Science in China Series C: Life Sciences

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KAI1/CD82 suppresses hepatocyte growth factor-induced migration of hepatoma cells via upregulation of Sprouty2

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We conducted a study concerning the suppressive mechanism of KAI1/CD82 on hepatoma cell metastasis. Hepatocyte growth factor (HGF) induces the migration of hepatoma cells through activation of cellular sphingosine kinase 1 (SphK1). Adenovirus-mediated gene transfer of KAI1 (Ad-KAI1) downregulates the SphK1 expression and suppresses the HGF-induced migration of SMMC-7721 human hepatocellcular carcinoma cells. Overexpression of KAI1/CD82 significantly elevates Sprouty2 at the protein level. Ablation of Sprouty2 with RNA interference can block the KAI1/CD82-induced suppression of hepatoma cell migration and downregulation of SphK1 expression. It is demonstrated that KAI1/CD82 suppresses HGF-induced migration of hepatoma cells via upregulation of Sprouty2.

KAI1/CD82, Sprouty2, migration, hepatocyte growth factor

KAI1/CD82 belongs to tetraspanin superfamily of transmembrane proteins, which are characterized by four membrane-spanning domains and play important roles in the regulation of cell migration, fusion, adhesion, differentiation and proliferation[1,2]. KAI1/CD82 acts as a suppressor of wide-spectrum tumor metastasis during the progression of different solid tumors including prostate, lung and liver cancers[3−5]. In malignant solid tumors, the presence of KAI1/CD82 predicts a better prognosis for cancer patients, and down-regulation or loss of KAI1/CD82 expression is constantly found in the clinically advanced stages[5,6]. KAI1/CD82 overexpression can inhibit cancer cell migration and invasion *in vitro* and suppress cancer metastasis in animal models.

KAI1/CD82 can inhibit the cell motility through modulating the cell motility-related signaling pathway or signal molecule function. Recently, Bandyopadhyay et al.[7,8] found that KAI1 interacts with the Duffy antigen receptor for chemokines (DARC) which transmits the inhibitory signal of cell proliferation and induces cell senescence by modulating the expression of TBX2 and p21. KAI1/CD82 suppresses integrin-induced cell invasion by regulating the cell growth factor receptor and its downstream molecules such as c-Met, EGF receptor, Src kinases and focal adhesion kinase (FAK)[9−11]. However, the exact molecular mechanism of the suppressor function of KAI1 remains elusive.

Ras/Erk signaling pathway plays a central role in the receptor tyrosine kinase (RTK) signal cascades. Sprouty2, a member of Sprouty family, has been identified as a negative feedback regulator of RTK signaling mainly by interfering with the Ras/Raf/mitogen- activated protein kinase cascade[12]. Several lines of evidence suggest Sprouty2 has the tumor suppressive function. Consistently, Sprouty2 is downregulated in several types of cancer, including breast, prostate and liver

Received March 2, 2007; accepted May 19, 2008
doi: 10.1007/s11427-008-0086-1
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Supported by the National Natural Science Foundation of China (Grant Nos. 30670954 and 30670885)

cancers[13 – 15]. Overexpression of Sprouty2 has been shown to suppress migration of cells via inhibition of ERK activation[16]. Sprouty protein can biochemically interact with membrane proteins, such as Cav-1[17]. Given the similar effect and position in the cytokine signaling, we hypothesize that Sprouty2 and KAI1/CD82 might have a functional overlapping in the suppression of cytokine signaling. Here, we characterize the functional role of Sprouty2 in the KAI1/CD82-induced suppressive effect on migration of hepatoma cells.

1 Materials and methods

1.1 Cell culture

SMMC-7721 cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Hycolone). Cells were passaged twice a week, and the cells at the logarithmical growth phase were used for all experiments. A replication-deficient recombinant adenovirus vector expressing KAI1 (Ad-KAI1) was constructed and expanded. An adenovirus vector carrying green fluorescent protein (GFP) was utilized to transfect SMMC-7721 cells as a negative control.

1.2 Assay of sphingosine kinase activity

Activation of SphK1 was measured as previously described with slight modifications[18]. Briefly, cells were harvested in buffer and lysed by freeze-thaw for three times. The protein concentration of the cell lysate was determined using bicinchoninic acid (BCA protein assay, Pierce). Samples were assayed for SphK1 activity by incubation with sphingosine (Sigma) and [γ-^{32}P] ATP (10 μCi, 20 mmol/L) containing $MgCl_2$ (200 mmol/L) for 30 min at 37℃. Reactions were stopped and the labeled lipids in the organic phase were separated by thin-layer chromatography on Silica Gel G60 (Merck). Labeled S1P was visualized by autoradiography.

1.3 Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline, and total cell lysates were prepared by rupturing the cells in lysis buffer. Cells were lysed by freeze-thaw for three times, and the insoluble material was removed by centrifugation at 12000 *g* for 20 min. Equal samples of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore). After being blocked, the PVDF membrane was incubated with the primary antibody and then with horseradish peroxidase-conjugated secondary antibody. The membrane was developed with enhanced chemiluminescence substrate (ECL, Amersham Pharmacia Biotech). Anti-SphK1 was from Calbiochem and Oncogene Company. Anti-Sprouty2 antibodies were from Santa Cruz Company.

1.4 Cell migration assay

Assessment of cell migration was performed as that recently described[19] with minor modifications. SMMC-7721 cells were dislodged after brief trypsinization. Cell suspension was washed extensively with RPMI 1640 containing 0.1% acid-free bovine serum albumin (migration medium) and resuspended in the same medium. 1×10^5 cells were dispersed into the upper chamber of Transwell compartment (Costar) with 8 μm pore size filter. 600 μL migration medium was filled in the lower chamber and the cells were allowed to adhere onto transwells for 2 h at 37℃. The medium in the lower chamber was then removed and cells were challenged by adding 600 μL of fresh migration medium with 50 ng/mL HGF or medium control to the lower chamber. Migration was allowed to proceed for 2 h at 37℃. The cells remaining attached to the upper surface of the filter were carefully removed with a cotton swab. Migrated cells were stained with crystal violet and examined by light micrography finally. The numbers of migrating cells in at least 10 consecutive fields were enumerated and their average was calculated. Data were expressed as the number of migrating cells per visual field.

1.5 RNA interference

The siRNA targeting human Sprouty2 and control was synthesized by Shanghai Gene Chemical Company. The silencing sequences for human Sprouty2 are as follows: sense-strand: 5′-AACAGTGATAGAAGAGACC-3′ and antisense-strand: 5′-GGTCTCTTCTATCACTGTT-3′; and non-silencing sequences are as follows sense-strand: 5′-TTCTCCGAACGTGTCACGT-3′ and antisense-strand: 5′-ACGTGACACGTTCGGAGAA-3′. These siRNA were cloned into the lentivirus vector with the GFP as a report gene. The virus was packaged in 293 cells. SMMC-7721 cells were transfected with the virus carrying sporuty2 siRNA or control virus, according to the recommendation of the manufacturer. GFP positive cells were sorted out by flow cytometry and expanded. The

expression of Sprouty2 was monitored by Western blotting analysis.

2 Results

2.1 Adenovirus-mediated KAI1/CD82 gene transfer suppresses HGF-induced migration and SphK1 activation of hepatocellcular carcinoma cells

SMMC-7721 cells were transfected with the recombinant adenoviral vector expressing KAI1 (Ad-KAI1) or control adenoviral vector. The expression of KAI1/CD82 was proven by Western blotting analysis or FACS (Figure 1). Then, the HGF-induced migration of these cells was examined. Our results showed that HGF at a concentration of 50 ng/mL could induce the migration of SMMC-7721 cells significantly, whereas overexpression of KAI1/CD82 could suppress HGF-induced migration of SMMC-7721 cells markedly (Figure 2). HGF induces the migration of hepatoma cells via multiple signals. It has been shown that SphK1 activation plays a vital role in HGF-induced cell migration[19]. In addition, HGF also activates SphK1 in SMMC-7721 cells (Figure 3(a)). SphK1 activation and expression in the SMMC-7721 cells transfected by Ad-KAI1 in different infection titer were investigated. The results show that the protein level of SphK1 was reduced in SMMC-7721 cells transfected with Ad-KAI1 (Figure 3(b)). Meanwhile, the cellular SphK1 activity was also decreased simultaneously (Figure 3(c)). These data indicate that KAI1/CD82 can suppress the HGF-induced activation of SphK1 and migration of hepatocellcular carcinoma cells.

2.2 KAI1/CD82 up-regulated the Sprouty2 protein level in SMMC-7721 cells

Sprouty2 is a negative regulatory molecule in HGF signaling cascades. The changes of Sprouty2 expression in SMMC-7721 cells transfected with KAI1/CD82 were analyzed by Western blotting. As shown in Figure 4, Sprouty2 protein was upregulated in KAI1/CD82 transfected cells. The upregulation of Sprouty2 was consistent with the increased efficiency of KAI1/CD82 gene transfer.

2.3 Sprouty2 mediated suppressive effect of KAI1/CD82 on hepatoma cell migration and SphK1 activation

To clarify the role of Sprouty2 in KAI1/CD82–induced SphK1 suppression of hepatoma cells, we employed a specific siRNA to block the expression of Sprouty2. SMMC-7721 cells were transfected with the lentiviral vectors carrying Sprouty2 siRNA. The transfected cells were selected by flow cytometry and expanded. As shown in Figure 5(a), the Sprouty2 protein was down-regulated significantly in transfected SMMC- 7721 cells. We also investigated that Sprouty2 deletion could abolish the suppressive effect of KAI1/CD82 on SphK1 expression in SMMC-7721 cells (Figure 5(b)). Sprouty2-depleted and control cells were transfected with Ad-KAI1 or control adenovirus. After being cultured for 48 h, these cells were stimulated by HGF at concentration of 50 ng/mL for migration assay. As illustrated in Figure 5(c), the number of migrated Sprouty2- deleted cells was significantly higher than that of control cells. These data indicated that Sprouty2 RNA interference attenuated the suppressive effect of KAI1/CD82 on HGF-induced cell migration.

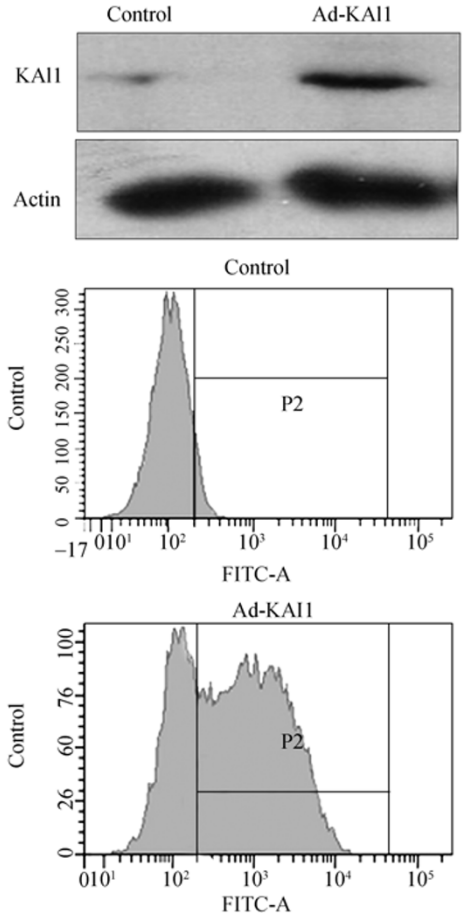

Figure 1 Adenorirus-mediated KAI1/CD82 expression by SMMC-7721 cells. SMMC-7721 cells were transfected with adenovirus-KAI1 or control adenoviral vector for 48 h at m.o.i of 100, then transfected cells were harvested for Western blotting analysis or FACS.

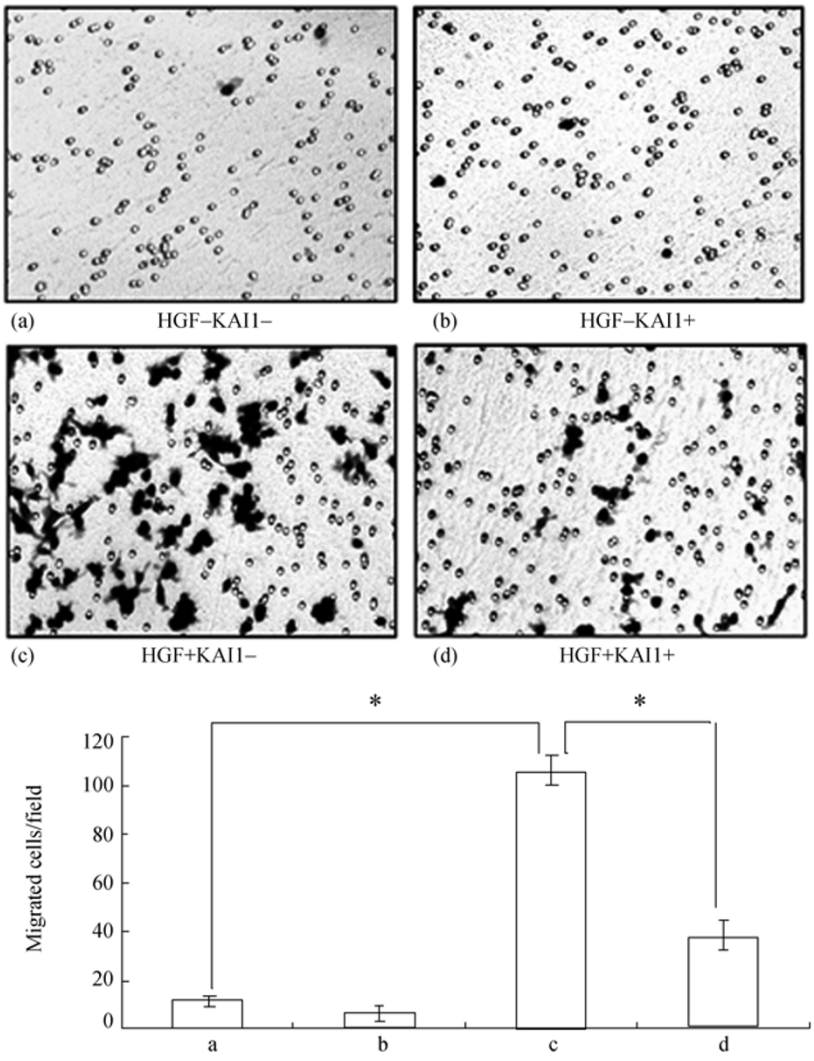

Figure 2 Adenovirus-mediated gene transfer of KAI1/CD82 suppresses the migration of SMMC-7721 hepatocellcular carcinoma cells induced by HGF. (a) SMMC-7721 cells were transfected with control adenoviral vector at m.o.i of 100. After being cultured for 48 h, the cells were plated in compartment without any stimulation. (b) SMMC-7721 cells were transfected with control adenovirus vector at m.o.i of 100. After being cultured for 48 h, the cells were plated and stimulated by HGF at 50 ng/mL for 2 h. (c) SMMC-7721 cells were transfected with adenovirus-KAI1 at m.o.i of 100. After being cultured for 48 h, the cells were plated and stimulated by HGF at 50 ng/mL for 2 h. Migrating cells were fixed, stained with 0.5% crystal violet, and photographied. (d) The migrated cells were counted in 10 consecutive fields. Each condition was tested in triplicate and data were represented as mean ± SD (*, $P < 0.01$).

3 Discussion

KAI1/CD82, a transmembrane glycoprotein, has been identified to play an important role in suppression of cancer cell metastasis. It attenuates the growth factor signaling in various cancer cells. However, the mechanism of this process is still ambiguous. In this study, we investigated its mechanism by using a model of HGF-induced migration of SMMC-7721 cells. HGF induces invasion of hepatoma cells via multiple signaling pathways, including MAPK, PI3K and ETS[19,20]. In addition, SphK1 activation is also involved in HGF-induced migration of hepatoma cells. SphK1 is a key enzyme catalyzing phosphorylation of sphingomyelin (SphK) to form sphingosine 1-phosphate (S1P) which acts as both an extracellular mediator and an intracellular second messenger in regulating cellular processes, such as growth, differentiation, motility, survival, and gene expression[21]. SphK1 is thought to be a signal transducer between growth factor and bioactive sphingolipids. It plays a key role in mediating cytokine-induced cell movement. Thus, we investigated the effect of KAI1/ CD82 on the cellular activity of SphK1. We found that adenovirus-mediated gene transfer of KAI1/CD82 could reduce SphK1 expression and decrease the SphK1 cellular activity. This gives the clue that suppression of KAI1/CD82 on cell migration

is associated with SphK/S1P signaling.

Sprouty (Spry) proteins comprise a family of structurally related negative-feedback regulators of RTK signaling. Because of their capability to interact with various signaling molecules, Spry proteins are thought to exert their inhibitory effects by interfering with the

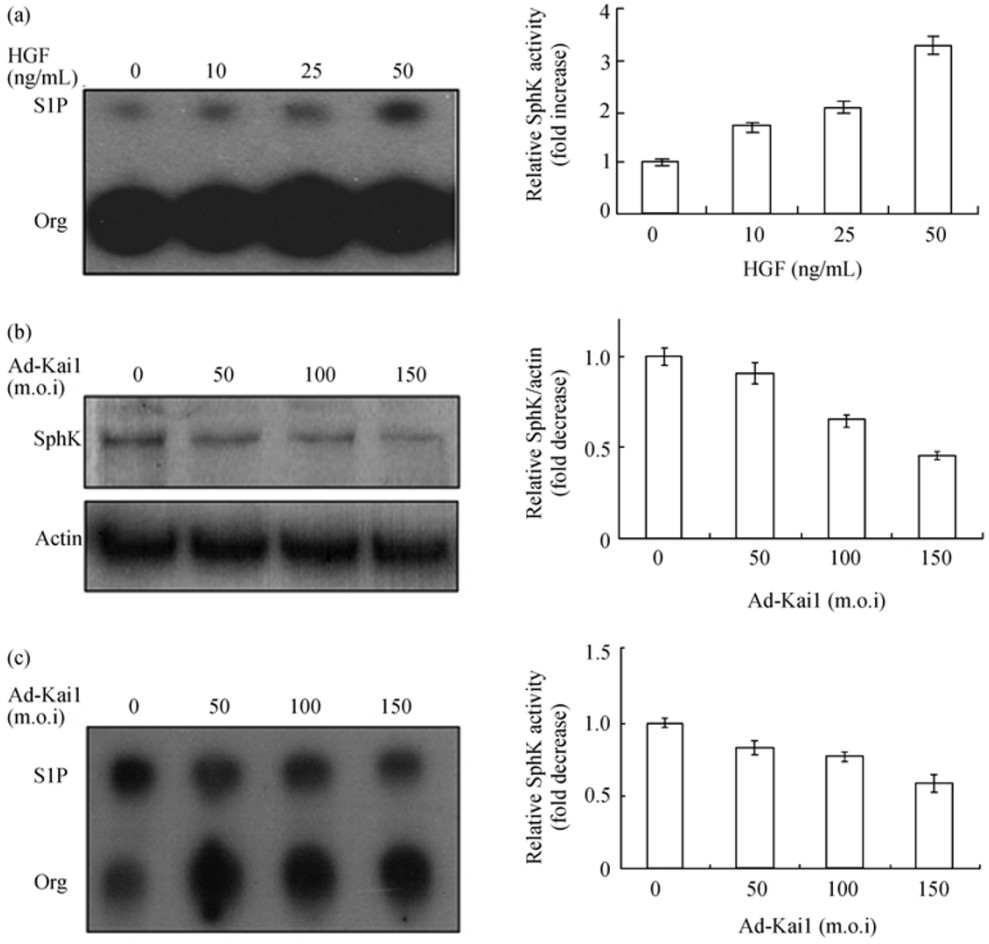

Figure 3 Adenovirus-mediated gene transfer of KAI1 suppresses SphK1 activation of SMMC-7721 cells. (a) Activation of SphK1 induced by HGF. SMMC-7721 cells were treated with HGF at different concentrations as indicated and the sphingosine kinase 1 activity in cell lysates was determined. The location of S1P visualized with autoradiogram demonstrating formation of [^{32}P] S1P, and [^{32}P] S1P levels indicate activation of SphK1. The spots at the origin (Org) are carryovers of labeled ATP. (b) KAI1/CD82 downregulated SphK1 expression. SMMC-7721 cells were transfected with adenovirus-KAI1 for 48 h at m.o.i of 0, 50, 100 and 150, then harvested for Western blotting analysis. (c) KAI1/CD82 suppressed SphK1 activity. The SMMC-7721 cells were treated as in Figure 3(b) and the SphK1 activity was assayed.

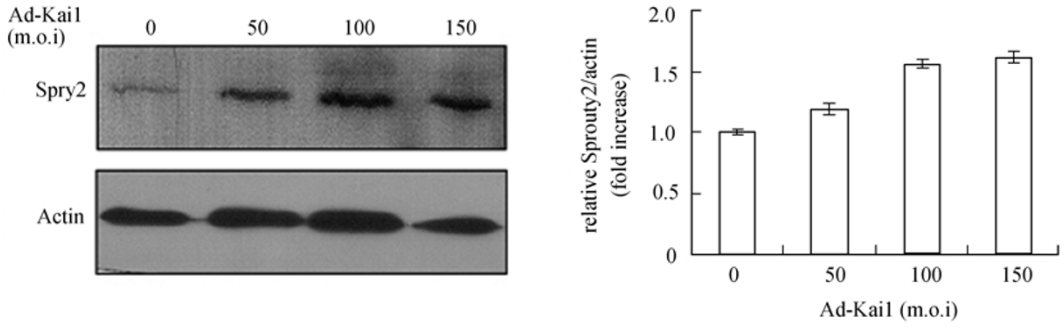

Figure 4 KAI1/CD82 upregulates the expression of Sprouty2 protein. SMMC-7721 cells were transfected with adenovirus-KAI1 for 48 h at m.o.i of 0, 50, 100 and 150, then transfected cells were harvested for Western blotting analysis.

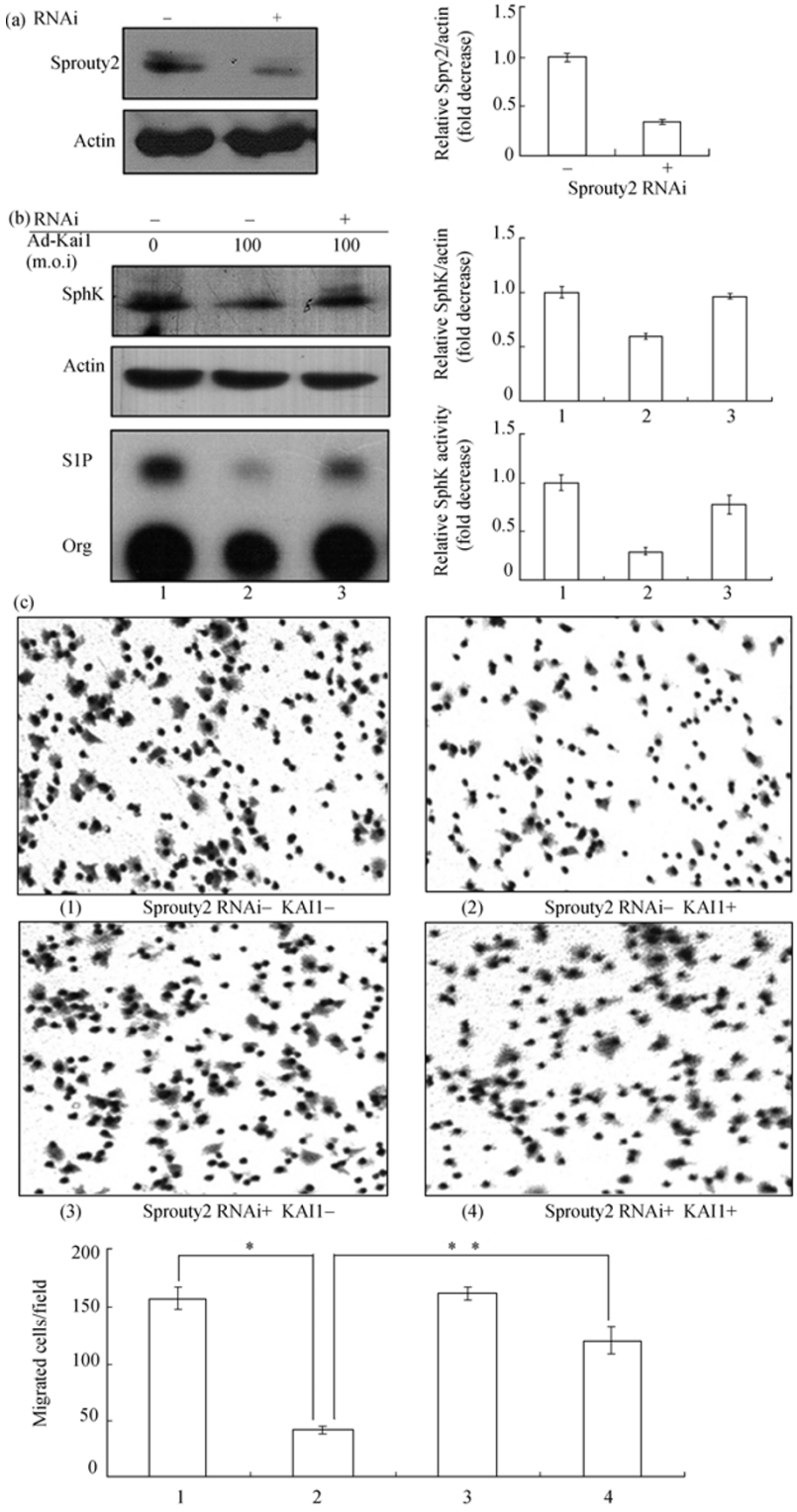

Figure 5 Depletion of Sprouty2 with siRNA attenuates the inhibitory effect of KAI1/CD82 in hepatoma cells. (a) SMMC-7721 cells were transfected with specific siRNA for Sprouty2 or control siRNA. Sprouty2 levels were analyzed by Western blotting. (b) Depletion of Sprouty2 reverses KAI1/CD82-induced downregulation of SphK1. SMMC-7721 cells of Sprouty2 silencing and control cells were transfected with Ad-KAI1 at an m.o.i 100 for 48 h. SphK1 levels were checked by Western blotting. (c) Depletion of Sprouty2 blocks KAI1/CD82-induced suppression of SMMC-7721 cell migration: 1, Control cells were transfected with empty adenovirus vector; 2, SMMC-7721 cells were transfected with Ad-KAI1 adenovirus vector; 3, Sprouty2 RNA interference cells were transfected with Ad-KAI1 adenovirus vector. The number of migration cells was counted in 10 consecutive fields. Each condition was tested in triplicate and data were represented as mean±SD (*, P<0.01; **, P<0.05).

formation of signaling complexes required for RTK-mediated signal flow. Sprouty2, one member of Sprouty family, is known to act downstream in many RTKs pathways including FGF, VEGF and HGF. It is found that Sprouty2 expression can be induced by HGF. The overexpressed Sprouty2 in the form of negative feedback can regulate HGF signaling[16]. Adenovirus-mediated gene transfer of KAI1/CD82 could inhibit the HGF-induced migration of SMMC-7721 cells. This gives the possibility that Sprouty2 relays the suppressive signals from the KAI1/CD82. Then it was characterized that KAI1/CD82 expression could induce the up-regulation of Sprouty2 in hepatoma cells. Strategy for RNA interference has been widely used to identify the gene function. The expression of Sprouty2 was blocked by a lentivirus vector carrying the specific siRNA. We further identified that depletion of Sprouty2 with siRNA attenuated the inhibitory effect of KAI1/CD82 in hepatoma cells, including both SphK1 expression and cell migration. These data indicated that the mechanism by which KAI1/CD82 inhibits tumor metastasis is via the Sprouty2 upregulation.

The authors thank Prof. WANG JiaXi for modifying this manuscript.

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