

Downregulation of Skp2 inhibits the growth and metastasis of gastric cancer cells in vitro and in vivo

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Abstract S-phase kinase-associated protein-2 (Skp2) is overexpressed in human cancers and associated with poor prognosis. Skp2 acts as an oncogenic protein by enhancing cancer cell growth and tumor metastasis. The present study has demonstrated that small hairpin RNA (shRNA)-mediated downregulation of Skp2 markedly inhibits the viability, proliferation, colony formation, migration, invasion, and apoptosis of human gastric cancer MGC803 cells, which express a high level of Skp2. In contrast, Skp2 shRNA had only a slight effect on the above properties of BGC823 cells, which express a low level of Skp2. In accord, knockdown of Skp2 suppressed the ability of MGC803 cells to form tumors and to metastasize to the lungs of mice, and the growth of established tumors, by inhibiting cell proliferation and enhancing cell apoptosis. In contrast, overexpression of Skp2 promoted tumorigenesis of BGC823 cells in mice. Skp2 depletion induced cell cycle arrest in the G₁/S phase by upregulating p27, p21, and p57 and downregulating cyclin E and cyclin-dependent kinase 2. Skp2 depletion also increased caspase-3 activity, impeded the ability of cells to form filopodia and locomote, upregulated RECK

(reversion-inducing cysteine-rich protein with kazal motifs), and downregulated matrix metalloproteinase (MMP)-2 and MMP-9 activity and expression. The results suggest that downregulating Skp2 warrants investigation as a promising strategy to treat gastric cancers that express high levels of Skp2.

Keywords S-phase kinase-associated protein-2 · Gastric cancer · Proliferation · Cell cycle · Metastasis · p27

Introduction

Gastric cancer is still the third most common cause of cancer-related death in men worldwide, though it is no longer the second most frequent cancer [1]. The 5-year survival rate of gastric cancer is <20 %, which reflects the fact that more than 70 % of new cases occur in developing countries. In such countries, 80 % of cases are already clinically advanced and/or remotely metastatic at the time of diagnosis, and hence, curative surgery is no longer an option for these patients [2]. Conventional adjuvant treatments have shown only modest effects on the survival of patients with advanced gastric cancer [3]. There is, therefore, an urgent need for novel treatments to treat gastric cancer.

S-phase kinase-associated protein 2 (Skp2) belongs to the ubiquitin–proteasome system and acts as the substrate-specific factor for the Skp1–Clu1–F-box (SCF) complex. SCF catalyzes the ubiquitylation of multiple functional proteins, such as the cyclin-dependent kinase inhibitors (CKIs) p27, p57, and p21, and thereby promotes their degradation [4]. Most of the SCF targets are tumor suppressor proteins, which are involved in many different cellular processes, including the cell cycle, cell proliferation, differentiation, apoptosis, and survival, indicating that Skp2 functions as an oncoprotein [5]. Skp2 is overexpressed by various types of

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human cancers including colorectal carcinoma [4], breast cancer [6], hepatocellular carcinoma [7], myxofibrosarcoma [8], oral squamous cell carcinoma [9], nonsmall cell lung carcinoma [10], as well as gastric cancer, and is significantly associated with poor prognosis [11, 12].

Emerging evidence indicates that Skp2 promotes cancer progression by enhancing cell growth, inhibiting apoptosis, regulating the cell cycle, promoting invasion and metastasis, inducing drug resistance, and participating in cross-talk with other major cancer signaling pathways [13–16]. Skp2-transfected gastric cancer cells are resistant to actinomycin D-induced apoptosis [11]. Conversely, Skp2 overexpression increases the expression of matrix metalloproteinase (MMP)-2 and MMP-9 and the invasive ability of lung cancer cells [17]. Therefore, inhibition of Skp2 has become a promising strategy to combat cancers. For instance, downregulation of Skp2 by RNA interference significantly inhibits the proliferation of a variety of tumor cell lines derived from breast [18], oral [19], lung [20], and colorectal [4] cancers and hepatocellular carcinoma. Further, deficiency of Skp2 profoundly restricts breast cancer metastasis [21]. However, it is not known whether Skp2 promotes the growth and metastasis of gastric carcinoma. This study seeks to determine whether downregulation of Skp2 inhibits the growth and metastasis of gastric carcinoma and to investigate the underlining mechanisms.

Material and methods

Cell culture and mice

Human gastric carcinoma cells MGC803, SGC7901, BGC823, AGS, NCI-N87, and HGC-27 were obtained from the Type Culture Collection Cell Bank (Chinese Academy of Sciences Committee, Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin, at 37 °C in a humidified atmosphere of 5 % CO₂. Six- to 8-week-old male nude BALB/c mice (H-2b) were obtained from the Animal Research Center, The First Affiliated Hospital of Harbin Medical University, China.

Antibodies and reagents

The antibodies (Abs) used in this study included primary Abs against Skp2, p27, p21, p57, cyclin-dependent kinase 2 (CDK2), cyclin E, MMP-2, MMP-9, RECK (reversion-inducing cysteine-rich protein with kazal motifs), metalloproteinase inhibitor 2 (TIMP-2), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), secondary horseradish peroxidase-conjugated rabbit antigoat, goat antimouse, and goat antirabbit Abs (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China).

Expression vectors and establishment of stable transfectants

The Skp2-small hairpin RNA (shRNA) pSuppressorNeo expression vector has been described previously [19]. Briefly, two oligonucleotides 5'-tcgaGGGAGUGACAAAGACUUUGgaguacugCAAAGUCUUUGUCACUCCCUUUUUU-3' and 5'-ctagAAAAAGGG AGUGACAAAGACUUUGcaguacucCAAAGUCUUUGUCACUCC-3' were annealed and introduced into the pSuppressorNeo vector. The first capitalized region corresponds to nucleotides 114–133 of human Skp2 (GenBank accession number NM_005983.2). A scrambled shRNA expression vector was used as the control. MGC803 cells were seeded in 10-cm plastic dishes and grown to 66 % confluence at which point they were transfected with 4 µg of pSuppressorNeo vector using Lipofectamine2000 (Invitrogen). Cells were detached with trypsin after transfection for 48 h and seeded in selection medium containing geneticin (G418; 500 µg/ml). Stable transfectants were selected at 4 weeks of culture. The complementary DNA encoding human Skp2 (GenBank NM_005983) was generated by reverse transcription PCR, sequenced and subcloned into pcDNA3.1 (Invitrogen) vector. Stable BGC823 transfectants (Skp2^{high}) that overexpress Skp2 were established as above, and overexpression of Skp2 was confirmed by Western blot analysis.

Cell viability assay

The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Beijing, China) was used to determine cell viability. Cells were seeded at 1×10^3 cells/well in 96-well plates. At different time points, the culture medium was replaced with 100 µl of fresh medium containing 10 µl of CCK-8 solution. The cells were further incubated for 2 h at 37 °C, and the optical density (OD) at 450 nm was measured. The experiment was repeated thrice.

Bromodeoxyuridine incorporation assay

Cell proliferation was measured using the 5-bromo-2-deoxyuracil (BrdU) labeling kit (Boster Biological Technology Ltd., China). Briefly, BrdU was added to the culture medium at the final concentration of 10 µM. BrdU-labeled indices were measured by visually scoring nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) in 50–100 cells in 20 independent visual fields. Thereafter, BrdU-positive cells were scored as a percentage of the total cell number. The experiment was repeated thrice.

Anchorage-independent growth assay

The soft agar colon formation assay was performed as described previously [22]. Cells (1×10^3) were seeded in

35-mm culture plates containing 0.5 % agar and RPMI-1640 medium with 10 % FCS and cultured for 2 weeks. The colonies were fixed with 4 % paraformaldehyde and stained with Giemsa stain. The plates were dried and photographed, and colonies containing more than 50 cells were counted. The experiment was repeated thrice.

Assessment of cell cycle and apoptosis

Cells were seeded at 5.0×10^5 cells/well in six-well plates, cultured for 48 h, and then harvested. The percentage of cells at G1 and S phases was determined with a cell cycle detection kit (BD Biosciences, Beijing, China) using flow cytometry with a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA). The above cells were incubated with 5 μ l of Annexin V and 5 μ l of propidium iodide (PI) for 15 min at room temperature in dark, according to the manufacturer's instruction (BD Biosciences, San Jose, CA, USA), and then subjected to flow cytometry to measure the apoptosis rate (percent) or viewed under a laser scanning confocal microscope (LSM-510 Meta, Carl Zeiss Jena GmbH, Jena, Germany). The experiments were repeated thrice.

Caspase-3 activity assay

A Caspase-Glo[®] 3/7 Assay kit (Promega, Beijing, China) was used to measure caspase-3 activity.

Migration and invasion assays

Transwells (8- μ m pore size) and the basement membrane matrix Matrigel were purchased from BD Bioscience (San Jose, CA, USA). For migration assays, 2×10^4 cells resuspended in 200 μ l of serum-free medium were seeded on the polycarbonate membrane in a transwell culture chamber, and the lower chamber was filled with 750 μ l of medium with 10 % FBS as a chemoattractant. After incubation for 12 h at 37 °C in a humidified atmosphere of 5 % CO₂, the transwell culture chamber was washed with phosphate-buffered saline (PBS), and the cells on the top surface of the polycarbonate membrane were removed. For invasion assays, a similar procedure as above was conducted, except that the transwell membrane was coated with 300 ng/ μ l of Matrigel, and the cells were incubated for 24 h at 37 °C. Cells that migrated to the bottom surface of the insert were fixed with methanol and stained by Giemsa stain. The cells were counted based on digital images of five fields taken randomly at $\times 200$. The experiments were repeated thrice.

Immunofluorescence detection of F-actin

Cells were washed with PBS, fixed with 4 % paraformaldehyde for 30 min, permeabilized with 0.1 % Triton X-100 in

PBS for 15 min, stained with a 50 μ g/ml fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma) in PBS for 40 min at room temperature, and viewed by fluorescence microscopy.

Western blot analysis

The methods have been described previously [23, 24]. Briefly, lysates of cells or tissues containing an equal amount of protein (20 μ g) were resolved on sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with primary Abs, followed by horseradish peroxidase-conjugated secondary Abs, and then developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co. Ltd., Beijing, China). Blots were stained with anti- β -actin Ab, and the relative protein level (percent) of each protein was normalized with respect to β -actin band density.

Gelatin zymography assay

Conditioned medium from an equal number of cells that had been incubated in serum-free medium for 48 h was collected and separated on 10 % acrylamide gels containing 0.1 % gelatin (Invitrogen). The gels were incubated in 2.5 % Triton X-100 solution at room temperature with gentle agitation to remove SDS, and soaked in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, and 0.5 mM ZnCl₂) at 37 °C overnight. After reaction, the gels were stained for 1 h with staining solution (0.1 % Coomassie Brilliant Blue, 30 % methanol, and 10 % acetic acid) and then destained in the same solution without Coomassie Brilliant Blue. The gelatinolytic activity of MMP-2 and MMP-9 was visualized as a clear band against a dark background of stained gelatin.

Animal experimental protocols

All surgical procedures and care administered to the animals were in accordance with institutional ethics guidelines.

Tumorigenicity study Cells (1×10^6) were injected subcutaneously into both sides of the flanks of mice. There were four mice per group; hence, each group of mice had eight tumors. The animals were monitored for tumor formation every week and killed 7 weeks later. Tumors were measured at the end of the experiment, and tumor volume was estimated by the formula: $\pi/6 \times a^2 \times b$, where a is the short axis, and b the long axis.

In vivo metastasis study Cells (1×10^6) were injected into groups of six mice via the lateral tail vein. The mice were

killed 7 weeks later, and their lungs were removed. The numbers of metastases on the lung surface were counted with a magnifying lens, and the lungs were weighed and subjected to hematoxylin and eosin (HE) staining.

Therapeutic effect study Cells (1×10^6) were injected subcutaneously into the left flanks of mice. When tumors reached $\sim 100 \text{ mm}^3$, the mice were assigned to two groups of six mice, which received an intratumoral injection of scrambled shRNA (control) or Skp2 shRNA, respectively. The shRNA transfection solution was prepared by mixing shRNA expression vector, Lipofectamine 2000 and serum-free medium. Each tumor received an injection of $50 \mu\text{l}$ shRNA transfection solution containing $200 \mu\text{g}$ shRNA. The tumors were measured every 4 days and the mice killed 28 days later.

Immunohistochemistry, assessment of cell apoptosis in situ, and quantitation of Ki-67 proliferation index

The methods have been described previously [23–25].

Statistical analysis

All data were expressed as the mean values \pm standard deviation. Comparisons were made with a one-way analysis of variance followed by a Dunnett's *t* test using SPSS software (version 17.0). $P < 0.05$ was considered statistically significant.

Results

Downregulation of Skp2 inhibits cell proliferation

The expressions of Skp2 and its downstream target, p27, were detected in six human gastric cancer cells. As shown in Fig. 1a, the expression levels of Skp2 varied in different cell lines. MGC803, SGC7901, and NCI-N87 cells expressed a higher level of Skp2 protein, whereas BGC823, AGS, and HGC-27 cells expressed a lower level of Skp2 protein. MGC803, SGC7901, and BGC823 cells were transfected with the Skp2 shRNA vector, and 48 h later, the expression of Skp2 was determined by Western blot analysis. As shown in Fig. 1b, c, the levels of Skp2 protein were highly significantly (both $P < 0.001$) reduced in both the MGC803 and SGC7901 cells and significantly ($P < 0.05$) reduced in BGC823 cells. Based on the above results, the MGC803 and BGC823 cells, which express a higher and lower level of Skp2 protein, respectively, were used for the following experiments. Both cell lines were transfected with Skp2 shRNA or the scrambled shRNA (control) and selected with

G418 to obtain stable transfectants of each. The MGC803 and BGC823 cells, which were stably depleted of Skp2, were cultured and their cell viability was measured with a CCK-8 kit. As shown in Fig. 1d, depletion of Skp2 highly significantly ($P < 0.001$) reduced the viability of MGC803 cells but had almost no effect on BGC823 cells, compared with the respective scrambled shRNA-transfected cells.

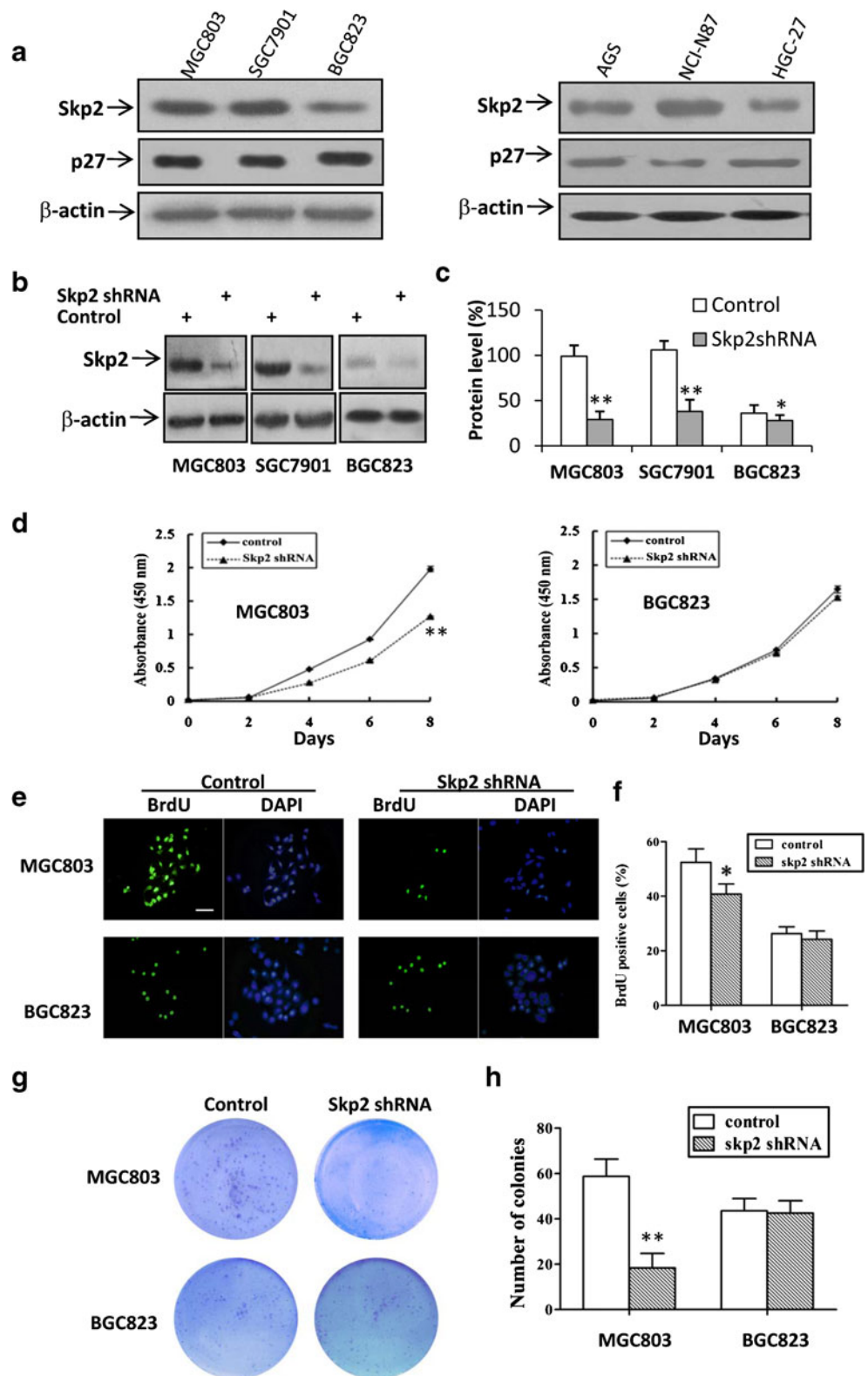
In accord with the results of cell viability, cell proliferation as measured by BrdU-DAPI staining was also decreased following downregulation of Skp2, as evidenced by a significant ($P < 0.05$) decrease in the percentage of BrdU-positive MGC803 cells (Fig. 1e, f). Specifically, $52.18 \pm 5.0\%$ of MGC803 cells transfected with the scrambled control shRNA were BrdU-positive, while only $40.8 \pm 4.0\%$ of the Skp2-depleted MGC803 cells were BrdU-positive. In contrast, the percentage of BrdU-positive Skp2-depleted BGC823 cells was not significantly different from that of the control cells (Fig. 1e, f). Similarly, the sizes and the numbers of colonies of Skp2-depleted MGC803 cells in soft agar plates in an anchorage-independent growth assay were highly significantly decreased compared with control MGC803 cells transfected with scrambled shRNA (Fig. 1g, h). In contrast, there was no change in the sizes and the numbers of colonies of Skp2-depleted BGC823 cells seeded into soft agar plates (Fig. 1g, h).

Downregulation of Skp2 induces cell cycle arrest in G1 phase by modulating the expression of regulators in the G1/S phase transition

We next investigated the effects of Skp2 depletion on the cell cycle as analyzed by flow cytometry. As shown in Fig. 2a, b, the fraction of cells at the G1 phase was significantly ($P < 0.05$) increased in Skp2-depleted MGC803 cells compared with matching control cells, but Skp2 depletion had almost no effect on the cell cycle progression of BGC823 cells.

To elucidate the mechanism accounting for the inhibition of cell cycle arrest by Skp2 depletion, we examined the expression of regulators of the G₁/S phase transition, including cyclin E and CDK2, and the Skp2 targets p27, p21, and p57. As shown in Fig. 2c and d, the protein levels of cyclin E and CDK2 were highly significantly ($P < 0.001$) decreased in Skp2-depleted MGC803 cells compared with those in the control cells. In contrast, the levels of p27 were highly significantly ($P < 0.001$) increased, and the levels of p21 and p57 were significantly ($P < 0.05$) elevated in Skp2-depleted MGC803 cells compared with the control cells (Fig. 2c, d). In contrast, with the exception that p21 was significantly ($P < 0.05$) upregulated in Skp2-depleted BGC823 cells, the protein levels of cyclin E, CDK2, p27, and p57 remained unchanged in Skp2-depleted BGC823 cells, compared with control cells (Fig. 2c, d).

Fig. 1 Downregulation of Skp2 inhibits the growth of gastric cancer cells in vitro. **a** The endogenous expression of Skp2 and p27 proteins in a panel of human gastric cancer cell lines was assessed by Western blot analysis. **b** The cells were transfected with scrambled shRNA (control) or Skp2 shRNA, and expression of Skp2 was evaluated by Western blot analysis. **c** The expression levels of Skp2 in **b** were normalized with that of β -actin. **d** MGC803 or BGC823 cells, stably transfected with scrambled shRNA (control) or Skp2 shRNA, were cultured and their viability was assessed using a CCK-8 assay. The optical density (OD) at 450 nm as a measure of cell viability was measured at the indicated time points. **e** The above cells in **d** were cultured for 4 days, and cell proliferation was further analyzed using a bromodeoxyuridine (BrdU) incorporation assay. BrdU (*green*) stains the nuclei of only proliferating cells, whereas DAPI (*blue*) stains the nuclei of all cells ($\times 200$ magnification). Magnification bar=200 μ m. **f** The percentage of BrdU-positive cells was plotted. **g** The above stable transfectants in **d** were seeded into soft agar to evaluate their ability to form colonies. **h** The numbers of colonies were plotted. A significant reduction from control is denoted by a *single asterisk* and a highly significant reduction by a *double asterisk*

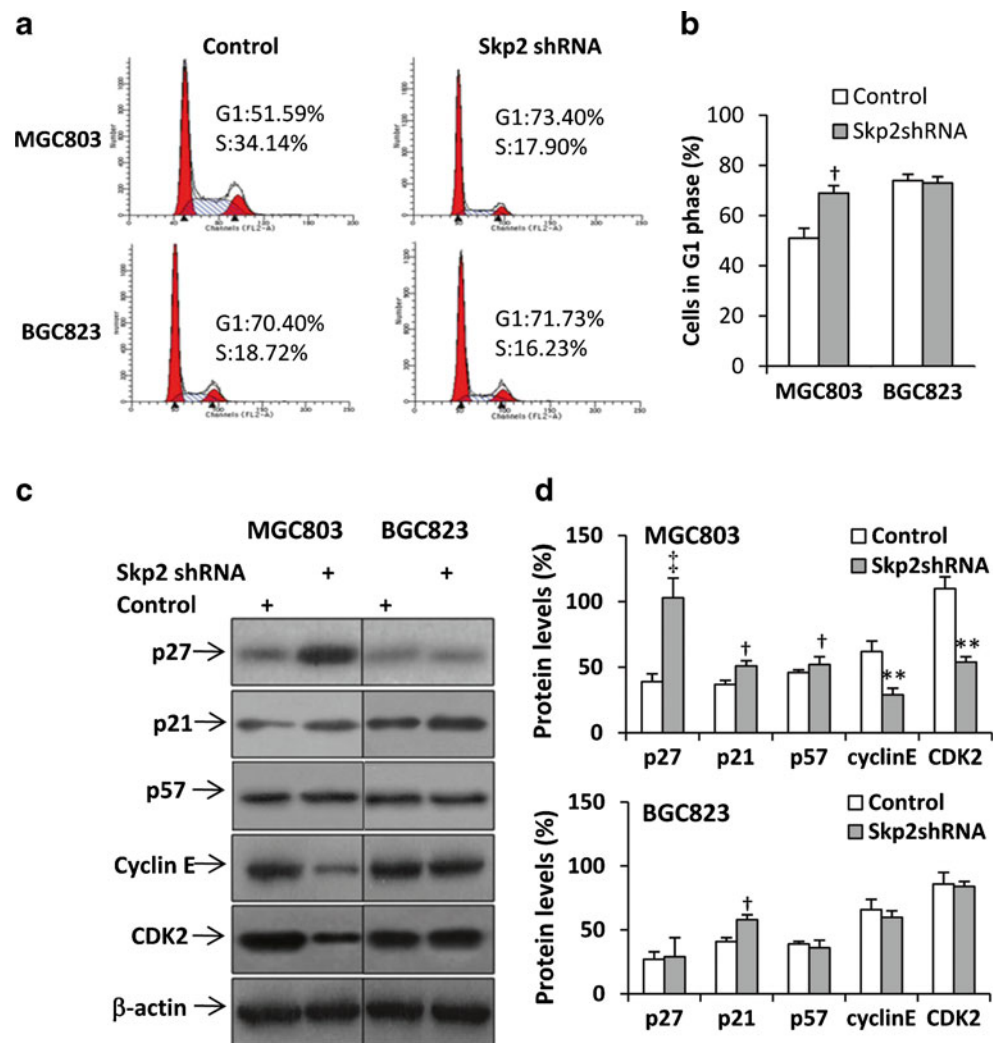


Downregulation of Skp2 induces cell apoptosis

As shown in Fig. 3a, b, Skp2 depletion highly significantly ($P < 0.001$) increased the apoptosis rate of MGC803 cells, and significantly ($P < 0.05$) increased the apoptosis rate of

BGC823 cells, as compared with the respective control cells. Skp2 depletion also highly significantly ($P < 0.001$) increased caspase-3 activity in MGC803 cells, and significantly ($P < 0.05$) increased caspase-3 activity in BGC823 cells (Fig. 3c). Representative photographs were taken of

Fig. 2 Downregulation of Skp2 induces cell cycle arrest. **a** MGC803 or BGC823 cells were transfected with scrambled (control) or Skp2 shRNAs and harvested 48 h later. The cells were subjected to flow cytometry to measure the stages of the cell cycle. **b** The percentages of cells in the G1 phase were plotted. **c** Expression of Skp2, p27, p21, p57, cyclin E, and CDK2 proteins in MGC803 or BGC823 cells in **a** was detected using Western blot analysis. **d** The density of each protein band was measured and normalized to that of β -actin. A highly significant reduction from control is denoted by a *double asterisk*. A significant increase from control is denoted by a *dagger* and a highly significant increase by a *double dagger*



scrambled shRNA-transfected (control) and Skp2 shRNA-transfected MGC803 cells, which had been stained with Annexin V/PI and examined by confocal microscopy. The early staged apoptotic Skp2 shRNA-transfected cells were stained green by Annexin V, and the nuclei of late-staged apoptotic cells were stained red by PI (Fig. 3d).

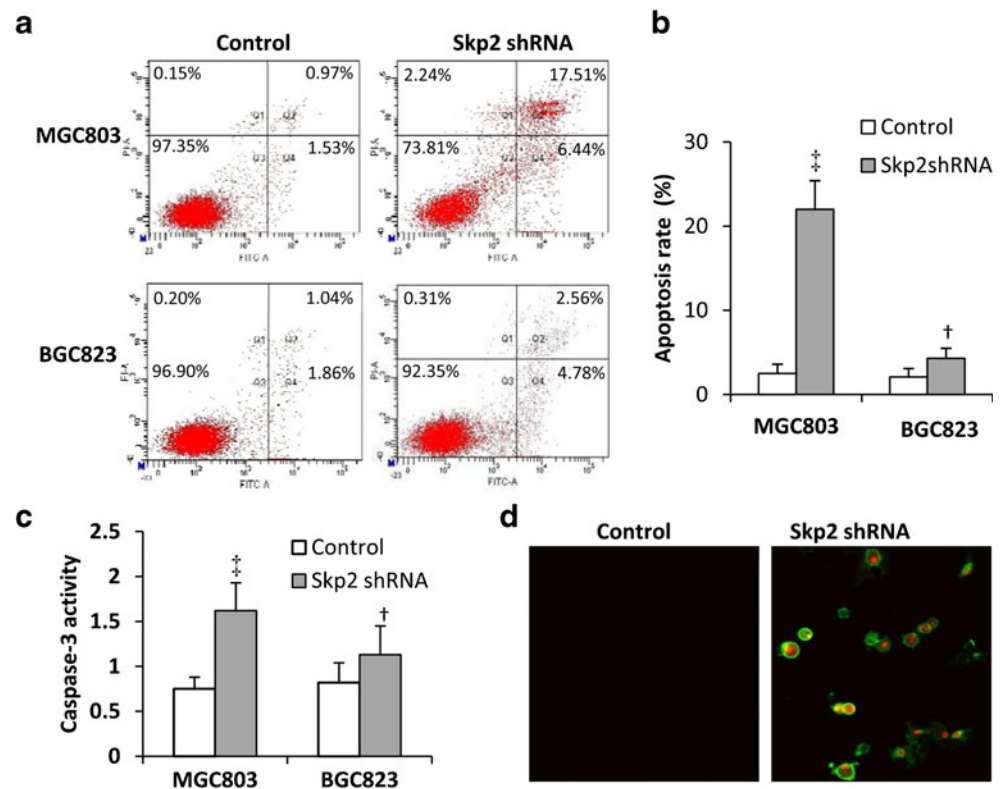
The above results indicate that downregulation of Skp2 greatly influences the proliferation, cell cycle, and apoptosis of MGC803 cells, but only slightly affects with respect to BGC823 cells. Therefore, downregulation of Skp2 in regulating tumorigenicity was only examined in MGC803 cells in the following experiments.

Downregulation of Skp2 inhibits cell migration and invasion

It has been demonstrated that high expression of Skp2 is correlated with the degree of malignancy of tumors [13, 15]; thus, we tested whether Skp2 deletion would reduce the ability of MGC803 cells to migrate and invade. The numbers of

migrated and invaded Skp2-depleted cells in transwell migration and invasion assays were highly significantly (both $P < 0.001$) lower than with scrambled shRNA-transfected control cells (Fig. 4a–c). F-actin was stained with FITC-conjugated phalloidin to determine whether Skp2 depletion alters the structure of the cytoskeleton, which is essential for cell motility. As shown in Fig. 4d, marked changes in the cell morphology were observed in Skp2-depleted MGC803 cells compared with controls. The number of Skp2-depleted cells containing extended filopodia was highly significant ($P < 0.001$) less than with control cells (Fig. 4e). We further measured the activity of MMP-2 and MMP-9, as there is a clear relationship between MMPs and cancer cell invasion [26]. A gelatin zymography assay showed that the activities of MMP-2 and MMP-9 were reduced in the Skp2-depleted cells, compared with control cells (Fig. 4f). In accord, the protein levels of MMP-2 and MMP-9 were also significantly (both $P < 0.05$) reduced by Skp2 depletion (Fig. 4g, h). In addition, the membrane-anchored matrix metalloproteinase-regulator RECK, a negative regulator of MMP-2 and MMP-9 and an inhibitor of

Fig. 3 Downregulation of Skp2 promotes tumor cell apoptosis. The MGC803 and BGC823 cells in Fig. 2 were stained with Annexin V/PI, and subjected to flow cytometry to measure cell apoptosis. **a** Representative dot plots from cytometrically analyzed cells are shown. The apoptosis rate (**b**) and caspase-3 activity (**c**) were plotted. **d** Representative photographs of MGC803 cells transfected with scrambled (control) or Skp2 shRNAs, stained with Annexin V/PI, and viewed by laser scanning confocal microscopy. A significant increase from control is denoted by a dagger and a highly significant increase by a double dagger



tumor metastases [27], was significantly upregulated by Skp2 depletion, whereas TIMP2, a tissue inhibitor of MMP-2 and MMP-9 [28], remained unchanged upon Skp2 depletion (Fig. 4g, h).

Downregulation of Skp2 inhibits tumorigenesis and tumor metastasis

Skp2-depleted MGC803 cells were subcutaneously injected into mice to investigate whether their biological behavior in vivo had been changed compared to the cells in vitro. As shown in Fig. 5a, Skp2-depleted MGC803 tumors grew to only $1,363.6 \pm 152.3 \text{ mm}^3$ in size at 7 weeks following inoculation, whereas the control tumors of $1,985.7 \pm 229.4 \text{ mm}^3$ were highly significantly ($P < 0.001$) larger. We further investigated the formation of metastases in the lungs of mice that had been intravenously injected with cancer cells. The mice were killed 7 weeks following injection of tumor cells, and their lungs were weighed. As shown in Fig. 5b, the mice injected with Skp2-depleted MGC803 cells had an average lung weight of $290.2 \pm 43.5 \text{ mg}$, which was significantly ($P < 0.05$) lighter than the average lung weight of $365.3 \pm 56.7 \text{ mg}$ of mice that received the control cells. Representative images were taken from HE-stained lung sections from the mice injected with Skp2-depleted MGC803 cells or control cells (Fig. 5c). The results indicate that Skp2 status affects tumorigenesis. In support of this finding, parental BGC823 cells or their stable transfectants (Skp2^{high}) were subcutaneously

injected into the mice. As shown in Fig. 5d, Skp2^{high} tumors grew to $1,639 \pm 206 \text{ mm}^3$ in size, which was significantly greater than the control tumors of $1,267 \pm 139 \text{ mm}^3$ at 5 weeks following inoculation.

Gene transfer of Skp2 shRNA inhibits tumor growth

Next, we investigated whether gene transfer of shRNA targeting Skp2 could show an inhibitory therapeutic effect on the growth of established tumors in mice. Tumors were established by subcutaneous injection of MGC803 cells into the flanks of mice. When tumors reached $\sim 100 \text{ mm}^3$, the mice were assigned to two groups, which received an intratumoral injection of either the scrambled (control) shRNA or the Skp2 shRNA, respectively. As shown in Fig. 5e, tumors in the control group grew remarkably fast reaching $1,747.2 \pm 164.5 \text{ mm}^3$ in size 4 weeks after treatment started. In contrast, the tumors treated with Skp2 shRNA were highly significantly ($P < 0.001$) smaller than the control tumors, reaching only $1,122.8 \pm 143.1 \text{ mm}^3$.

Tumor cell proliferation and apoptosis in situ

In accord with the in vitro results, Skp2 shRNA significantly downregulated the expression of Skp2, cyclin E, and CDK2 and upregulated the expression of p27 expression (Fig. 6a, b), in the tumors of mice shown in Fig. 5e. Skp2 shRNA-treated tumors had fewer Ki-67-positive cells and more apoptotic cells than scrambled shRNA-treated control

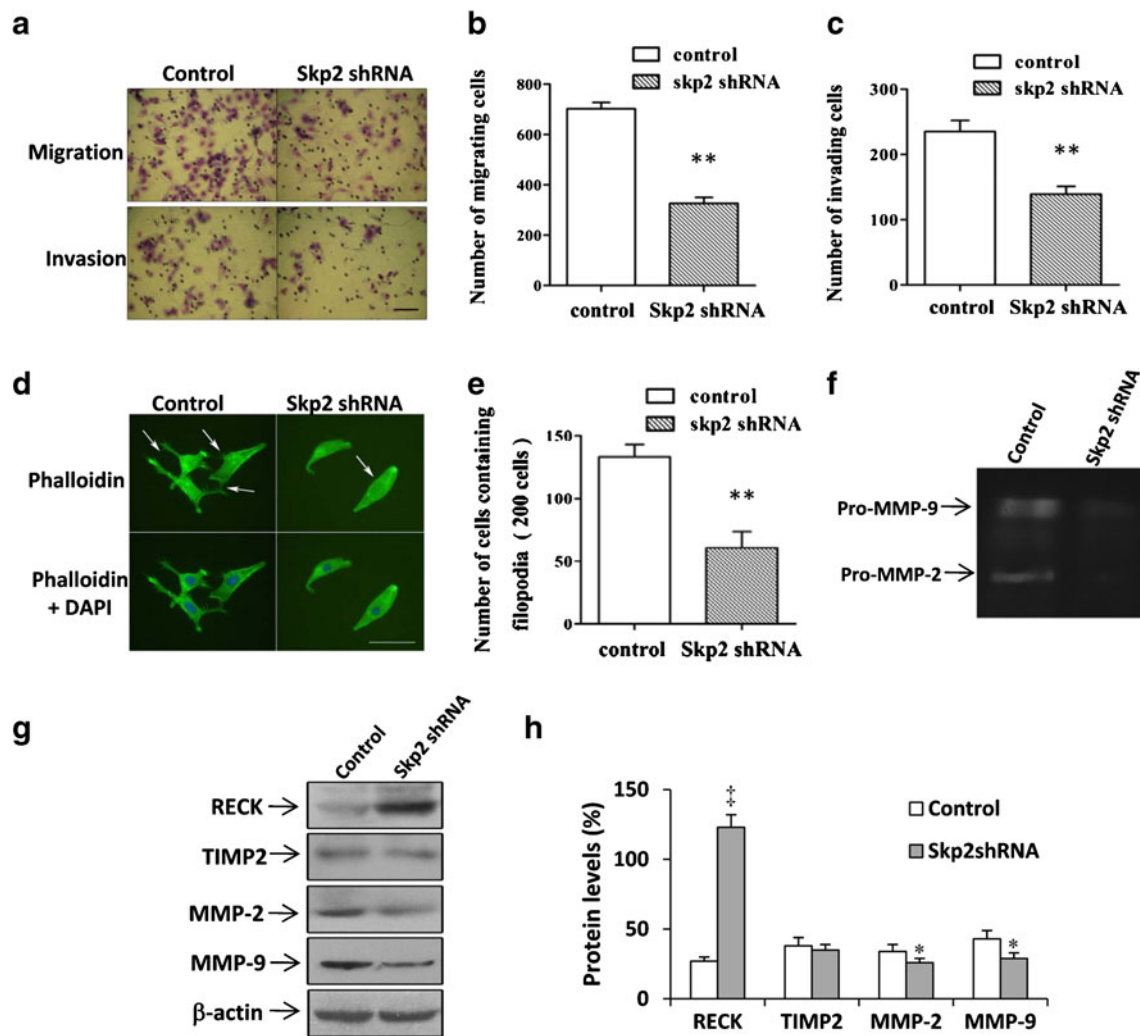


Fig. 4 Downregulation of Skp2 inhibits tumor cell migration and invasion. **a** MGC803 cells stably transfected with scrambled (control) or Skp2 shRNAs, were subjected to migration and invasion assays. Migrated or invaded cells were visualized using Giemsa staining ($\times 200$ magnification). Bar=200 μm . **b** Numbers of migrating cells were counted. **c** Numbers of invading cells were counted. **d** The above cells in **a** were immunostained with phalloidin for the presence of microfilaments (green), and the cell nuclei were stained with DAPI (blue; $\times 400$ magnification). The arrows point to filopodia. Bar=50 μm . **e** The number of cells containing filopodia were counted. **f** The conditioned

medium of MGC803 cells in **a** were subjected to a gelatin zymography assay to analyze the gelatinolytic activity of pro-MMP-9 (upper line, ~92 kDa) and active MMP-2 (lower line, ~66 kDa). **g** Lysates of the above cells in **a** were subjected to Western blot analysis to determine the expression of RECK, TIMP-2, MMP-2, and MMP-9. **h** The density of each protein band was measured and normalized to that of β -actin. A significant reduction from control is denoted by a single asterisk and a highly significant reduction by a double asterisk. A highly significant increase from control is denoted by a double dagger

tumors (Fig. 6c). The proliferation index of tumors treated with Skp2 shRNA was significantly ($P < 0.05$) lower by 43.3 % than that of the control tumors (Fig. 6d). The tumors treated with Skp2 shRNA had a highly significantly ($P < 0.001$) higher apoptosis index by 146.9 % than the control tumors (Fig. 6e).

Discussion

Skp2 acts as an oncoprotein by altering the cell cycle, proliferation, differentiation, apoptosis, and survival of cancer cells

[5]. The present study has, for the first time, systemically demonstrated that downregulation of Skp2 by RNA interference inhibits the viability, proliferation, colony formation, migration, and invasion, and induces apoptosis of human gastric cancer MGC803 cells that express high levels of Skp2. In contrast, knockdown of Skp2 had only a slight effect on the above properties of BGC823 cells, which express low levels of Skp2. In accord with the results obtained in vitro, Skp2 depletion significantly suppressed the ability of MGC803 cells to form tumors to metastasize to the lungs of mice and tumor growth by inhibiting cell proliferation and enhancing cell apoptosis, while overexpression of Skp2 in

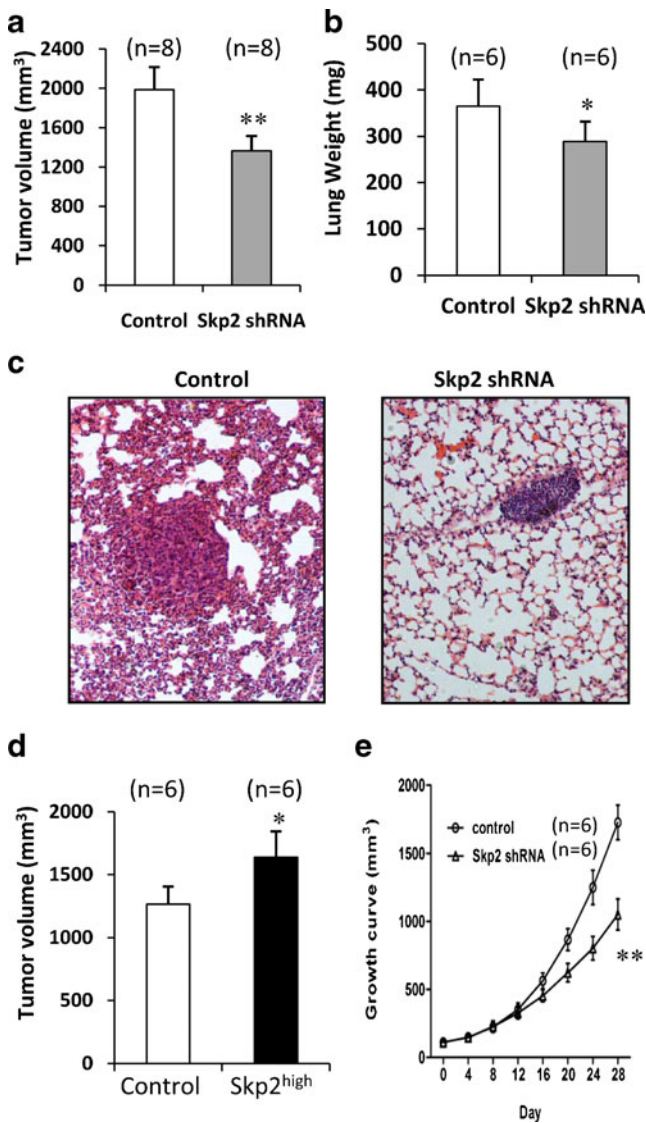


Fig. 5 Effects of Skp2 expression on tumorigenesis, metastasis, and tumor growth in mice. **a** MGC803 cells stably transfected with scrambled (control) or Skp2 shRNAs were subcutaneously inoculated into the mice. Tumor volumes were measured 7 weeks later. **b** The above cells were intravenously injected into the mice. The mice were killed 7 weeks later to harvest the lungs, which were weighed. **c** Representative photographs of HE-stained lung sections. **d** Parental BGC823 cells (control) or their stable transfectants (Skp2^{high}) were subcutaneously inoculated into the mice. Tumor volumes were measured 5 weeks later. **e** Mice were subcutaneously injected with MGC803 cells and tumors that formed were injected with scrambled (control) or Skp2 shRNAs when they reached ~100 mm³ in volume. The sizes of tumors were measured. “n” indicates the number of tumors examined. A significant difference from control is denoted by a *single asterisk* and a highly significant difference by a *double asterisk*

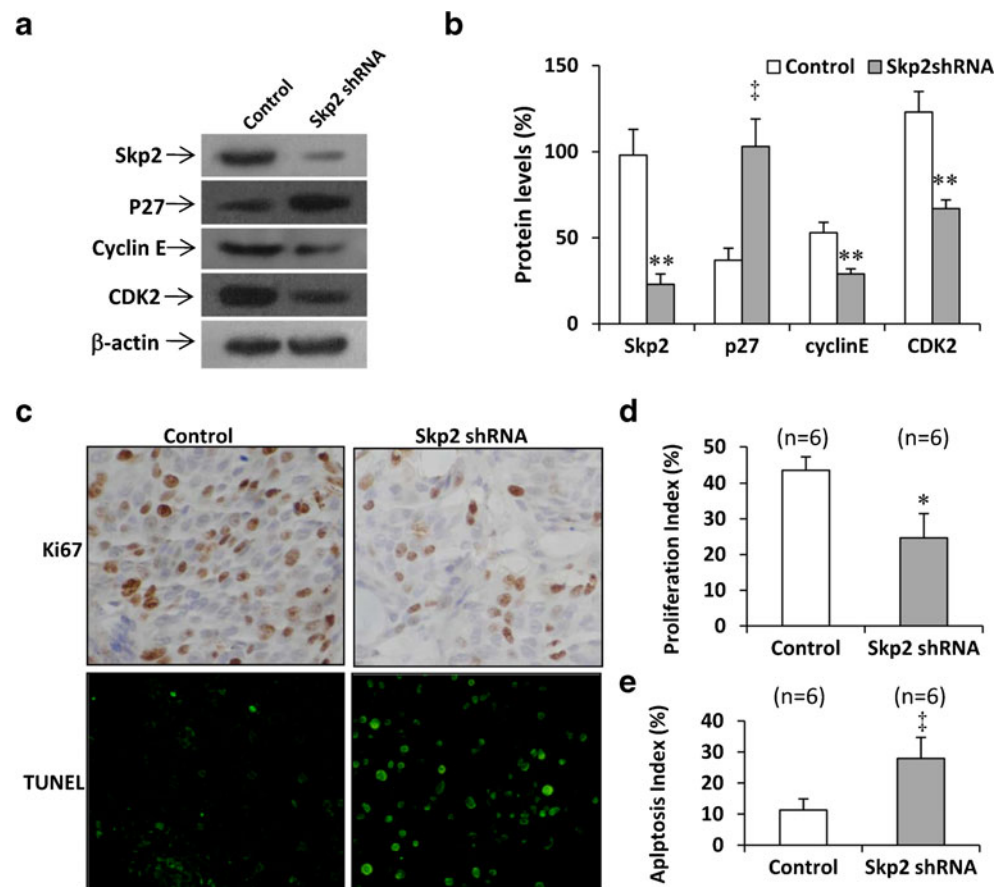
BGC823 cells enhanced tumorigenesis in vivo. The results indicate that downregulating Skp2 may warrant investigation as a promising strategy to treat gastric cancers that express high levels of Skp2. In one study, the majority (65 out of 98) of gastric cancer tissues were found to express high levels of Skp2, which was associated with a significantly poorer

prognosis [11]. In support of the present study, gastric carcinoma cells that were engineered to stably express high levels of Skp2 displayed an aggressive behavior, including a higher growth rate and invasive potential, and greater resistance to apoptosis [11].

A key function of Skp2 is to drive cell cycle progression by inducing the ubiquitination and degradation of p27 [29, 30]. Here, we have shown that Skp2 knockdown resulted in the cell cycle arrest of MGC803 cells due to effects on the expression of p21, p27, p57, cyclin E, and CDK2, which regulate the G₁/S phase transition [31]. p21, p27, and p57 are implicated in the negative regulation of cell cycle progression from G₁ to S phase by binding to and modulating the activity of CDKs. Conversely, the cyclin E/CDK2 complex promotes progression from G₁ to S phase by triggering the initiation of DNA replication, regulating genes that orchestrate cell proliferation and G₁ to S phase transition, and mediating cyclin E–CDK2-dependent p27 phosphorylation [32]. Notably, Skp2 depletion only slightly effected the cell cycle and proliferation of BGC823 cells and had no significant impact on the expression of p27, p57, cyclin E, and CDK2, except for a slight upregulation of p21 protein. The difference in the results obtained with the two human gastric cancer cell lines can be explained by the different levels of Skp2 protein they express. A low level of expression in the case of BGC823 cells may indicate that Skp2 plays an insignificant role in regulating the proliferation and cell cycle progression of these cells. It has been reported that the function of Skp2 is cell-type dependent, though Skp2 is responsible for the majority of p27 degradation [16]. Nevertheless, the stabilities of p21, p27, and p57 are differentially regulated in a manner that relies upon many factors, such as diverse extracellular stimuli, differences in subcellular context in different tissues and cells, CKI interactions with other regulators, and phosphorylation by distinct protein kinases [32].

Recent evidence has revealed that Skp2 mediates cancer cell anoikis [33] and apoptosis [34]. Here, we have shown that Skp2 depletion resulted in elevated cell apoptosis in response to increased expression of p27 and caspase-3 activity. In accord, it has been reported that Skp2 depletion induced cell apoptosis in lung cancer [20], gastric cancer [11], glioblastomas [35], oral cancer [36], and breast cancer [18]. The main mechanism accounting for the enhanced cell apoptosis due to knockdown of Skp2 is the increase in p27 expression, as overexpression of p27 is reported to trigger the apoptosis of carcinoma cells [37]. Downregulation of Skp2 also increases the activation of caspase 3 [20], as reported in the present study. Although not investigated here, Skp2 has also been shown to suppress the p53-dependent apoptosis of cancer cells via inhibiting the coactivator p300 [14]. Downregulating Skp2 expression increased DNA-damage-mediated apoptosis in multiple

Fig. 6 Effects of downregulation of Skp2 on tumoral gene expression, apoptosis, and proliferation in situ. **a** Tumor homogenates prepared from mice in Fig. 5e at day28 when the mice were killed were Western blotted to measure the expression of Skp2, p27, cyclin E, and CDK2. **b** The density of each protein band in **a** was measured and normalized to that of β -actin. **c** Illustrated are representative tumor sections prepared from the mice in Fig. 5d, when they were killed at day 28. The sections were stained with an anti-Ki67 Ab (*upper panel*) or with the TUNEL agent (*lower panel*). The tumor cell proliferation index (**d**) and apoptosis index (**e**) were quantified. “*n*” indicates the number of tumors examined. A significant reduction from control is denoted by a *single asterisk* and a highly significant reduction by a *double asterisk*. A highly significant increase from control is denoted by a *double dagger*



cancer cells, and conversely, Skp2 overexpression suppressed p53-mediated apoptosis [14]. Overexpression of Skp2 also inhibited transactivation of forkhead box protein O1 (FOXO1) and abolished the inhibitory effect of FOXO1 on the apoptosis of prostate cancer cells [38].

Distant metastasis is the leading cause of mortality in gastric cancer and is a major obstacle in its treatment. Skp2 promotes cancer cell migration, invasion, and metastasis [39, 40]. The present study has demonstrated that Skp2 depletion significantly reduces the metastasis of intravenously injected gastric cancer cells to the lungs of mice. In accord, Skp2 depletion using RNA interference markedly reduced the capacity of esophageal cancer cells to migrate or invade in vitro and significantly decreased the metastasis of tumors to the lungs of nude mice [33]. There are several mechanisms responsible for the metastasis-promoting activities of Skp2. First, Skp2 regulates the cytoskeleton, which plays an important role in maintaining cell shape and coordinating cell locomotion. Skp2 facilitates the formation of microfilaments, filopodia, and stress fibers, and coordinates cell motility, thus enhancing the invasive potential of cells [11]. We have shown herein that Skp2 depletion results in a reduction in the number of migrated and invaded cells, and impaired formation of filopodia. The increased expression of p27 as a result of Skp2 depletion may contribute to this activity, as elevated levels of p27 have been

shown to decrease cell motility and reduce the formation of filopodia [41]. Secondly, Skp2 overexpression increases the expression of MMP-2 and MMP-9, leading to cell invasion in lung cancer cells [17]. MMP-2 and MMP-9 selectively increase the degradation of extracellular matrix components, which facilitates tumor invasion and metastasis [42]. Here, we have demonstrated that the activity and expression of MMP-2 and MMP-9 were significantly reduced in Skp2-depleted MGC803 cells. RECK and TIMP-2 are critical negative regulators of MMP-2 and MMP-9 [27, 28]. TIMP-2 interacts with the integrin $\alpha 3 \beta 1$ to induce transcriptional activation of p27 in an MMP-independent manner [43]. Here, we have shown that Skp2 depletion upregulated RECK protein, but had no effect on TIMP-2 expression in MGC803 cells. RECK expression in colon carcinoma cells has been shown to result in cell cycle arrest accompanied by downregulation of Skp2 and upregulation of its substrate, p27 [44]. Thus, the results indicate that RECK and Skp2 may suppress one another. Overexpression of RECK leads to the accumulation of p27, which in turn results in inhibition of the cell cycle, and the activities and expression of MMP-2 and MMP-9.

In summary, the results suggest that downregulating Skp2 inhibits the growth and metastasis of gastric carcinoma and warrants investigation as a promising strategy to treat gastric cancers that express high levels of Skp2.

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Conflicts of interest None

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