Interference of Skp2 effectively inhibits the development and metastasis of colon carcinoma

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Abstract. Colon cancer is a common type of malignancy in the digestive system. The aim of the present study was to investigate the role of S-phase kinase-associated protein 2 (Skp2) in colon carcinoma and to identify whether depletion of Skp2 by Skp2-RNA interference (RNAi) attenuates the proliferation and migration of colon carcinoma. Three pairs of small interfering (si)RNA were designed based on the Skp2 gene sequence and the most effective one was used to silence the Skp2 gene in SW620 cells. Subsequent to the interference, quantitative polymerase chain reaction and western blot analysis were used for detecting the expression of Skp-2 mRNA and protein, respectively. The data demonstrated that the Skp2-siRNA effectively inhibited proliferation ($P<0.01$), increased the levels of apoptosis and induced G_0/G_1 phase arrest of the SW620 cells ($P<0.01$). Transfection of the Skp2 siRNA into SW620 cells effectively reduced Skp2 protein levels, while p27 protein levels increased. In the *in vivo* experiments, a lentiviral vector of the Skp2-RNAi transfected into SW620 cells markedly inhibited Skp2 expression, as detected by immunohistochemical analysis of nude mice. Additionally, tumorigenicity experiments showed that inhibition of Skp2 significantly increased the survival rate of nude mice. Thus, the *in vitro* and *in vivo* results demonstrated that interference of Skp2 expression significantly inhibited the proliferation and induced the apoptosis of SW620 cells. This suggests that Skp2 protein has an important role in the progression of colon cancer. Therefore, Skp2 may enable the early diagnosis of colon cancer and provide new insights into molecular targets for cancer therapy.

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Introduction

Colon cancer is a common type of malignancy in the gastrointestinal tract of the digestive system (1-4). Colon cancer is currently the fifth most common cause of cancer mortalities in China, mainly due to the recurrence of tumor metastasis (5,6). The incidence of colorectal cancer has exhibited a clear positive association with rich blood supply, rapid growth, high levels of infiltration and propensity to metastasize (7-10). Numerous studies have focused on the pathogenesis and development of colon cancer (11). With the development of molecular biology techniques, much of the progress in the understanding of colon cancer that has occurred concerns the molecular mechanisms of colon cancer (12). Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract. Benign adenoma initially develops from epithelial hyperplasia in normal colonic mucosa and then the potentially invasive and metastatic colon cancer forms (13). All of the progress that has occurred in the molecular mechanisms of colon cancer concerns the activation of oncogenes and inactivation of the tumor suppressor genes (14-16). Although much progress on the molecular mechanisms of colon cancer has been achieved, the molecular events remain to be fully elucidated.

Cell cycle regulation is critical for cell proliferation and tumorigenesis (17,18). The cell cycle is regulated by various factors, including cyclin, cyclin-dependent kinases (CDKs) and CDK interacting protein (cip)/kinase inhibitory protein (kip). S-phase kinase-associated protein 2 (Skp2) mainly induces the degradation of CDK inhibitors, including $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ (19-21). As an F-box protein, Skp2 is a key regulator for cell cycle progression. The expression levels of Skp2 are low in the G_0/G_1 phase, while they are elevated in S phase. The overexpression of the Skp2 gene may result in loss of control of the cells at the G_1/S checkpoint, which could induce the cells to continuously proliferate and divide. Thus, Skp2 is a tumor-promoting factor. A number of studies have revealed that the overexpression of Skp2 is associated with the progression of a variety of types of human cancer (22-25). Functional deletion of Skp2 leads to stabilization of CDK inhibitors, which can subsequently induce cell-cycle delay or arrest (26,27). However,

the role of Skp2 expression in the metastasis and prognosis of colon cancer remains controversial.

The aim of the study was to explore the role of Skp2 in colon carcinoma and to identify whether depletion of Skp2 by Skp2 RNA interference attenuates the proliferation and migration of colon carcinoma.

Materials and methods

Cell lines and small interfering (si)RNA. The SW620 colon cell line (American Type Culture Collection, Rockville, MD, USA) was grown in Dulbecco's modified Eagle's medium with 100 mM L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin. Skp2-siRNA and scramble siRNA were synthetized by Jima Corporation (Shanghai, China). Skp2 p45 shRNA (h) Lentiviral Particles were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Transfection. Cells were incubated in six-well plates ($3x10^5$ cells/well) overnight and were then transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) at a final RNA concentration of 100 nM, according to the manufacturer's instructions. The sequence of the Skp2-siRNA was as follows: Complementary oligonucleotides targeting Skp2, 5'-AGCTTTTCCAAAAAAGGGAGTGACAAAGACTTTG TCTCTTGAACAAAGTC-TTTGTCACTCCCG-3' and 5'-GA TCCGGGAGTGACAAAGACTTTGTTCAAGAGACAAAG TCTTTGTCACTCCCTTTTTTGGAAA-3'.

Western blot analysis. Whole cell extracts were prepared and separated by PAGE as previously described (28-30). The antibodies used included anti-Skp2 (Santa Cruz Biotechnology, Inc.), anti-p27 (Santa Cruz Biotechnology, Inc.), anti-β-actin and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) and were detected with an Enhanced Chemiluminescence Detection kit (Amersham Pharmacia Biotech, Amersham, UK).

Flow cytometric analysis. Annexin V-fluorescein isothiocyanate staining was used for a cell apoptosis assay as previously described (31). Propidium iodide (PI) staining was performed to analyze cell cycle progression as previously described (32). Briefly, $1x10^6$ colon cancer cells were washed three times in cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min. Following two further washes in PBS, PI and RNase A (Sigma-Aldrich, St. Louis, MO, USA) were added to a final concentration of 100 ng/ml each. After incubation for 15 min at room temperature, the cells were analyzed by flow cytometry (FACScan; BD Biosciences, Erembodegem, Belgium).

Scratch assay. The scratch assay was used to measure basic cell migration parameters. Briefly, cells were grown to confluence and a thin 'wound' was introduced by scratching with a pipette tip. The distance of which the cells at the wound edge had migrated into the wound space was measured following 0 and 12 h.

MTT assay. MTT assay kits were purchased from Sigma-Aldrich. The colon cancer cells were seeded in 48-well plates. After 6 h, the cells were transfected with siRNA specific for Skp2 or scramble siRNA for different time periods. The MTT solution was added to the cells (10% of total volume) and after a period of 4 h, the media was removed and replaced with acidified isopropanol and then the absorbance was read at 490 nm.

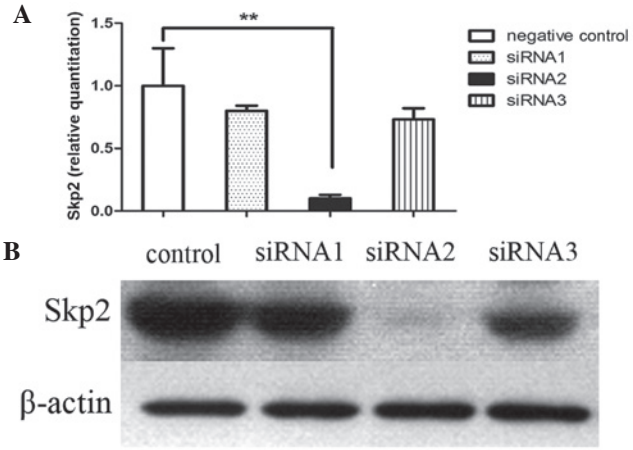

Figure 1. Identification of the most effective siRNA of the Skp2 gene in SW620 cells. The cells were plated in 48-well plates and three pairs of Skp2-siRNA were transfected into the SW620 cells. The expression levels of the Skp2 gene were detected 48 h later by (A) quantitative polymerase chain reaction and (B) western blot analysis. β-actin was used an internal reference. Results are presented as the mean ± standard error of the mean. **P<0.01, compared with the control group. Skp2, S-phase kinase-associated protein 2; si, small interfering.

Animals and grouping. Male BALB/c (nu/nu) mice were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, China) and housed under specific pathogen-free conditions. The mice were kept in a 12-h light and dark cycle. All animals were randomly divided into three groups (group A, control; group B, the group transfected with the lentiviral vector of Skp2-RNA interference (RNAi); and group C, scrambled siRNA group) and each group contained ten mice. All procedures were in accordance with the Declaration of Helsinki of the World Medical Association. The protocols were also approved by the Institutional Animal Care and Use Committee of Zhujiang Hospital of Southern Medical University (Guangzhou, China). The survival days were recorded and the survival rates were obtained using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical analyses. Data were entered into a database and analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA). The comparison of Skp2 and $p27^{kip1}$ mRNA expression following different treatments was conducted using a Student's t-test. Results are presented as the mean ± standard error of the mean. P<0.01 was considered to indicate a statistically significant difference.

Results

Skp2-siRNA2 is the most effective sequence for interfering with the Skp2 gene. To study the role of Skp2 as a therapeutic target for the treatment of colon cancer cells, three pairs of interfering RNAs (siRNA) were designed to specifically silence endogenous Skp2 expression and were transfected into SW620 cells. The expression levels of Skp2 were detected 48 h later by

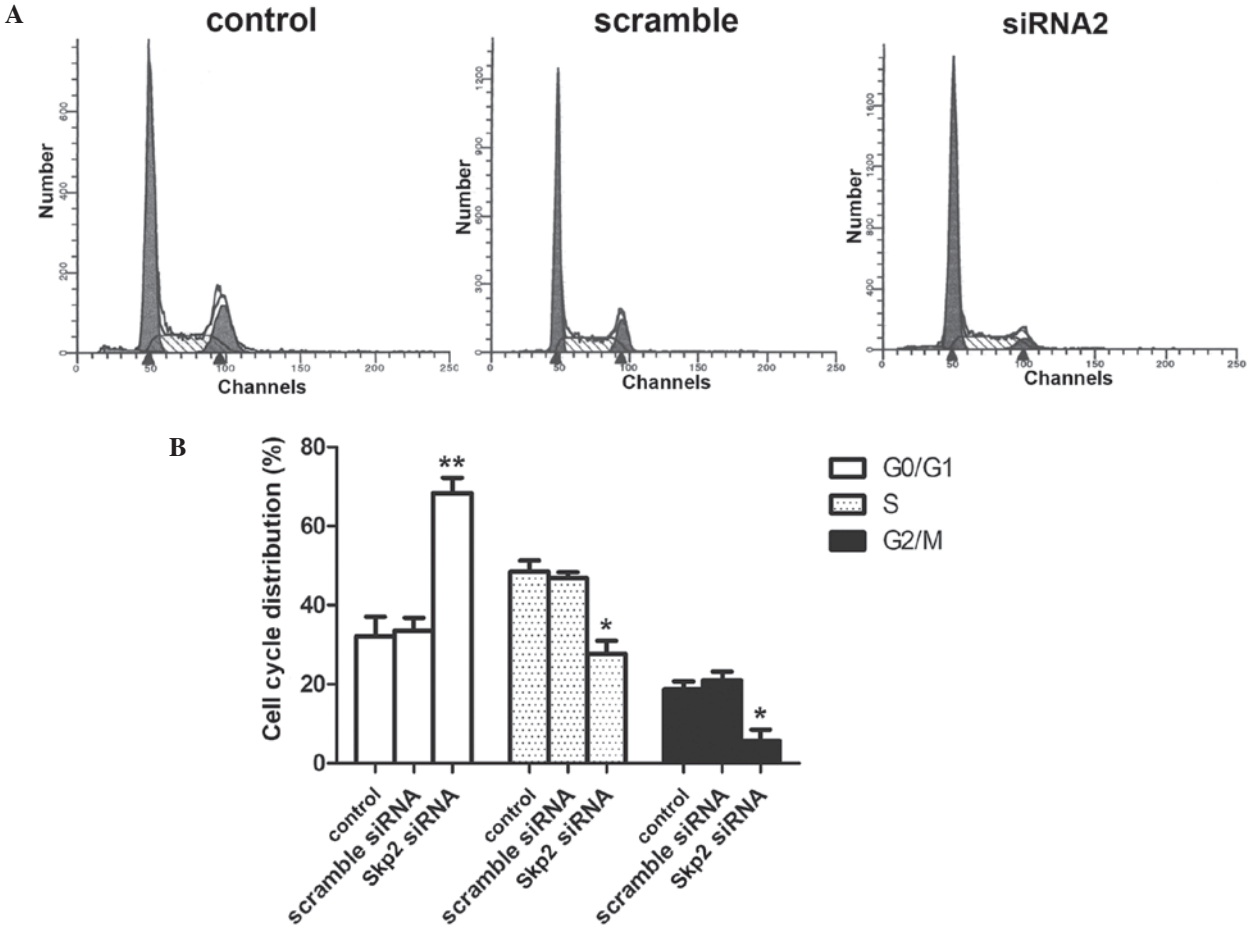

Figure 2. Distribution of the different groups of cells in the cell cycle phases. Cell cycle analysis of the SW620 cells was performed 48 h after transfection with Skp2-siRNA or scrambled siRNA. (A) The proportion of the cells in each phase was determined by flow cytometry of propidium iodide-stained cells and (B) the data were quantified. Results are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01, compared with the respective control group. si, small interfering; Skp2, S-phase kinase-associated protein 2.

quantitative polymerase chain reaction and β-actin was used as the positive control in the experiment. The results demonstrated that the Skp2-siRNA2 sequence was the most effective at silencing Skp2 expression (Fig. 1A). This result was consistent with the results detected by western blot analysis (Fig. 1B).

Skp2-siRNA induces cell cycle arrest in G_0/G_1 phase. In order to elucidate the mechanism of Skp2 siRNA, cell cycle analysis of SW620 cells was performed. As shown in Fig. 2, the data demonstrated an accumulation of colon cancer cells in G_0/G_1 phase, with a relative paucity of cells traversing through the S and G_2/M phases compared with those in the control groups.

Skp2-RNAi inhibits the migratory ability of SW620 cells. To determine the role of endogenously expressed Skp2 in the regulation of SW620 cell migration and proliferation, the *in vitro* scratch assay was used to measure cell migration. As shown in Fig. 3, the number of migrated SW620 cells was clearly reduced following transfection with Skp2-RNAi, compared with that of the control and the group transfected with scramble siRNA groups.

Transfection with Skp2-siRNA induces apoptosis of SW620 cells. Subsequently, whether treatment of cancer cells with siRNA specific for Skp2 was able to further induce apoptosis was investigated. As shown in Fig. 4, the apoptotic rates of colon cells transfected with Skp2-siRNA were significantly higher compared with those of the cells transfected with scrambled siRNA (38.90±4.5% for the Skp2-siRNA group compared with 8.2±1.8% for the scrambled siRNA group, n=10; P=0.0039).

Skp2-RNAi inhibits cell growth. In order to detect the effect of Skp2-siRNA on cell growth, an MTT assay kit was used to evaluate the proliferation of SW620 cells. As shown in Fig. 5, the optical $density_{490}$ values in the Skp2 siRNA group were significantly lower than the values in the control and scramble siRNA groups (P<0.01), which suggested that cell growth was significantly inhibited along with the downregulation of Skp2 expression levels.

Reducing the expression levels of Skp2 increases $p27^{kip1}$ expression levels in SW620 cells. In the present study, SW620 cells were depleted of endogenous Skp2 by RNAi with siRNA specific for Skp2 mRNA. The Skp2-depleted cells exhibited increased levels of endogenous p27 (Fig. 6). β-actin was used as an internal reference. In parallel, the results demonstrated that the Skp2-mediated degradation of $p27^{kip1}$ had an important role in cell proliferation and survival.

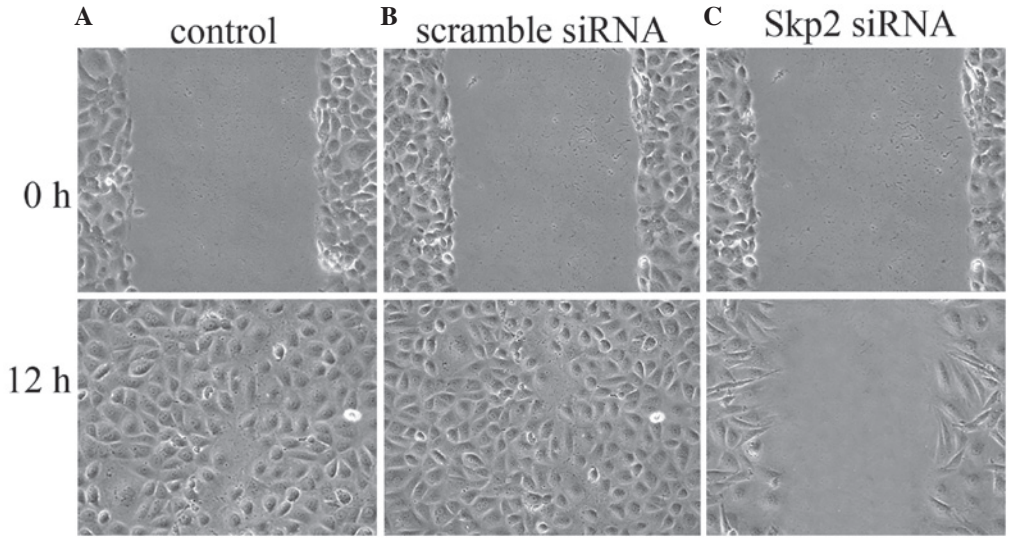

Figure 3. *In vitro* scratch assay. (A) Untreated group, (B) SW620 colon cancer cells treated with scrambled siRNA for 12 h after the scratch was performed and (C) SW620 cells transfected with Skp2-siRNA for 12 h after the scratch was performed. si, small interfering; Skp2, S-phase kinase-associated protein 2.

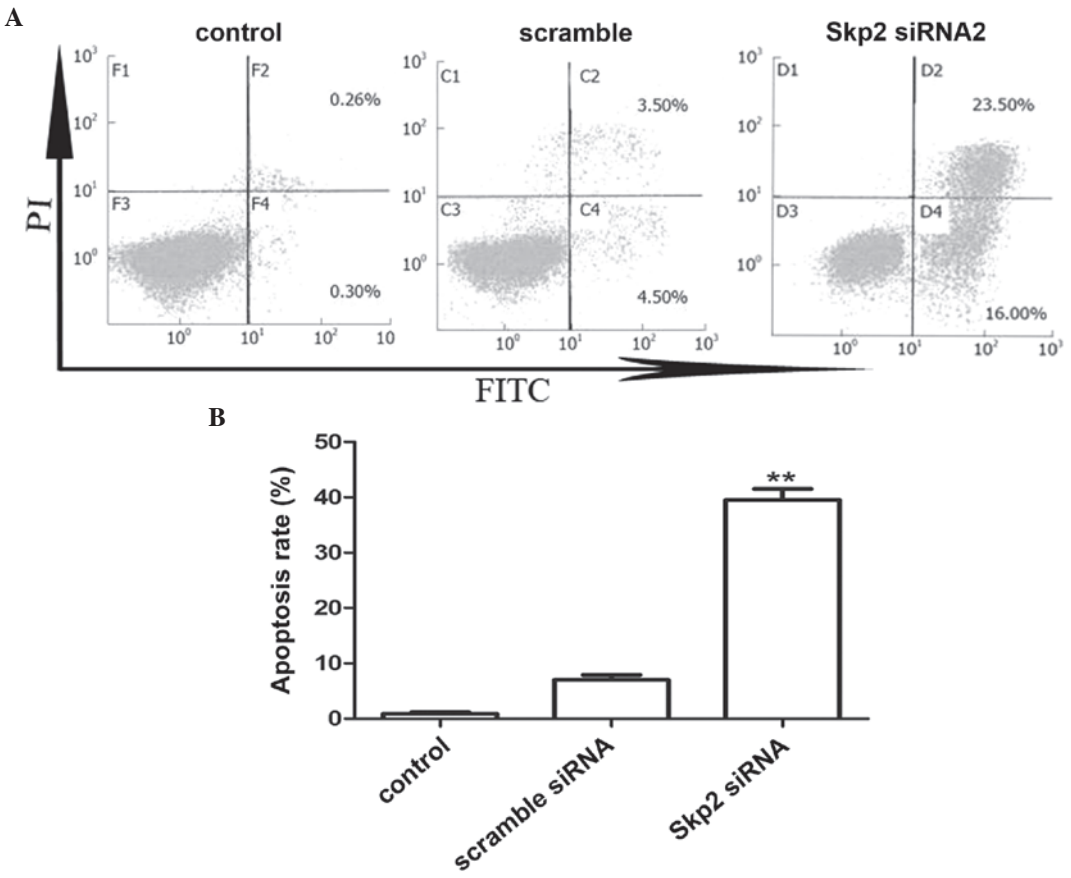

Figure 4. Induction of apoptosis was determined by PI-Annexin V staining. The cells were transfected with Skp2-siRNA and scrambled siRNA for 48 h. PI-Annexin V-positive cells were analyzed by (A) fluorescence-activated cell sorting and (B) the percentage of the PI-Annexin V-positive cells was quantified and shown in the histograms. Results are presented as the mean ± standard error of the mean. $^{**}P<0.01$, compared with the control group or the group treated with scrambled siRNA. Skp2, S-phase kinase-associated protein 2; si, small interfering; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Tumorigenicity experiments in nude mice. To further define the potential efficacy of Skp2-siRNA, a lentiviral vector of Skp2-RNAi was used and its activity against the proliferation and metastasis of colon carcinoma cells in a nude mouse model was evaluated. Paraffin-embedded samples were analyzed by immunohistochemical staining for Skp2 after challenging the animals with colon cancer cells for two weeks. The results revealed that Skp2-siRNA noticeably suppressed the expression of Skp2 in the tissues of nude mice (Fig. 7). Notably, the survival rates of the mice in the Skp2-RNAi group were

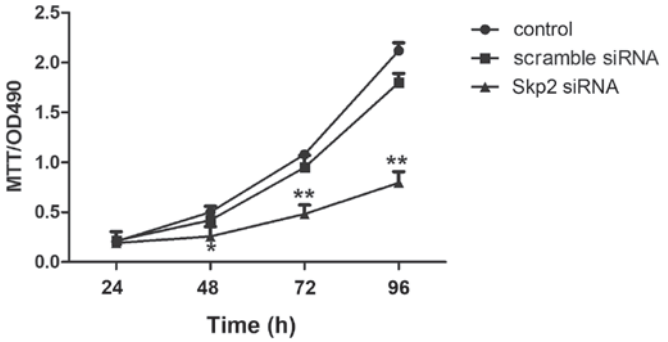

Figure 5. Inteference of Skp2 inhibited proliferation of SW620 cells, as detected by an MTT assay. SW620 cells ($1x10^5$) were planted in a 96-well plate and treated for different time periods in the presence of Skp2 siRNA or scrambled siRNA. The number of SW620 cells was measured. Untreated cells were used as the negative control. Data are shown as the mean ± standard error of the mean of at least three independent experiments on different individuals. **P<0.01 compared with the control group or the group treated with scrambled siRNA. OD, optical density; si, small interfering; Skp2, S-phase kinase-associated protein 2.

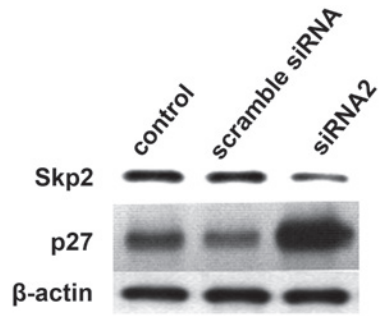

Figure 6. Expression levels of $p27^{kip1}$ were increased in SW620 cells. SW620 cells ($2x10^5$) were planted in a 24-well plate and transfected for 24 h in the presence of Skp2 siRNA or scrambled siRNA. Untreated cells were used as the negative control. Total cell lysates were analyzed for Skp2, p27 and β-actin by western blot analysis. β-actin was used as an internal reference. Skp2, S-phase kinase-associated protein 2; si, small interfering.

significantly higher than those in the scrambled siRNA and control groups (P=0.003 vs. scrambled group, P=0.006 vs. control group) (Fig. 8).

Discussion

In the present study, three pairs of Skp2 siRNA were designed to inhibit the endogenous Skp2 expression in colon cancer cells. A specific Skp2-siRNA which effectively reduced the endogenous expression levels of the Skp2 gene was successfully selected. Interference of Skp2 expression significantly inhibited the proliferation of SW620 cells compared with those in the control group, as detected by an MTT assay, which was consistent with the results detected in other cell lines, including HCT116, DLD-1 and DU145 (data not shown). The *in vitro* scratch assay results showed that SW620 cell growth was reduced in the Skp2-siRNA group compared with that in the control group, suggesting that silencing of Skp2 markedly reduced cell migration and proliferation, which was consistent with the results of the MTT assay.

As previously mentioned, Skp2 is an F-box substrate-recognition subunit of the Skp-Cullin-F-box protein (SCF)

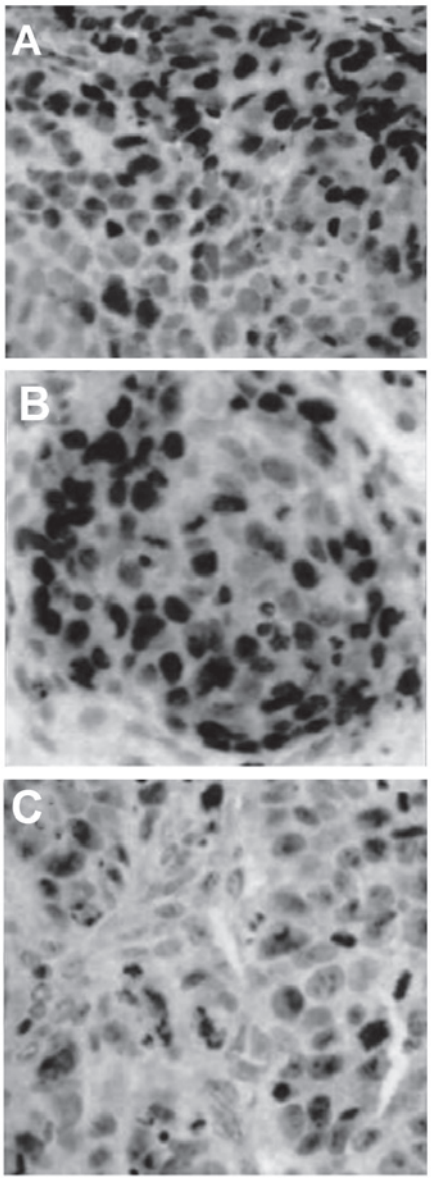

Figure 7. Paraffin-embedded samples were analyzed by immunohistochemical staining for Skp2. Skp2 protein was mainly located and expressed in the nucleus of the tumor cells. Skp2 expression in tumor tissues of the (A) control, (B) scrambled siRNA and (C) Skp2 siRNA groups (images of Skp2 staining were captured at x400 original magnification). Skp2, S-phase kinase-associated protein 2; si, small interfering.

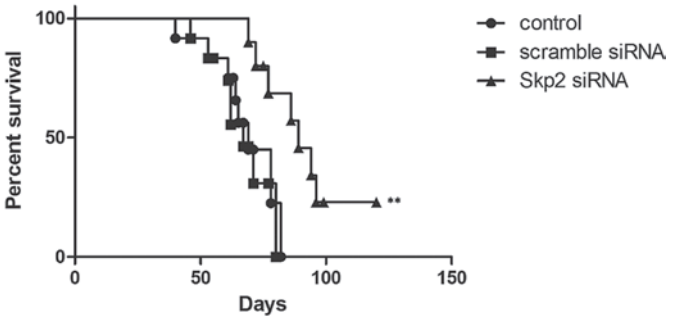

Figure 8. Survival rate of nude mice following lethal SW620 cell challenge. The nude mice were challenged subcutaneously with $5x10^5$ SW620 colon cancer cells in the flank area. The survival rate at 29 days was determined as follows: 100 x (number of survivors)/(number of challenged mice). Each group contained ten mice. **P=0.003 vs. scrambled group, P=0.006 vs. control group. si, small interfering; Skp2, S-phase kinase-associated protein 2.

ubiquitin-protein ligase complex which regulates the progression of the cell cycle by degrading the tumor suppressor gene $p27^{kip1}$ in a ubiquitin-mediated manner (17,18,33-35). In the present study, the effects Skp2 depletion by RNAi on cell cycle progression in colon cancer cells were also identified. The results demonstrated that loss of Skp2 resulted in a marked reduction in G_0/G_1 progression in colon cancer cells, whereas the number of cells in the G_2/M phase was reduced compared with those in the control group. Thus, the cell cycle was blocked in G_0/G_1 phase, and this delay was accompanied by an accumulation of $p27^{kip1}$. Elevated levels of Skp2 are usually accompanied by reduced levels of $p27^{kip1}$, which are considered to be associated with highly aggressive tumors and a poor prognosis in various types of cancer. The results of the present study also revealed that regulation of colon carcinoma proliferation by Skp2-siRNA is dependent on $p27^{kip1}$ protein expression.

Studies have previously proposed targeting of E3 ligases as a rational strategy to inhibit the progression of cancer by inhibition of proteasomes (36,37). Consistent with this theory, inhibition of SCF Skp2 in the present study blocked proliferation and migration of SW620 cells by inducing G_0/G_1 cell-cycle arrest and apoptosis. In addition to the *in vitro* inhibition of the proliferation and migration of SW620 cells, the antitumor effect of Skp2-RNAi on nude mice was also investigated in the present study through tumorigenicity experiments. All the results suggested that treatment with Skp2-RNAi represses the growth of metastatic tumors *in vivo*. Additionally, the immunohistochemical results demonstrated that a lentiviral vector of Skp2-RNAi effectively inhibited Skp2 expression in a murine model.

Thus, the results of the present study confirmed the hypothesis that Skp2 siRNA may be a useful therapeutic protocol for the treatment of colon carcinoma. Future studies may gradually elucidate the mechanism of Skp2 in colon carcinoma, and Skp2 may enable the early diagnosis of colon cancer and provide new insight into the molecular targets for cancer therapy.

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