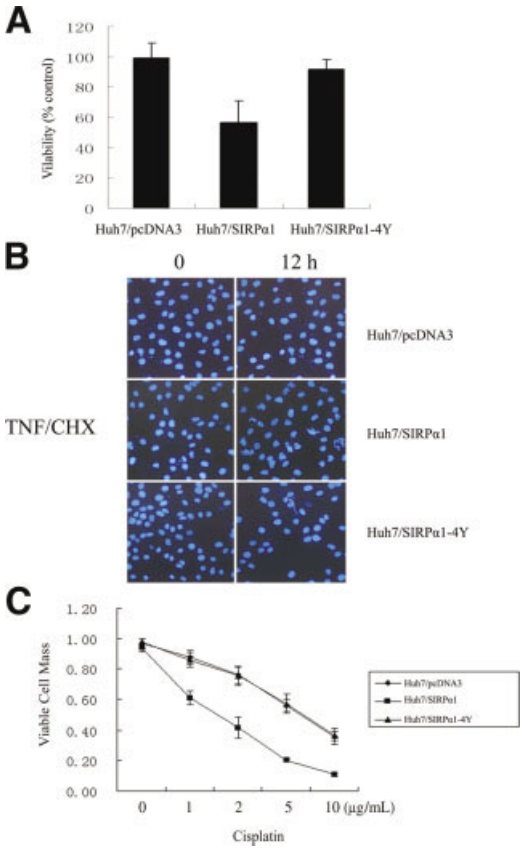

Fig. 3. Huh7/SIRPα1 cells are more susceptible to TNF-α or cisplatin-induced apoptosis. (A) Cells were transfected with a luciferase expression vector (pCMV-luc) for 24 hours, treated with TNF-α (25 ng/mL) and CHX (1 μg/mL) for 12 hours, and then harvested. Control cells were harvested after transfection without TNF-α treatment. The percentage of luciferase in the TNF-α–treated cells represents cell viability and has been normalized against that of the corresponding untreated control cells (100%). The data presented represent the mean of 3 independent experiments. (B) Nuclear condensation after 12 hours of TNF-α and CHX treatment was detected by fluorescence microscopy analysis of 3 cell pools stained with 1 μg/mL DAPI. (C) Huh7/SIRPα1 cells show lower survival after cisplatin treatment. Stable Huh7 transfectants were treated with cisplatin at different concentrations for 24 hours. Cell viability was determined by WST-8 and was shown as the percentage of living cells and as mean $\pm$ SE. All assays were carried out in triplicate and repeated at least 3 times.

Cisplatin is a DNA-reactive agent commonly used in chemotherapy protocols for the treatment of HCC. An assessment of growth effects of Huh7 cells subjected to 24 hour exposure to cisplatin was performed using WST-8 assay to measure viable cell mass. As shown in Fig. 3C, cells harboring wild type SIRPα1 were more sensitive than the empty vector control and the mutant counterpart to the growth inhibition effects of cisplatin.

SIRPα1 Negatively Regulates Growth Factor–Induced ERK1 Activation. Several groups have reported a modulation of MAPK activities by SIRPα1 or SHPS-1. To characterize the molecular mechanism responsible for the inhibitory effect of SIRPα1 on cell growth, we examined the effects of EGF and HGF, 2 major inducers of liver cell growth and proliferation, on the activity of ERK. As shown in Fig. 4A, EGF/HGF stimulation induced tyrosine phosphorylation of SIRPα1 and binding to SHP-2. Although both growth factors induced marked activation of P44 (ERK1) and P42 (ERK2) isoforms of ERK in Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells, their effects on ERK1 activity was substantially lower in the Huh7/SIRPα1 cells (Figs. 4B and C). Interestingly, TNF-α–induced ERK1 activation was similarly suppressed in Huh7/SIRPα1 cells (Fig. 4D), suggesting that growth factor- or cytokine-induced ERK1 activation was constitutively inhibited by SIRPα1 in Huh7 cells.

Because tyrosine-phosphorylated SIRPα1 was preferentially associated with SHP-2 that has been shown to positively regulate ERK activation, it is conceivable that SIRPα1-mediated ERK repression might be due to SHP-2 sequestration. To test this possibility, we transiently transfected the Huh7/SIRPα1 cells with empty vector or wildtype SHP-2 and examined ERK activity following EGF treatment. As expected, overexpression of SHP-2 restored ERK1 activation in Huh7/SIRPα1 cells, indicating that ERK activation was regulated by SIRPα1 via SHP-2.

SIRPα1 Suppressed NF-κB Transcriptional Activation. TNF-α and cisplatin have been reported to activate both the JNK and NF-κB pathways that transduced antiapoptotic signals in Huh7 cells.[22] To explore the mechanism of SIRPα1-mediated apoptosis induction, we first measured JNK and p38 activity in cells at different time points after stimulation with TNF-α or cisplatin. However, no significant difference was found in JNK or p38 activation after TNF-α or cisplatin stimulation (Fig. 5).

Having obtained these results, we shifted our attention to the NF-κB pathway. We monitored the NF-κB activity in stable Huh7 transfectants by measuring NF-κB–driven luciferase reporter activity. As shown in Fig. 6A, Huh7/SIRPα1 cells indeed displayed an evident decrease in basal NF-κB activity with respect to Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells. Interestingly, TNF-α failed to further enhance the NF-κB–driven reporter activity. Introduction of a super suppressor form of IκBα alone led to high levels of cell death (Fig. 6B). These observations indicated that the basal activity of endogenous NF-κB was important in maintaining the survival of Huh7 cells. Notablely, NF-κB–mediated transcription of Bcl-xL, an antiapoptotic member of Bcl-2 family, was also reduced in Huh7/SIRPα1 cells compared with controls (Fig. 6C). Since the antiapoptotic effect of NF-κB as well as that of Bcl-xL involves prevention of caspase activation,[23] we studied the cleavage of PARP, a caspase-3 substrate, by

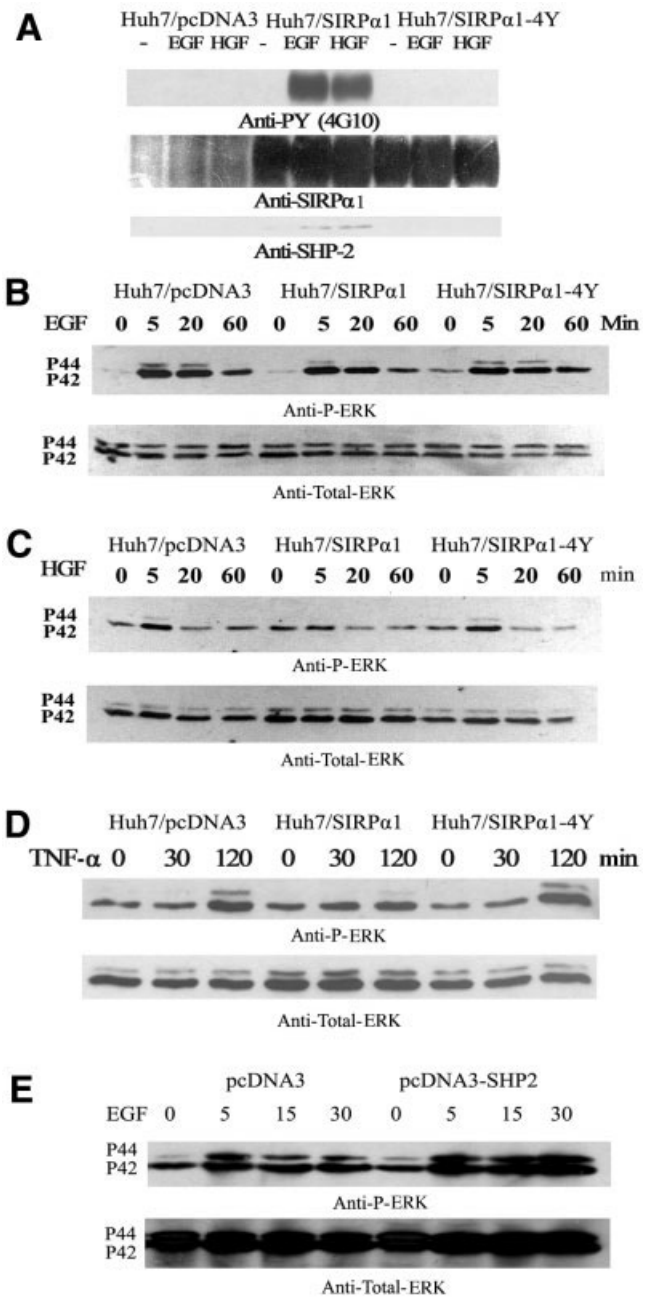

Fig. 4. SIRPα1 negatively regulates ERK1 activation in response to growth factors. (A) EGF or HGF stimulation induced tyrosine phosphorylation of SIRPα1 and its association with SHP-2. Cells were incubated for 5 minutes in the presence or absence of 50 ng/mL EGF or HGF, as indicated. Whole-cell extracts were then immunoprecipitated by anti-SIRPα1 antibody and subjected to immunoblot analysis with the indicated antibodies. (B and C) Cells were starved overnight and stimulated with EGF or HGF for the time indicated. The activated form of ERK1 and ERK2 were detected with antibodies specific for phosphorylated (P) ERK (upper panels), the same blot was also probed with antibodies to total ERK protein to ensure that the same amount of protein was present in each lane (lower panels). (D) Cells were starved overnight and stimulated with 25 ng/mL TNF-α for the time indicated. The activation of ERK1 and ERK2 was detected as described in (B) and (C). (E) Huh7/SIRPα1 cells were transiently transfected with a pcDNA3 vector or a wild-type SHP-2 construct. Twenty-four hours later, cells were treated with EGF and the phosphorylated (P) ERK was evaluated.

Western blot analysis. In Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells (Fig. 6D), the cleavage product was first detected 4 hours after TNF/CHX stimulation. In Huh7/SIRPα1 cells, in contrast, PARP cleavage had already started after 2 hours, indicating an early induction of caspase-3–like activity in the SIRPα1-expressing cells.

SHP-2 Was Involved in SIRPα1-Mediated NF-κB Suppression. To understand the molecular basis of SIRPα1-mediated NF-κB suppression, we assayed Akt and IKKα/β activities following the treatment of cells with TNF-α because both of them were required for TNF-α–induced NF-κB activation.[24] However, SIRPα1 expression did not affect their activation kinetics in response to TNF-α. Furthermore, IκBα proteolysis was also independent of SIRPα1 on TNF-α treatment (Fig. 7A). In addition, although wortmannin completely inhibited NF-κB–mediated transcription caused by addition of serum, the expression of SIRPα1 did not inhibit

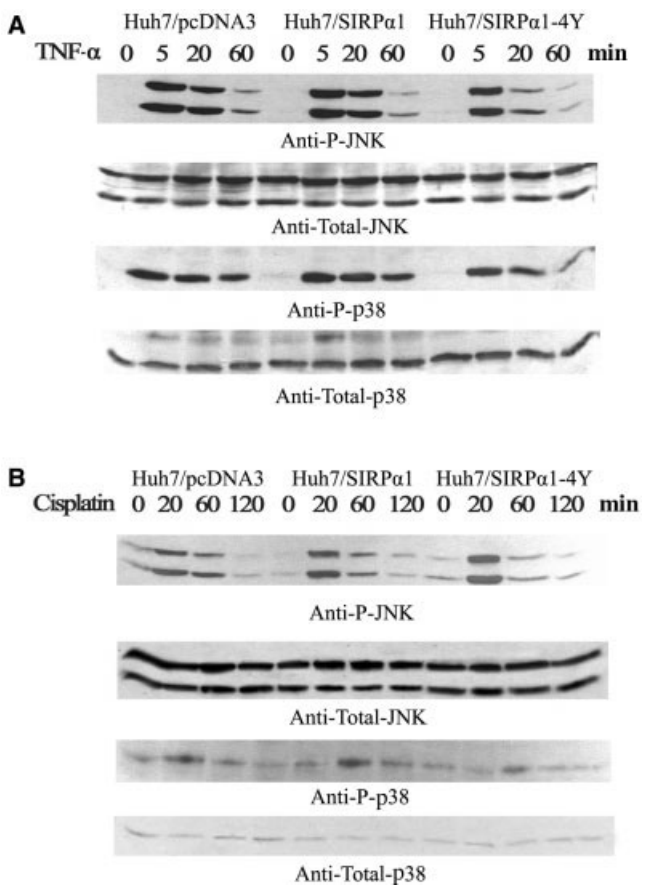

Fig. 5. Activation of JNK and p38 is normal by TNF-α or cisplatin. Serum-starved cells were treated with (A) 25 ng/mL TNF-α or (B) 20 μg/mL cisplatin for the indicated time periods. Whole-cell extracts were prepared from control and factor-treated cells. Equal amounts of cell lysates (50 μg total proteins) were subjected to immunoblot analysis with antibodies specific for the activated (phosphorylated [P]) forms of p54 and p46 JNK or of p38. The blots were also reprobed with antibodies to total JNK protein or p38 protein.

serum-induced NF-κB activation. The phosphorylation status of Akt after treatment with serum was consistently unchanged on SIRPα1 expression (Fig. 7B). These findings placed the defect in NF-κB activation regardless of the canonical PI3K/Akt pathway.

Because the SIRPα1-4Y mutant was not tyrosine phosphorylated and thus was incapable of binding the SHP-2 implicated in NF-κB activation,[14,25] we speculated that SIRPα1-mediated NF-κB suppression may also be due to SHP-2 sequestration by SIRPα1. To test this hypothesis, we analyzed NF-κB activity and survival of Huh7/

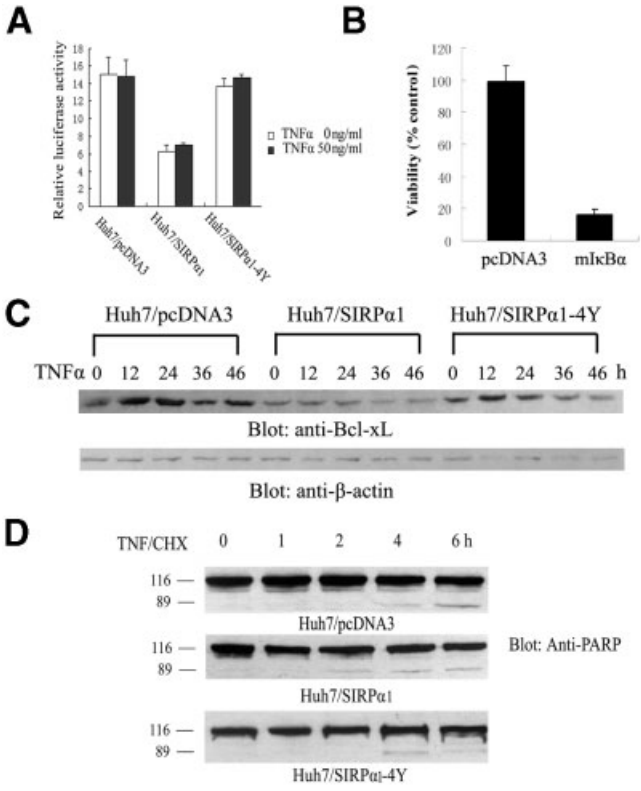

Fig. 6. SIRPα1 suppresses NF-κB transcriptional activation. (A) Cells were transfected with 100 ng of NF-κB–responsive luciferase reporter construct and 10 ng of pRL-TK plasmids as a control for transfection efficiency. Twenty-four hours after transfection, cells were treated with TNF-α (50 ng/mL) or left untreated, and luciferase activity was determined 8 hours after treatment. Data shown represent the mean of experiments performed in triplicate ± SD and are representative of at least 3 experiments with similar results. (B) The empty vector or mIκBα construct was cotransfected with the luciferase expression vector (pCMV-luc) into Huh7 cells at a ratio of 5:1. Twenty-four hours later, cells were lysed and subjected to luciferase assay. The percentage of luciferase in the transfected cells represents cell viability and has been normalized against that of the untransfected control cells (100%). (C) Western blotting of cell lysates was performed for Bcl-xL protein at the indicated times following TNF-α treatment. Normalization of protein loading was confirmed by β-actin staining. (D) Early caspase activation in SIRPα1 cells treated with TNF-α. Cells were treated with TNF-α (25 ng/mL) and CHX (1 μg/mL) for the indicated time. Whole-cell lysates were prepared and analyzed for PARP cleavage by immunoblotting with anti-PARP antibodies. Caspase activation is indicated by the appearance of 85 kd PARP cleavage product.

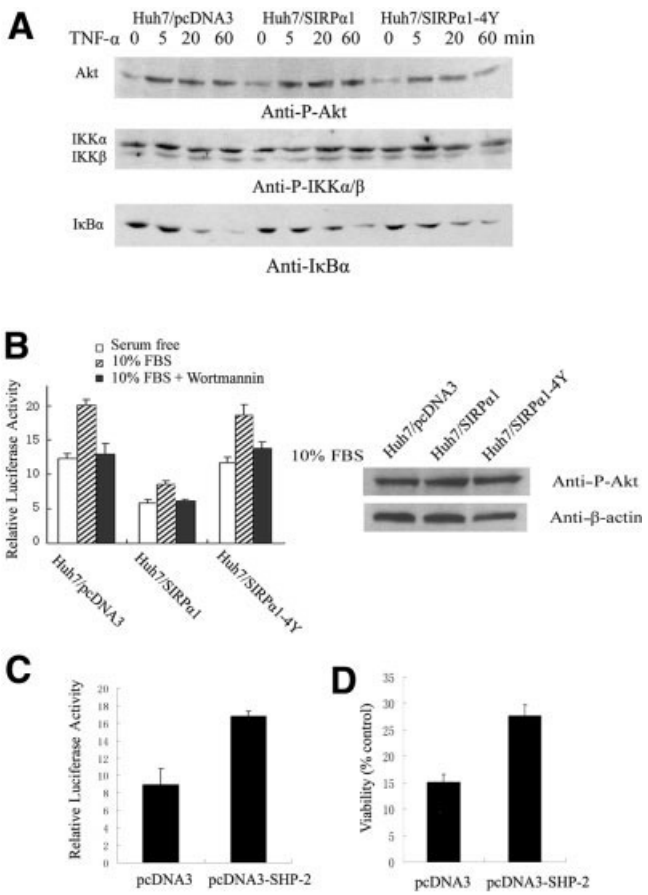

Fig. 7. SIRPα1-mediated NF-κB suppression is independent of Akt activation and can be rescued by SHP-2. (A) Serum-starved cells were stimulated with TNF-α (25 ng/mL) for the indicated time, and the cell lysates were prepared for immunoblot analysis with anti-P-Akt, anti-P-IKKα/β and anti-IκBα antibodies. (B) Cells were transfected with the NF-κB–responsive luciferase reporter construct and incubated overnight in serum-free medium followed by addition of 10% FBS. Wortmannin (100nM) added 30 minutes before serum suppressed serum-induced NF-κB activation (left); serum-induced Akt activation was analyzed by immunobloting with anti-P-Akt and β-actin antibodies (right). (C) The empty vector or SHP-2 construct were cotransfected with the NF-κB reporter plasmid into Huh7/SIRPα1 cells. Twenty-four hours later, the luciferase activity was analyzed as described in Methods and Materials. (D) Huh7/SIRPα1 cells were cotransfected with the empty vector or SHP-2 construct and the luciferase expression vector (pCMV-luc) at a ratio of 5:1 for 24 hours and treated with TNF-α (25 ng/mL) and CHX (1 μg/mL) for 24 hours. Cell viability was evaluated as described in the previous section.

SIRPα1 cells transiently overexpressing SHP-2. As shown in Fig. 7C, introduction of SHP-2 augmented NF-κB–driven reporter activity and, importantly, diminished TNF-induced cell death (Fig. 7D). These results indicated that SHP-2 was required for protection from SIRPα1-mediated TNF-α cytotoxicity.

SIRPα1 Regulated HCC Cell Spreading and Migration. Cell adhesion to extracellular matrix could induce tyrosine phosphorylation of SIRPα1 and its subsequent association with SHP-2, suggesting that this

protein also functioned in integrin-mediated signaling.[26,27] Next we examined the role of SIRPα1 in spreading of Huh7 cells on the extracellular matrix, a process promoted by integrins. As depicted in Fig. 8A, Huh7/SIRPα1 cells spread at a much reduced rate, with only about 30% of the cells spreading well by 60 minutes when more than 80% of cells containing empty vector or SIRPα1-4Y were well extended and displayed a flat morphology.

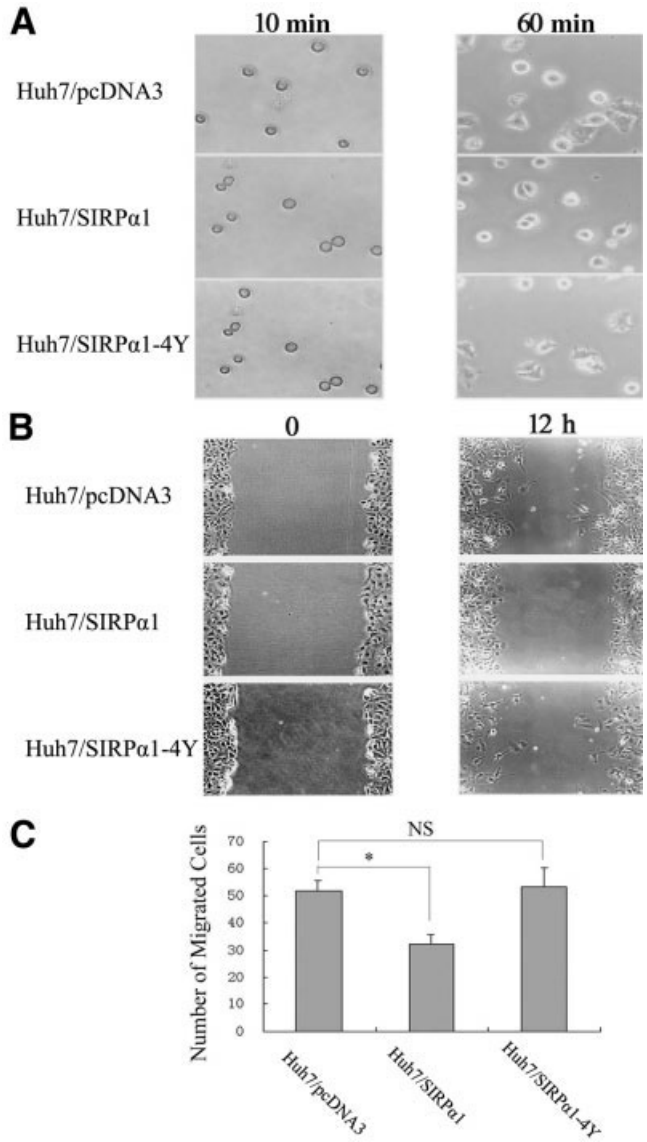

Fig. 8. SIRPα1-expressing cells exhibit reduced spreading and deficient migration. (A) Cells were plated on fibronectin-coated cell culture dishes, incubated at 37°C and then photographed at 10 and 60 minutes. The results are representative of 3 separate experiments. (Original magnification, ×200.) (B) Cells grown on fibronectin-coated dishes were wounded and maintained in serum-free medium with 40 ng/mL HGF to induce motility of Huh7 cells.[42] Cell migration into the wound was examined at 0 and 12 hours by phase-contrast microscopy. (Original magnification, ×100.) (C) Cells (1×10^5) were seeded on porous membrane coated with fibronectin in Boyden multiwell chambers. After 3 hours, cells that had migrated were stained with Giemsa solution and counted. Data represent means ± SD of triplicates from a representative experiment. NS, not significant. *$P < .05$ for the indicated comparisons (Student's *t* test).

To further explore the effect of SIRPα1 on cell motility, we examined cell migration by using "wound healing" and Boyden chamber assays. As shown in Figs. 8B and C, Huh7/SIRPα1 cells exhibited a reduced rate of cell migration compared to control cells. Taken together, these data suggest that functional phosphorylated SIRPα1 negatively regulates cell migration and cell spreading.

***Expression of Wild Type SIRPα1 Inhibited Tumorigenesis* In Vivo.** On the basis of the inhibitory effects of SIRPα1 on the proliferation, viability, and invasiveness of human HCC Huh7 cells *in vitro*, we then compared growth of the Huh7 clones after injection into athymic mice. Compared with empty vector or the SIRPα1 mutant, introduction of wild type SIRPα1 significantly inhibited tumor growth and prolonged the survival rate of tumor-bearing mice (Figs. 9A and B). Although the Huh7/SIRPα1-4Y cells showed an intermediate suppression between Huh7/pcDNA3 and Huh7/SIRPα1 cells, the survival of tumor-bearing mice in Huh7/SIRPα1-4Y and Huh7/pcDNA3 groups was not statistically significant. This observation confirmed the role of SIRPα1 in tumor growth inhibition.

Discussion

In this study, we found that expression levels of SIRPα1 were decreased in human HCC tissues and then characterized phenotypic alterations that resulted from SIRPα1 expression in the human HCC-derived Huh7 cell line. Effects were observed in the regulation of 3 important cellular activities: proliferation, apoptosis, and migration. We demonstrated that exogenous SIRPα1 expression significantly discouraged cell cycle progression and reduced the proliferation ability of these cells. Most strikingly, we demonstrated that SIRPα1 abrogated the colony formation ability of Huh7 cells and significantly suppressed tumorigenesis growth of xenografts in nude mice. In exploring the molecular mechanism responsible for SIRPα1-mediated cell growth inhibition, we found that ERK1 activity was diminished after EGF, HGF, or TNF-α stimulation in cells expressing SIRPα1. The mechanism of this selective effect is unknown; however, it is now clear that the cytoplasmic ITIM region of SIRPα1 is crucial for proper regulation of the Ras-ERK pathway by these growth factors. Although the role of tyrosine phosphorylation in the ITIM region of SIRPα1 is controversial with regard to the regulation of ERK activity, this tyrosine phosphorylation is generally indicative of receptor-mediated recruitment. In most cases, it is probable that SIRPα1 acts as a scaffolding molecule of SHP-2 and

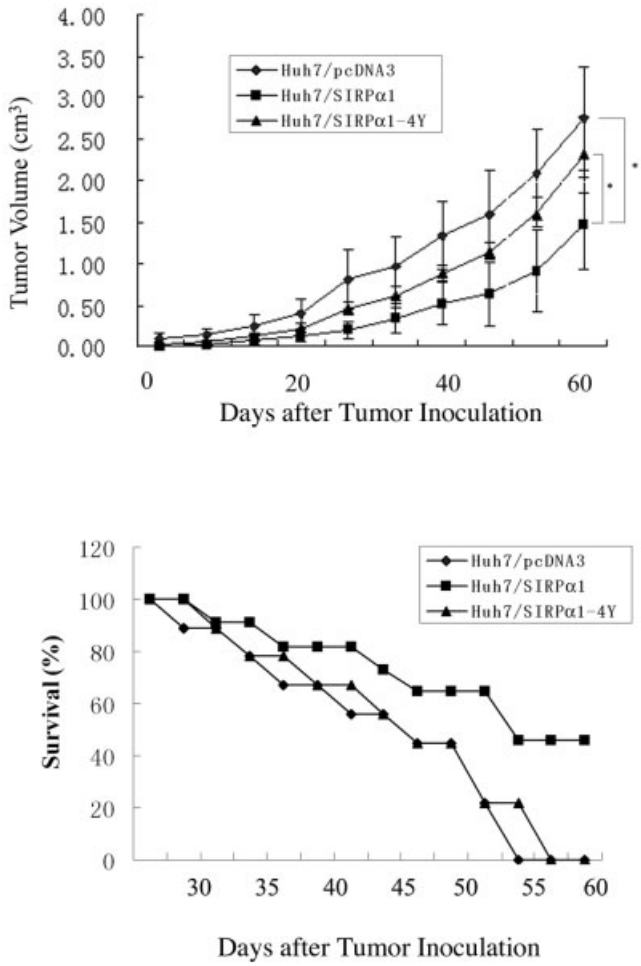

Fig. 9. SIRPα1 expression inhibited the tumorigenesis of Huh7 *in vivo*. (A) Cells (5×10^6) were subcutaneously injected into BALB/c nude mice for a total of 0.1 mL, and tumor volume was recorded weekly. Data represent the mean $\pm$SD of tumors derived from each cell line: Huh7/pcDNA3 (n = 9), Huh7/SIRPα1 (n = 11), and Huh7/SIRPα1-4Y (n = 9). The growth of tumor generated by inoculation of Huh7/SIRPα1 cells was significantly inhibited compared with that of Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells. *$P < .05$. (B) Mice inoculated with Huh7/SIRPα1 cells showed longer survival compared with the control mice ($P < .05$). There was no statistically difference between the survival of Huh7/pcDNA3- and Huh7/SIRPα1-4Y–bearing mice.

recruits it in the vicinity of the cell membrane. Thus, the nature of the SIRPα1 function may depend on the function of the phosphatase.[3] It has been widely established that the PTP activity of SHP-2 mediated positive signaling in the Raf/MEK/ERK pathway.[28–31] Our demonstration that overexpression of SHP-2 significantly upregulated ERK1 activity in Huh7/SIRPα1 cells indicated that SHP-2 was the necessary component of the growth factor–mediated ERK activation loop, and SIRPα1 might negatively regulate mitogenic signaling by sequestration of this tyrosine phosphatase from growth factor receptors.[1,32] This idea is supported by the higher affinity of SHP-2 for tyrosine-phosphorylated SIRPα1 rather than for autophosphorylated insulin and EGF receptors and p85 (the regulatory subunit of PI3K).[1,8,33]

This study confirms the negative regulatory function of SIRPα1 on cell proliferation and transformation,[1] but goes further in showing that expression of SIRPα1 may also be required for TNF-α– or chemotherapeutic agent-induced apoptosis in HCC cells. Previous studies suggested that activation of 2 signaling pathways, JNK and NF-κB, played a role in protecting Huh7 cells from apoptosis induced by TNF-α or a DNA-damaging agent.[22,34–37] Consistent with this, the present study suggests that the proapoptotic function of SIRPα1 is, at least in part, due to its inhibitory effect on NF-κB. Cytoprotection by NF-κB involves the activation of prosurvival genes, including Bcl-xL, that were previously shown to be up-regulated in HCC tissues, rendering HCC cells resistant to stress-induced apoptosis. Furthermore, down-modulation of Bcl-xL might be sufficient for the induction of apoptosis in response to cellular stress.[38] In agreement with its antiapoptotic role in HCC, reduced Bcl-xL expression was observed in Huh7/SIRPα1 cells, compared to Huh7/pcDNA3 and Huh7/SIRPα1-4Y cells, accompanied by early induction of caspase-3 activity; this might explain their increased susceptibility to apoptosis. This observation was consistent with another report that overexpression of SIRPα1 in glioblastoma cells resulted in reduced levels of Bcl-xL protein.[8]

During the course of the present study, ectopic expression of the cytoplasmic region of SHPS-1/SIRPα1 was shown to increase NF-κB–dependent transcription, presumably through promoting Akt phosphorylation; however, full-length SIRPα1 was shown to have an effect opposite to the truncated one.[9] The functional connection of SIRPα1 with the PI3K/Akt pathway was also previously reported.[8] In our study, however, expression of SIRPα1 failed to inhibit TNF-α– or serum-induced Akt activation, suggesting that separate pathways might exist for SIRPα1-mediated NF-κB suppression. Recently, it has been shown that SHP-2 is critical for linking EGF receptor to NF-κB transcriptional activity.[25] Furthermore, cells from mice lacking SHP-2 are unable to activate NF-κB in response to TNF and interleukin-1,[14] establishing SHP-2 as a key component of NF-κB signaling. Our observation that SHP-2 overexpression in Huh7/SIRPα1 cells rescued NF-κB activation and led to increased viability indicates that SIRPα1 may function as an adapter molecule in titrating SHP-2 signalosome, thereby leading to impaired NF-κB activation. The mechanistic insight to this possibility is an area of ongoing study. Taken together, these results demonstrate that SHP-2 likely acts as the molecular transducer of the ERK

repression and NF-κB suppression induced by wild type SIRPα1.

SIRPα1 also has a notable effect on cell migration and spreading. Huh7/SIRPα1 cells displayed significantly reduced ability to spread over fibronectin. Impaired cell migration on fibronectin was observed in Huh7/SIRPα1 cells but not in Huh7/SIRPα1-4Y cells, indicating that SIRPα1 functions to impair cell migration. Likewise, reduced cell spreading and migration was also noticed in glioblastoma U87MG cells overexpressing SIRPα1 by association between SIRPα1 and SHP-2.[8] This phenotype is quite similar to SHP-2–deficient cells or cells overexpressing a catalytically inactive mutant SHP-2, which exhibited impaired ability in cell spreading and migration, and it would suggest that SIRPα1 might work in concert with SHP-2 in the control of cell motility.[18,39] Given that Ras-MAPK activity appears to be required for cell migration on the extracellular matrix,[40,41] the impaired motility of cells expressing SIRPα1 might result from the inhibition of adhesion-induced ERK activation. However, recent data demonstrated that expression of wild type SHPS-1 promoted the migration of CHO-IR cells in response to insulin, revealing increased biochemical complexity in the regulation of cell motility by SIRPα1.[10] The possible explanation for this discrepancy might be the species difference between rat SHPS-1 and human SIRPα1 or the different cell line used. However, the mechanism by which SIRPα1 regulates cell motility remains unclear and warrants further investigation.

In summary, the data presented in this study substantiate the importance of SIRPα1 in the negative regulation of cell proliferation, survival, and migration in HCC cells. The heightened sensitivity of cells restoring SIRPα1 function could be exploited in the development of therapeutic regimens that may potentiate the antineoplastic effect of conventional cytokines or chemotherapeutic agents.

Acknowledgment: The authors thank Dr. A. Ulrich, Dr. Osamu Tetsu, Dr. G. S. Feng, Dr. C. Scheidereit, and Dr. Ya Cao for invaluable reagents, and Liang Tang for his technical assistance.

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