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Effects of RNAi-induced Skp2 inhibition on cell cycle, apoptosis and proliferation of endometrial carcinoma cells

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Abstract. The aim of the current study was to investigate the underlying mechanism of S-phase kinase associated protein 2 (Skp2) gene inhibition by lentivirus-mediated RNA interference (RNAi) on the cell cycle, apoptosis and proliferation of endometrial carcinoma HEC-1-A cells. A lentivirus shRNA vector targeting Skp2 was constructed and transfected into HEC-1-A cells. HEC-1-A cells transfected with a scramble sequence were used as negative controls. The mRNA and protein expression of Skp2, p27, cyclin D1 and caspase-3 were detected via reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The effects of Skp2 inhibition on the cell cycle, apoptosis and proliferation of HEC-1-A cells were detected using flow cytometry and a cell counting kit-8. Skp2 co-expression data was analyzed using Oncomine and TCGA databases. The positive recombinant viral clones were identified via PCR and confirmed via sequencing. The mRNA and protein expression of Skp2 were significantly decreased in HEC-1-A cells transfected with the lentiviral vectors compared with the negative control. In addition, there were no significant changes in the mRNA expression of p27 and cyclin D1; however, the protein levels of p27 and cyclin D1 were upregulated and downregulated, respectively, in HEC-1-A cells transfected with lentiviral vectors compared with negative controls. RNAi-induced Skp2 inhibition exerted an anti-proliferative effect by inducing cell cycle arrest, however cell apoptosis was not significantly affected. In the TCGA database, Skp2 expression positively associated with IGF2R, IGF2BP3, IGFBP1 and CCNF, while Skp2 expression negatively associated with IGF2, IGFBP6, IGFBP7 and IGFBP3. RNAi-induced Skp2 inhibition upregulated the protein expression of p27 and downregulated the protein expression of cyclin D1. The expression of Skp2 in endometrial cancer may therefore be regulated by the insulin-like growth factor 1 receptor signaling pathway.

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Introduction

Endometrial cancer is one of the most common invasive malignancies of the female genital tract. The incidence of endometrial cancer has gradually increased, approaching or even surpassing that of cervical cancer, posing a serious threat to women's health (1). Surgery is the standard treatment for endometrial cancer, followed by chemotherapy, radiotherapy or hormone (progesterone) therapy (1). However, the prognosis of patients with advanced endometrial cancer remains poor following standard treatment and is often associated with significant side effects, including myelosuppression, liver damage, radiation enteritis and radiation cystitis (1). In recent years, gene therapy has become increasingly common in clinical cancer research (2). Gene therapy has several advantages, which include high selectivity and efficacy on metastases in advanced cancer, making it a promising therapeutic strategy for the treatment of patients with advanced cancer and a possible alternative to surgery, radiotherapy and chemotherapy (2).

S-phase kinase-associated protein 2 (Skp2) is a substrate recognition component of the E3 ubiquitin-protein ligase complex SCF (Skpl-Cullin-F-box) that recognizes specific phosphorylated substrates and mediates the ubiquitination and subsequent degradation of cellular regulatory factors involved in regulating cell cycle progression (3), signal transduction and transcription. Several cell cycle regulators are substrates of the ubiquitin proteasome pathway, which include cyclin D1 and p27 (4). Skp2 is involved in cell signal transduction and transcriptional regulation and is closely associated with several oncogenes and tumor suppressor genes, indicating that Skp2 possesses oncogenic potential (5). Skp2 serves a regulatory role in several processes including cell cycle regulation,

growth, differentiation, proliferation, metastasis and apoptosis. Skp2 has also been reported to serve a role in drug resistance and is closely associated with the development of tumors, demonstrating a prognostic value in cancer (6-8). Skp2 is overexpressed in several types of human cancer, and it therefore targeting Skp2 may be a promising strategy for cancer treatment (9-11). Several studies have demonstrated that Skp2 expression is significantly increased in endometrial cancer compared with normal endometrium tissues (9-11). Skp2 is first produced in the G1/S phase of the cell cycle, increases in the S/G2 phase and decreases rapidly in the M phase (12,13). Skp2 thus exhibits time-dependent expression in the cell (12,13). Therefore, targeted therapy may increase the sensitivity of chemotherapy and radiotherapy at specific cell cycle phases.

In the current study, Skp2 was selected as an RNAi target gene. Specific RNAi lentiviral expression vectors were constructed to inhibit Skp2 expression at the mRNA level to determine the effect of Skp2 inhibition on the characteristics of the endometrial cancer cell line, HEC-1-A, including the cell cycle, apoptosis and proliferation. The aim of the current study was to investigate the underlying mechanism of Skp2 in endometrial cancer progression as a potential for targeted gene therapy.

Materials and methods

Materials. HEC-1-A and 293T cell lines were obtained from Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The lentiviral vector system [which includes the lentiviral vector pLV-green fluorescent protein (GFP), the lentivirus plasmid and two auxiliary packaging plasmids] was obtained from Shanghai GeneChem Co., Ltd. (Shanghai, China). T4 DNA ligase and restriction endonucleases were purchased from New England BioLabs, Inc., Ipswitch, MA, USA. A QIAGEN® Plasmid Extraction kit was purchased from Qiagen GmbH (Hilden, Germany). The PrimeScript™ RT reagent kit and SYBR® Premix Ex Taq™ were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). PCR primer synthesis was performed by Shanghai Shenggong Biology Engineering Technology Service, Ltd. (Shanghai, China). Mouse anti-human Skp2 monoclonal antibodies were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Mouse anti-human p27 monoclonal antibodies were purchased from Abcam (Cambridge, UK). Mouse anti-human cyclin D1 antibodies was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-human caspase-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A bicinchoninic acid (BCA) protein assay kit, radioimmunoprecipitation (RIPA) assay buffer, tetramethylethylenediamine, phenylmethylsulfonyl fluoride, a BeyoECL Plus kit, mouse anti-human β-actin antibodies, and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG secondary antibodies were all purchased from Beyotime Institute of Biotechnology (Haimen, China). The Cell Cycle Assay kit [which includes propidium iodide and RNaseA] was purchased from Nanjing KeyGen Biotech Co., Ltd (Nanjing, China; cat. no. KGA512). The Cell Counting Kit-8 (CCK-8) assay was purchased from Tongren Chemical Institute (Kyushu, Japan).

Recombinant lentiviral vector construction. Two Skp2 transcript variants (ACCESSION NM_032637 and NM_005983) were identified in GenBank (https://www.ncbi.nlm.nih.gov/gene/6502) and the target was designed according to the homologous region. According to the design principle of RNA interference sequences, a pair of negative control nonsense sequences and four pairs of shRNA sequences were designed using BLOCK-iT™ RNAi Designer software from Ambion (Thermo Fisher Scientific, Inc.; https://rnaidesigner.thermofisher.com/rnaiexpress/). The constructs were named accordingly: Skp2-NC, Skp2-1, Skp2-2, Skp2-3 and Skp2-4. The siRNA target sequence was: Skp2-NC, 5'-TTCTCCGAACGTGTCACGT-3'; Skp2-1, 5'-CCTTAGACCTCACAGGTAA-3'; Skp2-2, 5'-CCAACCATTGGCTGAACAT-3'; Skp2-3, 5'-CAGAAAGAATCTCCAGAAA-3' and Skp2-4, 5'-GTGTCATGCTAAAGAATGA-3'. BLASTN was used to align the sequences with the corresponding human genome database, and homologous sequences of other coding sequences were excluded. The double-strand DNA oligonucleotide containing the interference sequence was synthesized by Shanghai GeneChem Co., Ltd. and cloned into the AgeI and EcoRI digested lentiviral vector pLV-GFP (part of the aforementioned lentiviral vector system). Upon transformation into *E. coli* competent cells, the positive clones were identified by PCR using the following upstream and downstream primers: 5'-CCTATTTCCCATGATTCCTTCATA-3' and 5'-GTAATACGGTTATCCACGCG-3'. A stock solution containing 10x buffer, 0.5 mM $MgCl_2$, 2.5 mM dNTPs (Shanghai GeneChem Co., Ltd., Shanghai, China), 0.2 U/μl, Taq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China) and 0.4 μM primers (Shanghai GeneChem Co., Ltd., Shanghai, China). A total of 20 μl of PCR stock solution was added to each tube and the following thermocycling conditions were applied: 94˚C for 2 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, elongation at 72˚C for 30 sec and final extension at 72˚C for 7 min. Agarose gel electrophoresis was then used to analyze the result. PCR primers were designed to reside within 2 separate DNA fragments so that a positive PCR band of expected size reflected the correct joining of 2 DNA fragments. For effective colony PCR and subsequent analysis by agarose gel, PCR product sizes of ~343 bp were found to be optimal. The positive clones were selected and sent to Shanghai Meiji Biotechnology Co., Ltd., (Shanghai, China) for sequencing analysis.

Lentivirus packaging. The recombinant lentivirus plasmid and two auxiliary packaging plasmids (provided by the aforementioned lentiviral vector system) were purified using the QIAGEN Plasmid Extraction kit, according to the manufacturer's protocol. Recombinant lentiviral particles were generated using 293T cells which were co-transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 48 h transfection, 293T cell supernatants rich in lentivirus particles were collected. After the supernatant was obtained, virus titers were determined in 293T cells using an stepwise dilution method (14).

Cell culture and transfection of HEC-1-A cells. HEC-1-A cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml penicillin-streptomycin and 10%

newborn bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China), and cells were maintained at 37˚C in a 5% CO_2-humidified incubator. The following groups were included: The negative control group (LV-Skp2-NC) and the experimental groups (LV-Skp2-1, LV-Skp2-2, LV-Skp2-3 and LV-Skp2-4). Cells were seeded into six-well plates at a density of $1x10^5$ cells/well. Once cells reached 30% confluence, the recombinant lentiviruses LV-Skp2-1, LV-SKP-2, LV-Skp2-3 or LV-SKP-4 were used to transfect HEC-1-A cells (5 ·g/ml polybrene; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); multiplicity of infection, 10). Transfection efficiency was determined by observing GFP expression in HEC-1-A cells using an inverted fluorescence microscope (magnification, x40) under six fields of view.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HEC-1-A cells on day 4 or 5 following recombinant lentiviral transfection using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (2.5 ·g) was reversed transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. Subsequently, qPCR was performed using the SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. PRIMER3 5.0 software (PREMIER Biosoft International, Inc., Palo Alto, CA, USA) was used to design specific primers for Skp2, p27, Cyclin D1, and β-actin. The following primer pairs were used for qPCR: Skp2 forward, 5'-CCAGGAGATTCCAGACCT GAGT-3' and reverse, 5'-TGTCACTCCCTTTGCTCTTCA G-3' (212 bp); p27 forward, 5'-GGGGTATGAAGAGCTTGC TTTG-3' and reverse, 5'-GGGCAGTGAGGATAGGTTTCT G-3' (308 bp); cyclin D1 forward, 5'-TCAAATGTGTGCAGA AGGAGGT-3' and reverse, 5'-ATGGAGTTGTCGGTGTAG ATGC-3' (262 bp); β-actin forward, 5'-TCGTGCGTGACA TTAAGGAG-3' and reverse, 5'-AAGGTAGTTTCGTGGATG CC-3' (214 bp). RT-qPCR uses a two-step reaction according to the protocol of SYBR® PremixEx Taq™. The following thermocycling conditions were utilized: Initial denaturation at 95˚C for 15 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The relative mRNA expression of Skp2, p27 and cyclin D1 were quantified using the $2^{-\Delta\Delta Cq}$ method (15) and normalized to the internal reference gene, β-actin.

Western blot analysis. Total protein was extracted from HEC-1-A cells on day 7 following recombinant lentiviral transfection using RIPA lysis buffer and low-temperature centrifugation at 10,000 x g for 10 min at 4˚C. Total protein was quantified using a BCA assay and 50 ·g protein/lane was separated via SDS-PAGE on a 12% gel at 110 V. The separated proteins were transferred onto polyvinylidene difluoride membranes and blocked with 10% skim milk solution at room temperature for 2-3 h. The membranes were then incubated with primary antibodies against Skp2 (1:500; cat. no. 32-3300), p27 (1:500; cat. no. ab54563), cyclin D1 (1:1,000; cat. no. 2926), caspase-3 (1:200; cat. no. sc-396225) or β-actin (1:1,000; cat. no. AA128) overnight at 4˚C. The membranes were washed with Tris-buffered saline containing 0.04% Tween-20 (TBST), followed by incubation with HRP-labeled secondary antibodies (1:2,000; cat. no. A0216) for 2 h at room temperature. The membranes were washed with TBST prior to chemiluminescence imaging. Protein bands were visualized using the BeyoECL Plus (Beyotime Institute of Biotechnology) and protein expression was quantified using the ImageScanner Imaging System (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK) to process the film, Imagequant TL 7.0 image analysis software (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK) was utilized to analyze the molecular weight and net optical density of the protein band.

Flow cytometry. Cell cycle and apoptosis assays were performed using a Beckman Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Brea, CA., USA). HEC-1-A cells were seeded into 6-well plates at density of $1x10^5$ cells/well and upon reaching 30% confluence, the negative control virus LV-Skp2-NC and recombinant lentiviruses LV-Skp2-3 and LV-Skp2-4 were transfected into HEC-1-A cells. A total of 6 days following infection, EDTA-free trypsin was added and the cells were harvested. Following centrifugation at 1,000 x g for 5 min, cell pellets were washed twice with pre-cooled PBS and fixed with 70% pre-cooled ethanol at -20˚C for >12 h. Following further centrifugation at 1,000 x g for 5 min, ethanol was removed and cells were washed twice with PBS. Cells were resuspended with PBS, then propidium iodide and RNaseA were added at a final concentration of 20 and 50 μg/ml, respectively, according to the instructions of the Cell Cycle Assay kit. Samples were then incubated for 30 min at 37˚C in the dark. Analysis of the flow cytometric data were performed using System IITM 3.0 software (Beckman Coulter, Inc.). All experiments were performed in triplicate.

Cell proliferation assay. Cell proliferation was analyzed using a CCK-8 assay (Tongren Chemical Institute). HEC-1-A cells were seeded into 96-well plates at density of $5x10^3$ cells/well in high glucose DMEM supplemented with 100 U/ml penicillin-streptomycin and 10% newborn bovine serum and once the cells reached 30% confluence, the negative control virus LV-Skp2-NC and recombinant lentiviruses LV-Skp2-3 and LV-Skp2-4 were transfected into HEC-1-A cells. The medium was replaced with DMEM supplemented with 10% newborn bovine serum (90 ·l/well) and 10 ·l CCK-8 reagent at 12, 36, 60, 84, 108, 132, and 156 h time points following infection and cells were incubated at 37˚C in a 5% CO_2-humidified incubator for 30 min. The absorbance was measured at a wavelength of 450/630 nm with a microplate reader (Bio-Rad Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Three biological and three technical replicates were performed.

Bioinformatics analysis. Skp2 co-expression gene data was analyzed using Oncomine (www.oncomine.org) from published data obtained from obtained from Wu *et al* (16). The heatmap and the association between Skp2 and co-expressed genes in the cohort of patients with endometrial cancer in the TGCA database were analyzed using UCSC Xena (xena.ucsc.edu), and the expression data for each gene was used for further analysis.

Statistical analysis. Data presented as the mean ± standard deviation. All statistical analyses were performed using SPSS statistical software (version 17.0; SPSS, Inc., Chicago, IL,

USA). One-way analysis of variance followed by a Dunnett's post hoc test was used to analyze differences among multiple groups. A Student's t-test was used to analyze differences between the expression level of specific genes in the Skp2-high and Skp2-low endometrial cancer samples. P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency of recombinant lentivirus. Transfection efficiency was examined in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors LV-Skp2-1, LV-Skp2-2, LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. The percentage of cells expressing GFP (the transfection efficiency) observed under a fluorescent microscope was >70% compared with cells observed under light microscopy (data not shown).

Skp2 expression following lentiviral transfection. The relative Skp2 mRNA expression level was determined via RT-qPCR in HEC-1-A cells on day 5 following transfection with recombinant lentiviral vectors (LV-Skp2-1, LV-Skp2-2, LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC). The mRNA expression of Skp2 was significantly decreased in cells transfected with LV-Skp2-3 or LV-Skp2-4 compared with the negative control, LV-Skp2-NC (P<0.01; Fig. 1A). These results indicate that recombinant lentiviruses can specifically inhibit the expression of Skp2 mRNA, with inhibition rates of 54.39±3.19, 69.87±2.88, 80.36±2.61 and 86.46±0.77%, respectively (data not shown). The relative protein expression of Skp2 was determined by western blot analysis in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors. The protein expression of Skp2 was significantly decreased in cells transfected with all four lentiviral vectors compared with cells transfected with the negative control (P<0.01; Fig. 1B and C). These results suggest that all four recombinant lentiviruses can influence Skp2 protein inhibition, with inhibition rates of 5.11±0.68, 38.16±0.85, 53.04±1.51 and 55.34±0.97%, respectively (data not shown). Furthermore, the inhibitory effect of LV-Skp2-3 and LV-Skp2-4 was greater than LV-Skp2-1 or LV-Skp2-2 (Fig. 1C). The two recombinant lentiviral vectors LV-Skp2-3 and LV-Skp2-4 could therefore inhibit the expression of Skp2 at the mRNA and protein level, achieving the greatest effect on Skp2 gene silencing.

Effect of Skp2 inhibition on cell cycle and apoptosis. Flow cytometry was used to detect the effect of RNA interference of (RNAi)-induced Skp2 inhibition on the cell cycle and apoptosis of HEC-1-A cells following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. The proportion of cells in the G_0/G_1 phase was significantly decreased, while the proportion of cells in the G2/M phase was significantly increased in cells transfected with LV-Skp2-3 or LV-Skp2-4 compared with LV-Skp2-NC (P<0.05; Table I). In addition, there were no significant differences observed in the rate of apoptosis in cells transfected with LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC, 2.59±0.10, 2.68±0.15 and 2.54±0.25%, respectively (Fig. 2A). Furthermore, there were no significant differences in the protein expression of caspase-3 in cells transfected with LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC (Fig. 2B and C).

Table I. Effect of recombinant lentivirus interference on the cell cycle, as detected via flow cytometry.

Group	G_0/G_1	S	G_2/M
LV-Skp2-NC	40.00±4.90	25.77±2.55	34.23±2.35
LV-Skp2-3	32.63±0.83[a]	25.23±0.65	42.13±0.25[a]
LV-Skp2-4	30.26±1.55[a]	26.40±0.80	43.33±0.75[a]

Data presented as the mean ± standard deviation. [a]P<0.05 vs. LV-Skp2-NC. LV, lentivirus; Skp2, S-phase kinase associated protein 2.

Effect of RNAi-induced Skp2 inhibition on cell proliferation. Cell proliferation was assessed in HEC-1-A cells following transfection with the recombinant lentiviral vectors LV-Skp2-3 and LV-Skp2-4. Although there were no significant differences at 12, 36 and 60 h, cell proliferation at 132 and 156 h was significantly decreased in cells transfected with LV-Skp2-3 or LV-Skp2-4 compared with LV-Skp2-NC (P<0.01; Fig. 3A). In addition, cell proliferation significantly decreased at 84 and 108 h in cells transfected with LV-Skp2-4 compared with LV-Skp2-NC (P<0.05; Fig. 3A). The relative mRNA expression of cyclin D1 was determined via RT-qPCR in HEC-1-A cells on day 4 and 5 following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. The results revealed that there was no significant change in the mRNA expression of cyclin D1 (Fig. 3B). The relative protein expression of cyclin D1 was detected via western blotting in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors (Fig. 3C). The protein expression of cyclin D1 was significantly decreased in cells transfected with LV-Skp2-4 compared with LV-Skp2-NC (P<0.01; Fig. 3D).

Changes in p27 expression following letiviral transfection. The relative mRNA expression of p27 was determined via RT-qPCR in HEC-1-A cells on day 4 and 5 following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. The results revealed that there was no significant change in the mRNA expression of p27 (Fig. 4A). The relative protein expression of p27 was detected via western blotting in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors (Fig. 4B). The protein expression of p27 was significantly increased in cells transfected with LV-Skp2-3 or LV-Skp2-4 compared with LV-Skp2-NC transfected cells (P<0.01; Fig. 4C).

Skp2 co-expressed genes. To further determine the underlying regulatory mechanism of Skp2 in endometrial cancer, data mining was performed using the Oncomine and TCGA databases. The co-expression data obtained from Wu *et al* (16) was analyzed using Oncomine. The results demonstrated that the genes involved in the insulin-like growth factor 1 receptor (IGFR) signaling pathway were enriched in the Skp2 co-expression gene dataset (data not shown). Heatmaps of these genes were generated and analyzed using UCSC Xena browser (Fig. 5A). The results demonstrated that the expression of Skp2 was associated with insulin-like growth factor 2 receptor (IGF2R), insulin-like growth factor 2 mRNA binding

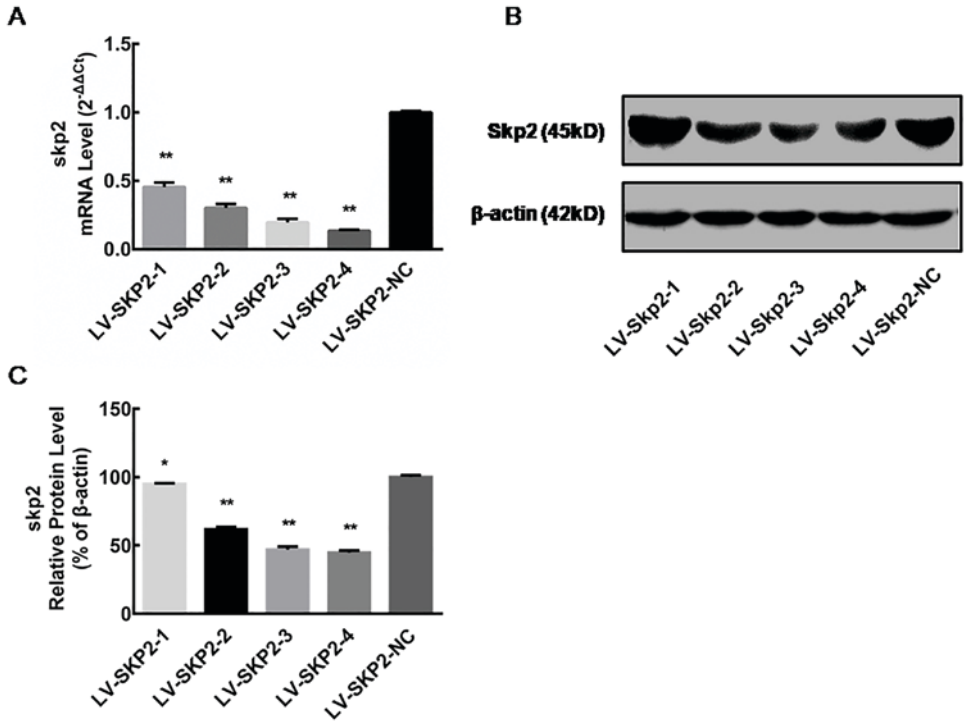

Figure 1. Skp2 expression was significantly decreased following lentiviral transfection. (A) Relative Skp2 mRNA levels was determined via reverse transcription-quantitative polymerase chain reaction in HEC-1-A cells on day 5 following transfection with recombinant lentiviral vectors LV-Skp2-1, LV-Skp2-2, LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. (B) Relative protein levels of Skp2 was determined via western blot analysis in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors. (C) Quantification of Skp2 protein expression. *P<0.05; **P<0.01 vs. LV-Skp2-NC. Skp2, S-phase kinase associated protein 2; mRNA, microRNA; LV, lentivirus.

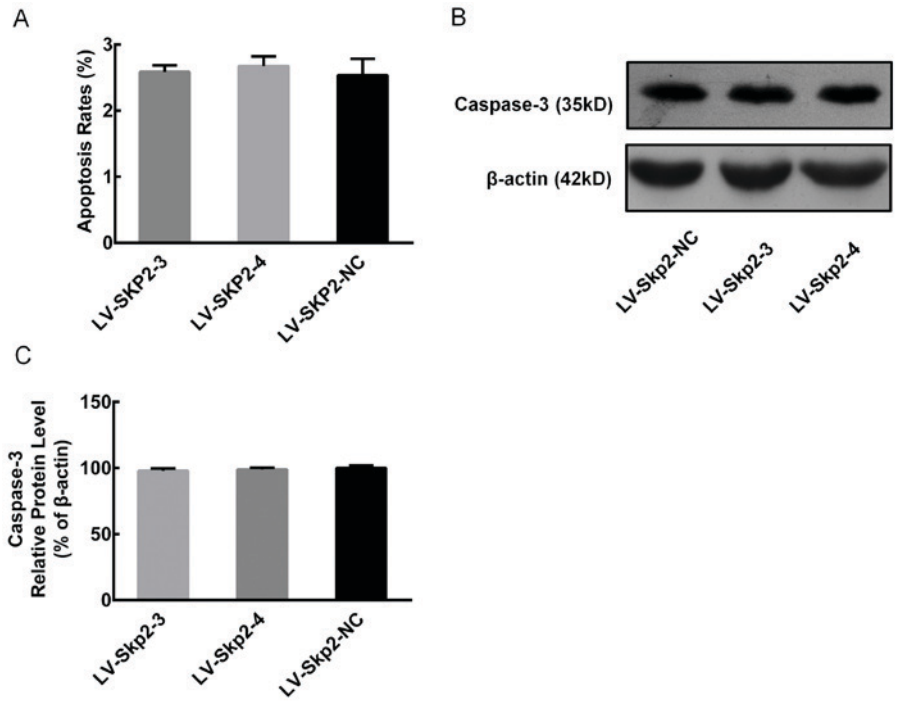

Figure 2. Effect of RNAi-induced Skp2 inhibition on cell apoptosis. (A) Flow cytometry was used to detect the effects of RNAi-induced Skp2 inhibition on cell apoptosis in HEC-1-A cells following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. (B) The relative protein expression of caspase-3 was determined via western blot analysis in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors. (C) Quantification of Caspase-3 protein expression. RNAi, RNA interference; Skp2, S-phase kinase associated protein 2; LV, lentivirus.

protein 3 (IGF2BP3), insulin-like growth factor 2 mRNA binding protein 1 (IGFBP1) and cyclin F (CCNF), while no association was observed with insulin-like growth factor 2 (IGF2), insulin-like growth factor binding protein 6 (IGFBP6),

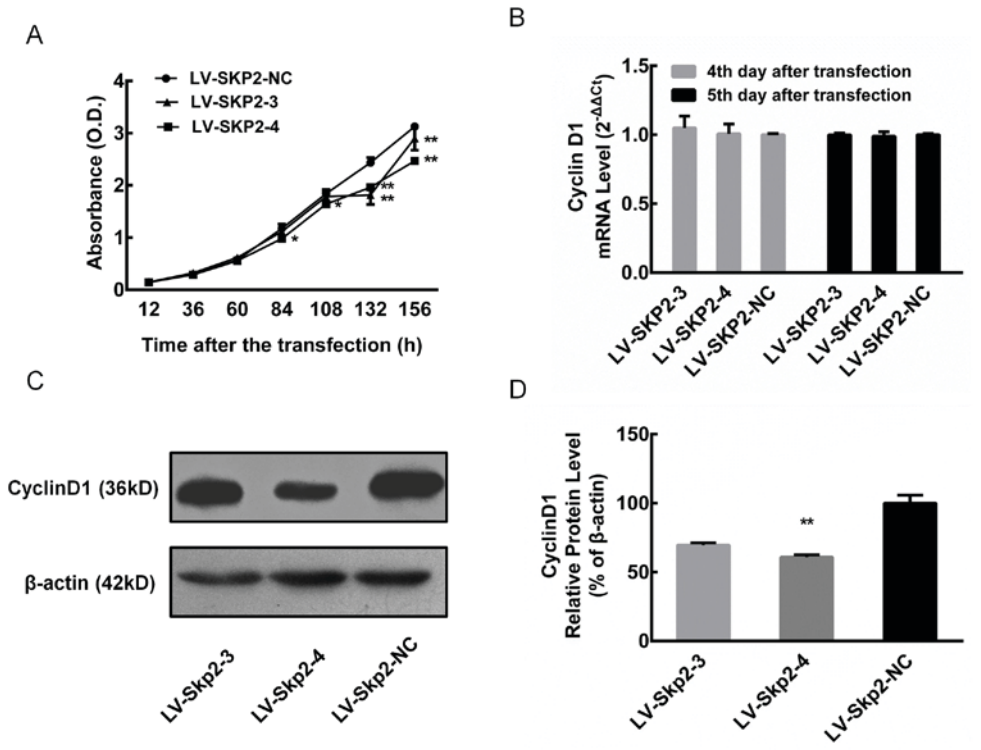

Figure 3. Effect of RNAi-induced Skp2 inhibition on cell proliferation. (A) Cell proliferation was examined in HEC-1-A cells following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. (B) Relative cyclin D1 mRNA levels were determined via reverse transcription-quantitative polymerase chain reaction in HEC-1-A cells on day 4 and 5 following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. (C) The relative protein expression of cyclin D1 was determined via western blot analysis in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors. (D) Quantification of Cyclin D1 protein expression. *P<0.05; **P<0.01 vs. LV-Skp2-NC. RNAi, RNA interference; Skp2, S-phase kinase associated protein 2; mRNA, microRNA; LV, lentivirus.

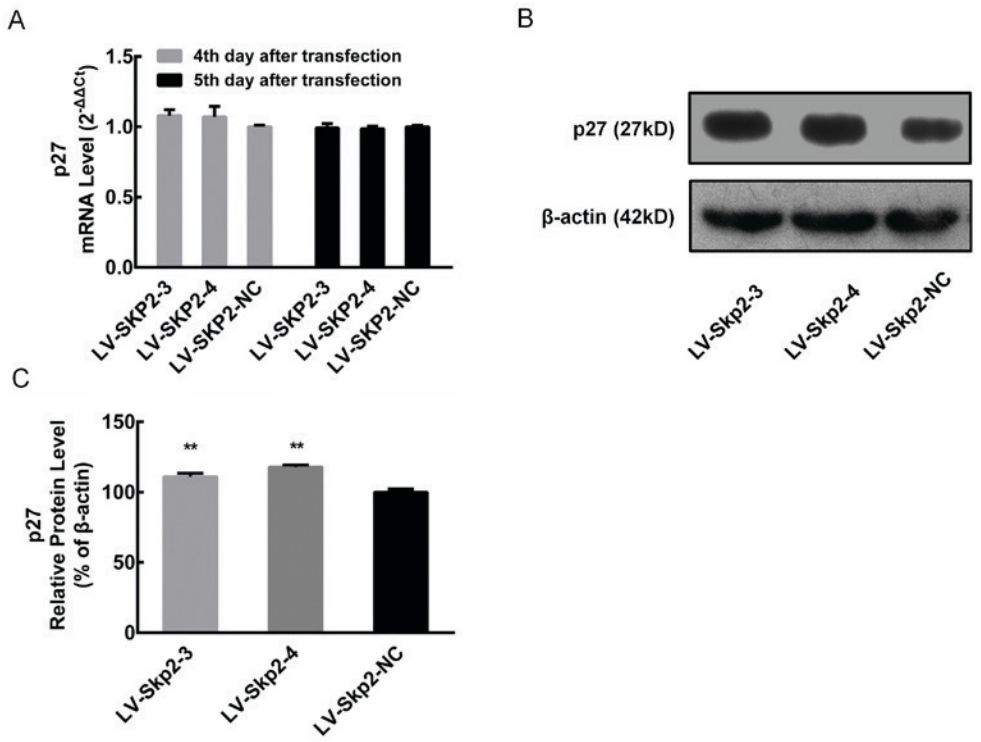

Figure 4. Effect of p27 expression following lentiviral transfection. (A) Relative p27 mRNA levels were determined via reverse transcription-quantitative polymerase chain reaction in HEC-1-A cells on day 4 and 5 following transfection with recombinant lentiviral vectors LV-Skp2-3, LV-Skp2-4 or LV-Skp2-NC. (B) The relative protein expression of p27 was determined via western blot analysis in HEC-1-A cells on day 7 following transfection with recombinant lentiviral vectors. (C) Quantification of p27 protein expression. **P<0.01 vs. LV-Skp2-NC. mRNA, microRNA; Skp2, S-phase kinase associated protein 2; LV, lentivirus.

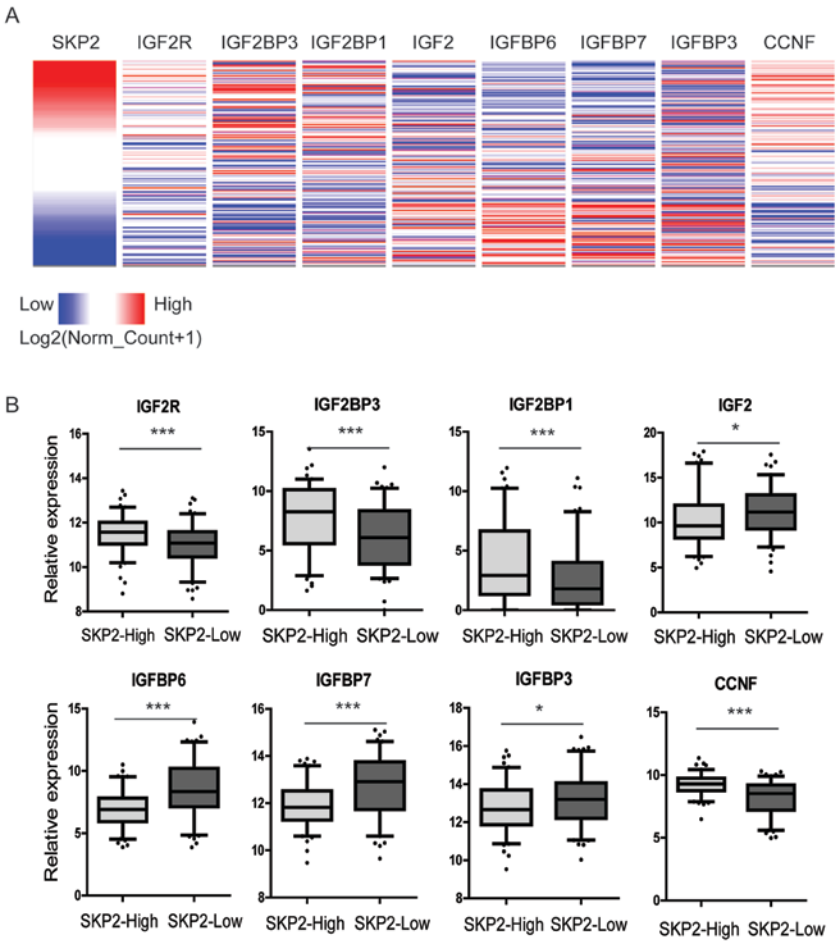

Figure 5. Skp2 co-expressed with genes in the IGFR signaling pathway. (A) Heatmap identification of gene co-expression patterns for Skp2, IGF2R, IGF2BP3, IGF2BP1, CCNF, IGF2, IGFBP6, IGFBP7 and IGFBP3 in the TCGA-EC dataset. (B) The relative gene expression of these genes in Skp2-High and Skp2-Low endometrial cancer samples. *P<0.05; ***P<0.001 as indicated. Skp2, S-phase kinase associated protein 2; IGFR, insulin-like growth factor 1 receptor; IGF2R, insulin-like growth factor 2 receptor; IGF2BP3, insulin-like growth factor 2 mRNA binding protein 3; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; CCNF, Cyclin F; IGF2, insulin-like growth factor 2; IGFBP6, insulin-like growth factor binding protein 6; IGFBP7, insulin-like growth factor binding protein 7; IGFBP3, insulin-like growth factor binding protein 3.

insulin-like growth factor binding protein 7 (IGFBP7) and insulin-like growth factor binding protein 3 (IGFBP3; data not shown). In addition, there was no association observed between Skp2 and insulin like growth factor 1, IGFR and insulin-like growth factor 2 mRNA binding protein 2 (data not shown). The gene expression data for the cohort of patients (n=201) with endometrial cancer in the TGCA database were analyzed. The 201 TCGA-EC samples were divided into two groups: Skp2-High (n=100) and Skp2-low (n=101), according to the expression of Skp2 and the expression of each co-expressed gene. In the Skp2-High group, the expression of IGF2R (P<0.001), IGF2BP3 (P<0.001), IGF2BP1 (P<0.001) and CCNF (P<0.001) were significantly increased, while the expression of IGF2 (P<0.05), IGFBP6 (P<0.001), IGFBP7 (P<0.001) and IGFBP3 (P<0.05) were significantly decreased compared with the Skp2-low group (Fig. 5B). Taken together, these results suggest that Skp2 may be regulated by the IGFR signaling pathway.

Discussion

The ubiquitin-proteasome system is an important mechanism for the regulation of protein content in eukaryotes via protein degradation, which involves three sequential reactions catalyzed by a ubiquitin-activating enzyme, a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3) (10). In eukaryotic cells, protein degradation is a highly selective and efficient ATP-dependent process, which occurs in the cytoplasm and nucleus (17). Substrate proteins are ubiquitinated, which specifically target the substrate protein for degradation by the 26S proteasome (18). Protein degradation regulates several cellular processes including cell cycle progression. SCF (Skpl-Cullin-F-box) is a multisubunit E3 ubiquitin-protein ligase complex, consisting of F-box proteins, Skp1, Cullins, Rbx1, Nedd8 and Skp2 (13,18). Although RNAi is a powerful gene-silencing process, there are relatively few studies that have assessed the application of RNAi technology towards Skp2 gene regulation in endometrial cancer. The aim of the current study was to investigate the expression of Skp2 in endometrial cancer and examine the effect of RNAi-induced Skp2 inhibition on the cell cycle, apoptosis and proliferation of the endometrium cancer cell line HEC-1-A.

Cyclins are a family of regulatory proteins that control cell cycle progression. Cyclins bind to and activate cyclin-dependent kinases (Cdk), which control cell cycle processes via the phosphorylation of its target substrate, consequently driving

the cell through G1 to S and G2 to M phase (19). If the G1-S or G2-M cell cycle checkpoints are blocked, cell proliferation may be inhibited, resulting in slow cell growth (19). In normal cell cycle progression, cyclin D1 forms a complex with Cdk4. Cyclin D1:Cdk4 phosphorylates and inactivates the retinoblastoma (RB) protein during early G1 phase to release E2F transcription factors, which initiate DNA synthesis, enabling cell cycle progression from the G1 to S phase during cell proliferation (20). Cyclin D1 overexpression occurs in several types of malignant tumor and dysregulated cyclin D1 expression can lead to the abnormal regulation of RB by Cdk4 (21). In addition, cyclin D1 can induce the expression of cyclin E and the activation of the Cyclin E-Cdk-2 complex in resting cells, causing the abnormal initiation of the cell cycle, which leads to the persistent proliferation of malignant cells (19). Several studies have demonstrated that cyclin D1 is closely associated with the occurrence, development, metastasis, prognosis and recurrence of several types of tumor (22-24).

p27 specifically binds to various cyclin-Cdk complexes and their monomers, blocking RB phosphorylation and inhibiting cell proliferation (25). In addition, p27 is also involved in cell-cell adhesion, promoting cell differentiation and inducing apoptosis (26,27). Several studies have demonstrated that the low expression of p27 occurs in several types of malignant tumor and is closely associated with tumor invasion, metastasis, staging and prognosis (28-30). In tumors, p27 is associated with a reduction or loss of protein expression, while gene mutations are rare (31). The low expression of p27 also attenuates the inhibition of positive regulatory cell cycle proteins, resulting in excessive cell proliferation and tumor development (32).

It is generally considered that Skp2 expression is negatively associated with p27 expression (33,34); however, Kim *et al* (35) reported that in 332 untreated patients with cervical cancer, there was no correlation between Skp2 and p27. Additionally, Handra-Luca *et al* (36) and Fagan-Solis *et al* (25) reported that there was no correlation between Skp2 and p27 in salivary gland epidermoid carcinoma and breast cancer, respectively. Several studies have also demonstrated that low expression levels of p27 in malignant tumors are associated with high expression levels of cyclin D1 (37-39), although the correlation between p27 and cyclin D1 remains unclear. A previous study revealed that p27 and cyclin D1 are negatively correlated (37); however, Jonason *et al* (40) reported that cyclin D1 has a specific function in the post-transcriptional regulation of p27. There may therefore be a feedback pathway between p27 and cyclin D1, to maintain a dynamic balance between the positive and negative regulators of the cell cycle. However, this feedback pathway may not be a unidirectional regulatory pathway, but rather, an intricate regulatory system distributed among various members. An abnormality in one or more of these members may be involved in tumor development and progression, however this remains to be confirmed (41).

In the current study, the application of lentivirus-mediated RNAi technology was used to inhibit Skp2 gene expression. RT-qPCR results revealed that the inhibition of Skp2 expression did not affect the mRNA expression of p27 and cyclin D1. However, downregulation of Skp2 increased the expression levels of p27 and decreased the expression levels of Cychin D1. Flow cytometry demonstrated that RNAi-induced Skp2 inhibition exerts an anti-proliferative effect by inducing cell cycle arrest in the G2/M phase, with no evidence of apoptosis. The inactive 35 kDa subunit (but not the cleaved, activated 17 kDa subunit) of caspase-3 was detected. However, there was no difference in the protein expression of caspase-3 following RNAi-induced Skp2 inhibition compared with the control group. These results indicate that the inhibition of Skp2 expression does not promote apoptosis of HEC-1-A cells. Therefore, RNAi-induced Skp2 inhibition does not affect the mRNA expression level of p27, but it does upregulate its protein expression by reducing the ubiquitin degradation of P27 protein.

Tsvetkov *et al* (42) revealed that SCF-Skp2 binds to phosphorylated Thr187 in p27 to specifically target p27 for degradation via a ubiquitin-dependent process during cell-cycle progression. In the current study, the inhibition of Skp2 expression did not increase the protein expression of cyclin D1, but instead downregulated cyclin D1 expression, which indicated that cyclin D1 may not be a substrate of the SCF complex, but a protein closely associated with Skp2. A study by Carrano *et al* (43) revealed that no direct biochemical data obtained using a reconstituted *in vitro* ubiquitination system, was available to verify whether cyclin D1, p21 and E2F-1 were true Skp2 substrates. However, It was likely, that Skp2 targets other substrates, including p27, for ubiquitination (43). Furthermore, the previous study revealed that two different Skp2 antisense oligonucleotides decrease Skp2 expression and concomitantly increase the levels of endogenous p27 (43). The study reveals that the phosphatase and tensin homolog/phosphoinositide 3-kinase signaling pathway and Cyclin D1 control a novel pathway that regulates the assembly of the SCF-Skp2 complex by modulating cullin neddylation and cullin associated and neddylation dissociated 1 binding at the G1/S cell cycle transition (37). Cyclin D1 regulates cyclin-dependent kinase inhibitor 1B (CDKN1B) abundance at a post-translational level, inhibiting the Skp2 promoter, Skp2 abundance and inducing CDKN1B phosphorylation at Ser10 (41). Therefore, it was hypothesized that cyclin D1 downregulation may be due to the reduced stability of other factors in the regulatory network affected by Skp2 expression. In the current study, inhibition of Skp2 led to the upregulation of p27 and G2/M arrest, which in turn inhibited the proliferation of HEC-1-A cells. These results correlated with other previous studies (44,45). Nakayama *et al* (44) demonstrated that Skp2 regulates the stability of p27, which participates in the regulation of G2/M phase. Pagano *et al* (45) also revealed that p27 inhibited Cdk1 activity at the G2/M phase, which is an important regulatory factor for G2-M checkpoints. In addition to p27, several cell cycle regulatory proteins also serve as substrates for SCF complexes, including cyclin A, cyclin B, p21 and p53, which are degraded via this pathway and are regulators of the G2-M checkpoint (45). Wu *et al* (46) identified small molecule inhibitors specific to SCF-Skp2 activity using *in silico* screens targeted at the binding interface for p27. These compounds selectively inhibited Skp2-mediated p27 degradation by reducing p27 binding through key compound-receptor contacts (46). In cancer cells, these compounds induce p27 accumulation in a Skp2-dependent manner and promote cell-type specific blocks in the G1 or G2/M phases (46). Furthermore, although the expression of p27 was upregulated in the current study, cell

apoptosis was not affected. This may be due to the inhibition of Skp2 affecting the stability of unknown factors, offsetting the pro-apoptotic effect of p27. RNAi-induced Skp2 inhibition affects the stability of downstream factors, but may also affect the stability of upstream factors via feedback regulation, thereby causing changes in multiple factors along the entire cell regulatory network.

The results of the current study demonstrated that lentivirus-mediated RNAi technology can effectively inhibit the expression of Skp2, thereby upregulating the protein expression of p27 and inhibiting the proliferation of human endometrial cancer HEC-1-A cells. The current study verified the use of *in vitro* experiments to assess the mechanism underlying endometrial cell growth via Skp2 inhibition. Therefore, these methods may be used to further investigate the role of Skp2 in tumorigenesis in future studies. Furthermore, Skp2 is thought to be a potential target for gene therapy in patients with endometrial cancer (47). However, multi-level and multi-factor interactions involved in cell proliferation, apoptosis and cycle regulation need to be examined further and considered in an integrative manner. In addition, as there may be several factors underlying disease etiology, the effect of Skp2 on the expression levels of cyclin D1 and p27 as well as on cell proliferation, apoptosis and cycle regulation, and the association between Skp2 expression and IGFR signaling require further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL, XZ and PS designed the study. HL and XZ collected the data. HL, GR, XS and XC analyzed the data. HL and GR prepared the manuscript and PS revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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