Small interfering RNA targeting of S phase kinase–interacting protein 2 inhibits cell growth of oral cancer cells by inhibiting p27 degradation

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Abstract

S phase kinase – interacting protein 2 (Skp2), an F box protein, is required for the ubiquitination and consequent degradation of p27. It is well known that reduced expression of p27 is frequently observed in various cancers including oral squamous cell carcinoma and is due to an enhancement of its protein degradation. Our previous study showed that overexpression of Skp2 was frequently found in oral squamous cell carcinoma and inversely correlated with p27 expression. Recently, a technique known as RNA interference has been successfully adapted to mammalian cells. In the present study, we investigated if small interfering RNA (siRNA)-mediated gene silencing of Skp2 can be employed in order to inhibit p27 down-regulation in oral squamous cell carcinoma. We used a siRNA plasmid vector, which has an advantage over synthetic siRNAs in determining the effects of decreasing the high constitutive levels of Skp2 protein in oral squamous cell carcinoma. We showed that Skp2 siRNA transfection decreased Skp2 protein and induced the accumulation of p27 protein in oral squamous cell carcinoma cells. Moreover, p27 protein in Skp2 siRNA-transfected cells is more stabilized than that in control siRNA-transfected cells. Interestingly, Skp2 siRNA inhibited the cell proliferation of oral squamous cell carcinoma cells both *in vitro* and *in vivo*. Our findings suggest that siRNA-mediated gene silencing of Skp2 can be a novel modality of cancer gene therapy for suppression of p27 down-regulation. [Mol Cancer Ther 2005;4(3):471 – 6]

Introduction

SCF^{Skp2} was identified as the E3 ubiquitin ligase that targets p27 for ubiquitination (1–3). SCF complexes represent an evolutionarily conserved class of E3 enzymes containing four subunits: Skp1, Cul1, one of many F box proteins, and Roc1/Rbx1 (4). Skp2, an F box protein, is required for the ubiquitination and consequent degradation of p27 both *in vivo* and *in vitro*. Binding of Skp2 to p27 phosphorylated on Thr^{187} has been shown. In addition, *in vitro* ubiquitination of recombinant p27 can be induced by the addition of purified Skp2 and cyclin E/cyclin-dependent kinase (Cdk) 2 or cyclin A/Cdk2 complexes to G_1 cell extracts. Antisense oligonucleotides to Skp2 or overexpression of a dominant-negative Skp2 mutant stabilize p27 protein *in vivo* (1). These findings indicate that Skp2 is specifically required for p27 ubiquitination and that Skp2 is a rate-limiting component of the machinery that ubiquitinates and degrades phosphorylated p27. Skp2 is frequently overexpressed in tumor cell lines, and forced expression of Skp2 in quiescent fibroblasts induces DNA synthesis (5). We and other groups have previously found that Skp2 overexpression was frequently observed in oral squamous cell carcinoma (6, 7). Skp2 overexpression is also found in other types of malignant tumors including lymphomas (8, 9), breast carcinomas (10), colorectal carcinomas (11), small cell lung cancers (12), and gastric carcinomas (13). Furthermore, Skp2 expression increases significantly during malignant progression from epithelial dysplasia to invasive oral squamous cell carcinoma (7). Importantly, Skp2 overexpression was well correlated with down-regulation of p27 protein in oral squamous cell carcinoma (6).

It is well known that reduced expression of p27 is frequently found in various cancers, and the lack of p27 is suggested to be due to an enhancement of its degradation (14). p27 is Cdk inhibitor and mediates G_1 arrest induced by transforming growth factor-β, contact inhibition, or serum deprivation in epithelial cell lines (15, 16). The increase in the cellular abundance of p27 upon induction of cell quiescence is primarily due to a decrease in the rate of its degradation (17). p27 is polyubiquitinated both *in vivo* and *in vitro* and a lower amount of p27 ubiquitinating activity is present in proliferating cells compared with quiescent cells (17). Furthermore, p27 ubiquitination requires its phosphorylation on Thr^{187} (18). In fact, aggressive human cancers such as colon cancers,

lymphomas, and astrocytic brain tumors express low levels of p27 because of its decreased stability (19–22). We also found that reduced expression of p27 was shown in 87% of oral squamous cell carcinoma cases and was well correlated with its malignancy including metastasis and poor prognosis (23). These previous findings suggest that enhanced p27 degradation observed in oral squamous cell carcinoma might be due to increased levels of Skp2, and that Skp2 overexpression may play an important role for the development of oral squamous cell carcinoma. In the present study, therefore, we examined if small interfering RNA (siRNA)-mediated gene silencing of Skp2 can be employed in order to inhibit p27 down-regulation in oral squamous cell carcinoma.

Materials and Methods

Cell Culture

Two oral squamous cell carcinoma cell lines (HSC3 and HSC4) were used in this study. These cell lines were provided by the Japanese Cancer Research Resources Bank. They were routinely maintained in RPMI 1640 (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and 100 units/mL penicillin-streptomycin (Life Technologies, Grand Land, NY) under conditions of 5% CO_2 in air at 37°C. For experiments, they were grown to subconfluence in this medium. Cells were lysed as described previously (6), and cell lysates were used for Western blot analysis.

Proteasome Inhibitor Treatment

We used the specific proteasome inhibitor Z-Leu-Leu-Leu-CHO (ZLLL) to examine the proteasome-mediated degradation activity in oral squamous cell carcinoma cell lines. ZLLL was obtained from the Peptide Institute, Osaka, Japan. The compound was dissolved in an amount of dimethyl sulfoxide required to establish a stock solution of 10 mmol/L. ZLLL was added to the oral squamous cell carcinoma cells at the final concentrations of 25 μmol/L for 5 hours.

Plasmids and Synthetic siRNA

Vector pSuppressorNeo generates biologically active siRNAs from the U6 promoter (Imgenex, San Diego, CA). Synthetic two-oligonucleotide primers(5′-tcgaGGGAGUG-ACAAAGACUUUGGgaguacugCAAAGUCUUUGUCA-CUCCCUUUUU-3′ and 5′-ctagAAAAAGGGAGUGACAA AGACUUUGGcgaguacucCAAAGUCUUUGUCACUCCC-3′) containing *Xho*I and *Xba*I overhangs were annealed and then were introduced into pSuppressorNeo vector. Oligo-nucleotide sequences correspond to a 19-nucleotide sequence from Skp2 (nucleotides 114-133), which are separated by an 8-nucleotide linker (lowercase letters) from the reverse complement of the same 19-nucleotide sequence (Fig. 1C). We also added transcriptional termi-nation (UUUUU) to the end of oligonucleotide. We used circular control plasmid, which contains a scrambled sequence that does not show significant homology to rat, mouse or human gene sequences, as a control. Transfection

was done in 60 mm plates using 2 μg of vector and 10 μL of Fugene 6 reagent (Roche, Penzberg, Germany). After 48 hours of transfection, cells were treated with G418 (Life Technologies) for 2 weeks as a selective marker. After G418 treatment, we cloned several stably transfectant cells. Each clone was screened for expression of Skp2 by Western blot analysis.

Western Blot Analysis

We examined the expression of Skp2 and p27 protein in oral squamous cell carcinoma cell lines and tissue samples by Western blot analysis. Western blotting was carried out as we described previously (24). We used an anti-Skp2 monoclonal antibody (Zymed Laboratories Inc., South San Francisco, CA), an anti-p27 mouse monoclonal antibody (Transduction Laboratories, Lexington, KY), an anti-Cul1 polyclonal antibody (Zymed Laboratories), and an anti-Cdk4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Thirty micrograms of protein were subjected to 10% PAGE followed by electroblotting onto a nitrocellulose filter. For detection of the immunocomplex, the enhanced chemiluminescence Western blotting detec-tion system (Amersham, Aylesbury, United Kingdom) was used.

p27 Protein Stabilization

For p27 protein stabilization, we measured its half-life by treatment with cycloheximide (cycloheximide, Sigma, St. Louis, MO) for 0, 30, 60 and 120 minutes. After cyclohex-imide treatment, cells were collected and the expression of p27 protein was examined by Western blot analysis as described above.

Protein Kinase Assay

For cdk2/cyclin E associated kinase activity, 100 μg of the lysate was precleared in lysis buffer with 40 μL of 1:1 slurry of protein A-agarose (Roche) for 30 minutes at 4°C. Samples were incubated with 1 μg of anti-cdk2 polyclonal antibody (Santa Cruz Biotechnology) for 3 hours at 4°C and subsequently centrifuged. The precipitates were washed thrice with lysis buffer and thrice with kinase buffer [50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $MgCl_2$, and 1 mmol/L DTT]. The precipitates were then sus-pended in 35 μL of kinase buffer containing 6 μg of histone H1 (Sigma, type III-S) followed by a 5-minute preincuba-tion at 30°C. Subsequently, 5 μL of a 60 mol/L [γ-^{32}P]ATP solution (3 μCi) were added, and the kinase reaction was carried out at 30°C for 10 minutes. The reaction was stopped by adding 20 μL of 4× Laemmli's sample buffer and boiling. The samples were subjected to 10% SDS-PAGE, followed by autoradiography.

Cell Proliferation and BrdUrd Incorporation

Cells (5.0×10^3) were plated onto a 24-well multiwell plate (3047, Falcon, Becton Dickinson, Franklin Lakes, NJ) and allowed to attach for 24 hours. The culture medium was then replaced with fresh medium. After then, trypsinized cells were counted by Cell Counter (Coulter Z1, Coulter, Florida) at 0, 2, 4, and 6 days. For bromodeoxyuridine (BrdUrd) incorporation, cells growing on coverslips were incubated with 10 μmol/L BrdUrd (Sigma) for 3 hours. The cells were fixed in cold methanol/acetone 1:1 for 10 minutes. In brief,

the cells were sequentially incubated in 1.5 mol/L HCl for 10 minutes. Then cells were washed thrice with PBS and incubated with the mouse anti-BrdUrd-fluorescein primary antibody (Roche) for 1 hour. The cells were washed four times with PBS. The nuclei were simultaneously stained with 10 μg/mL of 4′,6-diamidino-2-phenylindole. Cells with different BrdUrd-incorporation patterns were checked and counted with a conventional fluorescence microscope (Zeiss, Tokyo, Japan).

Tumorigenicity Assays

Briefly, cells (1.0×10^7) were injected s.c. into multiple sites in athymic (nude) mice. The animals were monitored for tumor formation every week and sacrificed 1 month later. Tumor length (L) and width (W) were measured at the end of the experiment, and tumor volume was estimated by the formula ($L \times W^2$) / 2.

Terminal Deoxynucleotidyl Transferase – Mediated Nick End Labeling Assay

Fragmented DNA was labeled with a digoxigenin-conjugated UTP by using terminal deoxytransferase. Positive nuclei were visualized by immunohistochemistry with an ApopTag labeling and detection kit (Intergen, Purchase, NY) according to the manufacturer's protocol, and nuclei were counterstained with hematoxylin. For

Figure 1. Skp2 siRNA induced p27 accumulation in oral squamous cell carcinoma cells. **A,** Skp2 and p27 expression in oral squamous cell carcinoma cells. Cdk4 expression was used as a loading control. Thirty micrograms of protein were subjected to Western blot analysis as described in Materials and Methods. **B,** accumulation of p27 after proteasome inhibitor treatment. Cells were treated with proteasome inhibitor, ZLLL (25 μmol/L) for 5 h. The expression of p27 protein was analyzed by Western blot analysis. Cul1 and Cdk4 expressions were used as a loading control. **C,** to suppress p27 degradation, we tried to inhibit the Skp2 expression by siRNA in HSC3 cells. Synthetic two-oligonucleotide primers (5′-tcgaGGGAGUGACAAAGA-CUUUGgaguacugCAAAGUCUUUGUCACUCCCUUUUU-3′ and 5′-ctagAAAAAGGGAGUGACAAAGACUUUGcaguacucCAAAGUCUUUGUCACUCCC-3′) containing XhoI and XbaI overhangs were annealed and then were introduced into pSuppressorNeo vector. Oligonucleotide sequences correspond to a 19-nucleotide sequence from Skp2 (nucleotides 114-133), which are separated by an 8-nucleotide linker (lowercase letters) from the reverse complement of the same 19-nucleotide sequence. Transcriptional termination (UUUUU) was added to the end of the oligonucleotide. **D,** Skp2 siRNA transfection induced the p27 accumulation in HSC3 cells. Transfection was done in 60 mm plates using 2 μg of vector and 10 μL of Fugene 6 reagent (Roche). After 48 h of transfection, cells were treated with G418 (Life Technologies) for 2 wks as a selective marker. After G418 treatment, we cloned stably transfectant cells. In Skp2 siRNA transfectant cells, expression of Skp2 and p27 was examined by Western blot analysis. Thirty micrograms of protein were subjected to Western blot analysis as described in Materials and Methods. We used circular control plasmid, which contains a scrambled sequence that does not show significant homology to rat, mouse, or human gene sequences, as a control. Cul1 expression was used as a loading control. **E,** half-life of p27 protein in control and Skp2 siRNA-transfected cells. To measure the half-life of p27 protein, we treated cycloheximide (50 μg/mL) for 0, 30, 60 and 120 min and then examined the expression of p27 by Western blot analysis. The signal intensity was measured by densitometric scanning and relative expression levels (p27/Cul1 ratio) were presented.

quantification, 10 random fields per section were documented by photomicroscopy, and the percentage and number of terminal deoxynucleotidyl transferase–mediated nick end labeling–positive epithelial cell nuclei relative to the total number of epithelial cell nuclei were calculated.

Results

Skp2 siRNA Induced p27 Accumulation in Oral Cancer Cells

Our previous study showed that Skp2 overexpression is frequently observed and plays an important role for p27 down-regulation in oral squamous cell carcinoma (6). Skp2 is well known as an important factor for p27 ubiquitination. In the present study, therefore, to suppress the ubiquitin-mediated degradation of p27 frequently shown in oral squamous cell carcinoma, we inhibited Skp2 expression by siRNA in HSC3 cells. HSC3 cells showed high expression of Skp2 and down-regulation of p27 (Fig. 1A). Accumulation of p27 was observed in HSC3 cells after proteasome inhibitor, ZLLL treatment, suggesting that down-regulation of p27 found in HSC3 cells may be brought about by high levels of Skp2 protein (Fig. 1B). In contrast, HSC4 cells showed low expression of Skp2 and high expression of p27 (Fig. 1A). Protein levels of p27 did not show any remarkable changes after ZLLL treatment in HSC4 cells (Fig. 1B). In order to block Skp2 expression selectively by siRNA, 19-nucleotide target sequence, separated by an 8-nucleotide spacer from its reverse complement sequence, was introduced into the siRNA generating pSuppressor-Neo vector system (Fig. 1C). Skp2 siRNA was transfected into HSC3 cells. After selection using G418, we could get two stable clones of Skp2 siRNA. Skp2 siRNA transfection induced the down-regulation of Skp2 protein (Fig. 1D). As we expected, accumulation of p27 protein was observed in Skp2 siRNA transfectant cells. Moreover, p27 protein in Skp2 siRNA transfectant cells is more stabilized than that in control siRNA transfectant cells (Fig. 1E).

Skp2 siRNA Inhibited the Cell Proliferation of Oral Cancer Cells *In vitro*

We examined the effect of Skp2 siRNA transfection on cell proliferation of oral cancer cells. Skp2 siRNA transfectant cells grew *in vitro* slower than control siRNA transfectant cells (Fig. 2A). Moreover, Skp2 siRNA transfectant cells showed significantly less BrdUrd-positive cells in comparison with control cells (Skp2 siRNA 1 versus control siRNA cells, $P = 0.043$; Skp2 siRNA 2 versus control siRNA cells, $P = 0.030$; Fig. 2B). Then, we asked if the proliferation inhibitory effect is caused by inhibition of Cdk2 kinase activity through p27 accumulation. After cell extracts were immunoprecipitated with an antibody against cdk2, cdk2 kinase activity was measured by using histone H1 as a phosphorylation substrate. Skp2 siRNA inhibited cdk2 kinase activity in HSC3 cells (Fig. 2C).

Skp2 siRNA Inhibited the Cell Growth of Oral Cancer Cells *In vivo*

Next, to identify the growth inhibitory effect *in vivo*, tumorigenicity of Skp2 siRNA and control siRNA trans-

fectant cells was assayed in each of three nude mice by s.c. injection of 1.0×10^7 cells per injection site (Fig. 3). We used Skp2 siRNA 2 cells which showed remarkable growth inhibitory effects in these experiments. The average tumor size of Skp2 siRNA transfectant cells was smaller (13.8 ± 2.8 mm^3), as compared with the control siRNA transfectant cells (25.7 ± 4.2 mm^3; $P = 0.100$; Fig. 3A). Microscopically, the tumors obtained by injection of Skp2 siRNA transfectant cells were generally well encapsulated by the fibrous connective tissue (Fig. 3B). Moreover, keratinization with necrosis was observed in the center of the tumor mass. In contrast, the tumors obtained by injection of control siRNA transfectant cells infiltrated the surrounding tissues without fibrous encapsulation (Fig. 3B). We also examined the rate of apoptotic cells in tumors by terminal deoxynucleotidyl transferase–mediated nick end labeling assay. Tumors due to injection of Skp2 siRNA-transfected cells showed more apoptotic cells in comparison with control tumors ($P = 0.08$; Fig. 3C).

Discussion

It is well known that reduced expression of p27 is frequently found in various cancers including oral squamous cell carcinoma, and the lack of p27 is suggested to be due to an enhancement of its degradation (14). SCF^{Skp2} was identified as the E3 ubiquitin ligase that targets p27 for ubiquitination (1–3). Skp2 is an F box protein and is a specific factor for the ubiquitination and consequent degradation of p27. Skp2 is frequently overexpressed in tumor cell lines, and forced expression of Skp2 in quiescent fibroblasts induces DNA synthesis (5). In addition, Skp2 overexpression was frequently found and well correlated with down-regulation of p27 in various tumors (6–13). These findings indicate that Skp2 is an oncogene. This observation is supported by the findings that (*a*) Skp2 cooperates with activated N-Ras in tumorigenesis in an *in vivo* model (25), (*b*) Skp2 cooperates with H-Ras^{G12V} to malignantly transform primary rodent fibroblasts both *in vitro* and *in vivo* (7), and (*c*) cotransfection with Skp2 and cyclin E promoted abundant hepatocyte replication and hyperplasia of the liver *in vivo* (26). Overall, recent studies in mouse and human tumors underscore the importance of Skp2-mediated degradation of p27 in cancer development (25, 27). Therefore, we believe that Skp2 siRNA can be a novel modality of cancer therapy for inhibition of p27 degradation.

In recent years, siRNA has become a specific and powerful tool to turn off the expression of target genes (28). With this study, we have shown for the first time that down-regulation of Skp2 is an effective and specific approach to inhibit the kinase activity of Cdk2 through p27 accumulation and inhibit proliferation in tumor cells. We successfully applied RNA silencing to inhibit the expression of Skp2 by using expression vector. Yokoi et al. (29, 30) have previously reported that down-regulation of Skp2 by Skp2-antisense treatment induced apoptosis and
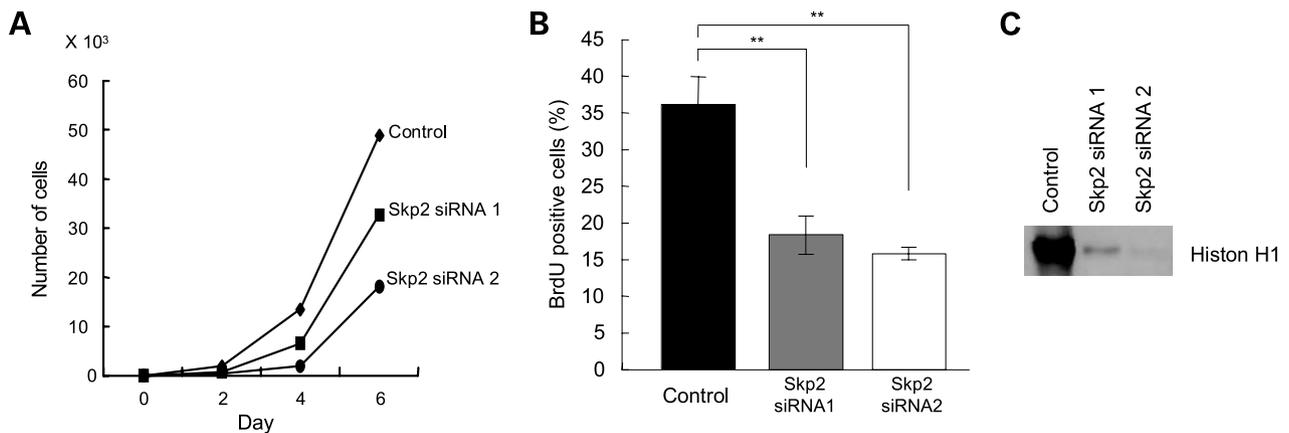
Figure 2. Skp2 siRNA inhibited the cell proliferation of oral squamous cell carcinoma cells *in vitro*. **A,** the effect of Skp2 siRNA transfection on cell proliferation *in vitro*. Cells (5.0×10^3) were plated onto a 24-well multiwell plate and counted by Cell Counter at 0, 2, 4, and 6 d. The graph shows the average of three separate experiments. **B,** BrdUrd incorporation in Skp2 siRNA-transfected cells. For BrdUrd incorporation, cells growing on coverslips were incubated with 10 μmol/L BrdUrd for 3 h. Incorporated BrdUrd was detected with antibodies as described in Materials and Methods. *Columns,* mean; *bars,* ± SD; **, *P* < 0.05. Correlation was analyzed by Fisher's exact test. **C,** Cdk2 kinase activity in Skp2 siRNA-transfected cells. After cell extracts were immunoprecipitated with an antibody against cdk2, cdk2 kinase activity was measured by using histone H1 as a phosphorylation substrate.

inhibited invasion and migration in lung cancer cells. Although we could not observe apoptosis in Skp2 siRNA transfectant cells, the tumors from injection of Skp2 siRNA-transfected cells showed more apoptotic cells in comparison with control tumors by terminal deoxynucleotidyl transferase–mediated nick end labeling assay (Fig. 3C). Moreover, control siRNA-transfected cells showed higher invasiveness in comparison with Skp2 siRNA transfectant cells *in vivo* (Fig. 3B). Importantly, the siRNA plasmid vector used in this study has an advantage over synthetic siRNAs in determining the effects of decreasing the high

constitutive levels of Skp2 protein in oral squamous cell carcinomas, because with the transfection of siRNA plasmid vector, it may be possible to knock down the gene continually.

It has recently been reported that degradation of Skp2 is mediated by ubiquitin ligase anaphase promoting complex/cyclosome and its activator Cdh1 (31, 32). As described above, overexpression of Skp2 is frequently observed and is associated with p27 down-regulation in oral squamous cell carcinomas. Although amplification of the *Skp2* locus was found at least in small cell lung tumors
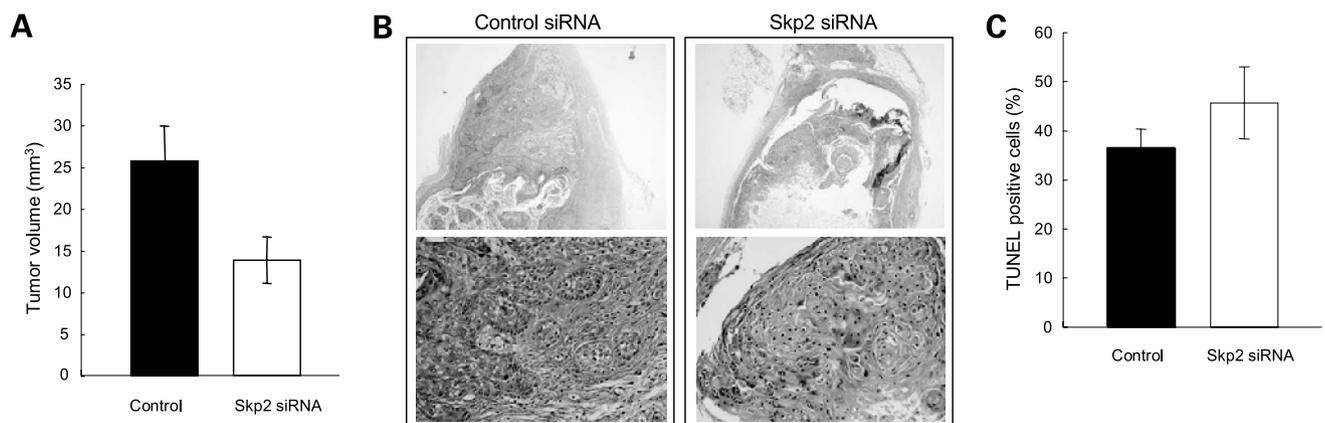
Figure 3. Tumorigenicity of Skp2 siRNA transfected cells. **A,** the effect of Skp2 siRNA transfection on cell growth *in vivo*. Tumorigenicity of Skp2 siRNA-transfected and control siRNA-transfected cells were assayed in three nude mice by s.c. injection of 1.0×10^7 cells per injection site. Tumor length (*L*) and width (*W*) were measured at the end of the experiment, and tumor volume was calculated by the formula ($L \times W^2$) / 2. The average of tumor volume of Skp2 siRNA-transfected cells was 13.8 ± 2.8 mm^3 and that of control siRNA-transfected cells was 25.7 ± 4.2 mm^3. *Columns,* mean; *bars,* ± SD. **B,** histology of tumors by injection of Skp2 siRNA and control siRNA-transfected cells. **C,** the rates of apoptosis in tumors by injection of Skp2 siRNA and control cells. The rates of apoptosis were examined by terminal deoxynucleotidyl transferase–mediated nick end labeling assay. The percentage and the number of terminal deoxynucleotidyl transferase–mediated nick end labeling-positive epithelial cell nuclei relative to the total number of epithelial cell nuclei was calculated in both tumors. *Columns,* mean; *bars,* ± SD.

(12), the mechanism for the overexpression of Skp2 in cancer is not yet known. Therefore, there is a possibility that abnormalities in the degradation of Skp2 may be involved in high levels of Skp2 proteins in human cancers. The clarification of this mechanism will be of interest.

Acknowledgments

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