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Original Article

S-phase kinase-associated protein 2 impairs the inhibitory effects of miR-1236-3p on bladder tumors

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Abstract: We have previously demonstrated that miR-1236-3p has the robust ability to up-regulate p21 expression by targeting the p21 promoter, thus inhibiting bladder cancer progression. Microarray experiments displayed that miR-1236-3p significantly increased the expression of the oncogenic F-box protein S-phase kinase-associate protein 2 (Skp2) while activating p21 expression in bladder cancer cells. Here, we confirmed that Skp2 was over-expressed following transfection with miR-1236-3p. Further, we demonstrated that miR-1236-3p and its sequence homology dsRNA, dsRNA-245 (which is completely complementary to the p21 promoter), both are able to potently induce p21 expression. We found that dsRNA-245 did not induce changes in Skp2 expression, while miR-1236-3p could increase Skp2 expression; this influence was independent of p21 activation. Moreover, transfection of miR-1236-3p or dsRNA-245 into bladder cancer cells significantly inhibited cell proliferation and clonegenesis and induced cell cycle arrest mainly by regulating p21 expression. However, the growth inhibition caused by dsRNA-245 was more effective than that caused by miR-1236-3p. This difference in effect size is mainly related to the miR-1236-3p-induced expression of Skp2. In summary, our results provided evidence that both endogenous and exogenous small RNAs might function to induce p21 expression by interacting with the same promoter region, therefore impeding bladder cancer cell growth. Additionally, our results indicated that microRNA activation can activate the expression of some tumor suppressor genes as well as some oncogenes. This indicated the need for the further study of clinical applications of RNA activation.

Keywords: RNA activation, miR-1236-3p, dsRNA-245, bladder cancer, Skp2, p21, proliferation

Introduction

Bladder cancer (BCa) is the most common malignancy of the urinary tract. There were approximately 79,030 newly diagnosed cases and 16,870 deaths across both genders in the United States in 2017 [1]. Furthermore, BCa is the primary cause of death out of all urinary tumors in China [2]. Non-muscle invasive cancers have a high risk of recurrence and progression after local therapies, while muscle invasive cancers often have a poor prognosis even with systemic treatment [3-5]. BCa initiation, proliferation and metastasis have largely been associated with the down-regulation of specific tumor suppressor genes, so targeted gene therapy may be a promising strategy for cancer treatment [6]. However, its it unclear whether miRNAs can up-regulate the expression of unknown genes in human BCa cells. Further studies are also needed to determine whether miR-1236-3p and dsRNA-245 can up-regulate Skp2 expression and inhibit BCa proliferation.

MicroRNAs (miRNAs) are a group of small endogenous non-coding RNAs (ncRNAs) of approximately 19-24 nucleotides that post-transcriptionally regulate target genes [7]. Cells use miRNAs to negatively regulate gene expression by repressing translation or directing sequence-specific degradation of target mRNAs [8-11]. In this regard, miRNAs are considered to be the key regulators of gene expression. Double-stranded RNAs (dsRNAs) or miRNAs complementary to specific gene promoter sequences can induce the expression of target genes [12, 13].

Table 1. Sequences for dsRNAs and siRNAs used in the present study

Synthesized RNAs	Sense (5'-3')	Antisense (5'-3')
dsP21-242	AGGGACUGGGGGAGGAGGG	CCCUCCUCCCCCAGUCCCU
dsP21-243	GAGGGACUGGGGGAGGAGG	CCUCCUCCCCCAGUCCCUC
dsP21-244	CGAGGGACUGGGGGAGGAG	CUCCUCCCCCAGUCCCUCG
dsP21-245	GCGAGGGACUGGGGGAGGA	UCCUCCCCCAGUCCCUCGC
Control miRNA	ACUACUGAGUGACAGUAGA	UCUACUGUCACUCAGUAGU
siP21	CUUCGACUUUGUCACCGAG	CUCGGUGACAAAGUCGAAG
siSKP2	UCUAAGCCUGGAAGGCCUG	CAGGCCUUCCAGGCUUAGA

There are many miRNAs and dsRNAs that can suppress the proliferation and metastasis of BCa cells. In a previous study, we demonstrated that miR-370-5p, miR-1180-5p and miR-1236-3p positively regulate p21 expression and that the overexpression of these 3 miRNAs potently inhibits the proliferation of BCa cells [14]. Moreover, after transfection of miR-1236-3p into BCa cells, gene chip analysis showed that miR-1236-3p activated p21 expression and increased the expression of Skp2. To determine whether and by what mechanism miR-1236-3p can induce Skp2 expression in human BCa cells, we synthesized four dsRNAs (dsRNA-242, dsRNA-243, dsRNA-244, and dsRNA-245) corresponding to the miRNA target sequence and determined that only dsRNA-245 could activate p21 expression. We observed that the miR-1236-3p-induced inhibition of BCa cell proliferation was slightly weaker than that caused by dsRNA-245, mainly due to Skp2 overexpression.

Materials and methods

Tissue samples

BCa tumor specimens and normal tumor-adjacent tissues were obtained from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) after informed consent and ethics committee approval. After surgical removal, fresh samples were trimmed and cryopreserved in liquid nitrogen for further analysis or fixed in 4% paraformaldehyde immediately and then paraffin-embedded for immunohistochemistry (IHC).

MiRNA, dsRNA and recombinant lentivirus

MiRNA mimics and (double-stranded RNAs) dsRNAs were synthesized by RiboBio Co., Ltd. (Guangzhou, China). A small interfering RNA (siSkp2) was utilized to silence Skp2 expression, and a control miRNA, which lacks of significant homology to all known human sequences, was used as a negative control [12, 15]. Lenti-miR-1236, Lenti-dsRNA-245 and Lenti-dsControl were purchased from GenePharma (Shanghai, China). The sequences of the custom dsRNAs are listed in **Table 1**.

Cell culture, miRNA and dsRNA transfection, and lentiviral infection

The human BCa cell lines 5637 and T24 (ATCC) were cultured in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA). The day before transfection, cells were plated in growth medium without antibiotics at a density of 30-50%. Both dsRNAs and miRNAs were transfected into BCa cells at a final concentration of 50 nM using Lipofectamine RNAiMax (Invitrogen, USA) according to the manufacturer's protocol.

Lenti-miR-1236 and Lenti-dsRNA-245 were used to overexpress miR-1236-3p and dsRNA-245, respectively, following infection of 5637 cells according to the manufacturer's instructions. Lenti-dsControl served as a negative control. Fluorescence expression was observed at 48-72 h after infection. Then, cells were reseeded into new plates for further experiments.

RNA isolation, quantitative real-time PCR (qRT-PCR) and miRNA analysis

TRIzol reagent (Invitrogen, USA) was used to extract total RNA according to the manufacturer's protocol. Then, 500 ng of RNA was reverse transcribed into cDNA using a Takara reverse transcription kit (Takara, China) according to the manufacturer's instructions. The resulting cDNA was amplified by SYBR Premix Ex Taq II (Takara, China) on an Mx3000P instrument (Stratagene, USA). All the primers included in this study were offered by Invitrogen (Shanghai, China) and are listed in **Table 2**. Gene expression levels were assessed by relative quantification using GAPDH as the endogenous reference gene. All experiments were performed in triplicate.

Table 2. Primers used in this study

Name	Sequences (5'-3')	Assay used for
p21 (S)	GCCCAGTGGACAGCGAGCAG	PCR
p21 (AS)	GCCGGCGTTTGGAGTGGTAGA	PCR
GAPDH (S)	TCCCATCACCATCTTCCA	PCR
GAPDH (AS)	CATCACGCCACAGTTTCC	PCR
CDK1 (S)	TGGGGTCAGCTCGTTACTCA	PCR
CDK1 (AS)	CACTTCTGGCCACACTTCATTTA	PCR
p27 (S)	ACGGGAGCCCTAGCCTG GAGC	PCR
p27 (AS)	TGCCCTTCTCCACCTCTTGCC	PCR
SKP2 (S)	GGTGTTTGTAA GAGGTGGTATCGC	PCR
SKP2 (AS)	CACGAAAAGGGCTGAAATGTTC	PCR
Control miRNA (S)	CCCGGGGTCTACTGTCTC	PCR
Control miRNA (AS)	CAGTGCAGGGTCCGAGGTAT	PCR

Protein extraction and western blotting analysis

Total proteins were extracted using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Roche, Switzerland) according the manufacturer's instructions. Then, 50 μg of each protein sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) and then incubated with primary antibodies, including those against Skp2 (1/1000) (Affinity, USA), P21 (1/1000) (BD Biosciences), P27 (1/1000) (Affinity, USA), CDK1 (1/1000) (Boster, China), GAPDH (1/500) (Boster, China) and β-actin (1/500) (Boster, China), overnight at 4°C. After three washes, the membranes were incubated with second antibodies and visualized using an enhanced chemiluminescence (ECL) assay kit (Millipore, USA).

Cell growth assay

Cell viability was detected daily for five days using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method (Promega, USA) according to the manufacturer's protocol. MTS was added to cells, and then the plate was incubated for 2 h at 37°C at each time point. Absorbance was measured on a microplate reader (Bio-Rad, USA) at 490 nm.

Clonogenic survival assay

BCa cells were reseeded in 6-well plates at approximately 1000 cells per well after transfection, and the medium was replaced every 3 days. The colonies were then fixed 10 days later and stained with 0.5% crystal violet (Sigma, USA) for 30 min at room temperature. The colony formation rate was calculated using the following equation: colony formation rate = number of colonies/number of seeded cells × 100%.

Cell cycle analysis by flow cytometry

Cells were collected 72 h after transfection and fixed with 70% ethanol at 4°C overnight. Then, the fixed cells were washed and incubated with RNase A (0.1 mg/mL) for 30 min at room temperature. Cellular DNA was stained with propidium iodide (PI) (0.05 mg/mL) and analyzed on a FACSort flow cytometer (BD Biosciences, USA). All experiments were repeated 3 times, and a total of 10,000 events were analyzed for each sample. The data were processed using CELL Quest software (BD Biosciences, USA).

Nude mice and tumorigenicity assay

Five-week-old male BALB/c-nude mice were obtained from Hua Fukang Biological Technology Company Limited (Beijing, China). All nude mice were housed in an animal facility with a specific-pathogen free (SPF) environment. The mice were kept in a ventilated, dry and clean environment. The room temperature was maintained at 20~25°C, and humidity was maintained at 40-70%. The mice were allowed *ad libitum* access to water and food under automatic day/night control (12:12 h). Animal protocols were approved by the Laboratory Animal Ethics Committee of Experiment Animal Center of the Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China).

Approximately 5×10^6 infected 5637 cells with Lenti-miR-1236, Lenti-dsRNA-245 or Lenti-dsControl were injected subcutaneously into the right flank of male BALB/c-nude mice. Tumor size (length and width) was measured using calipers every 3 days for 28 days. Then, tumor volume was calculated using the follow-

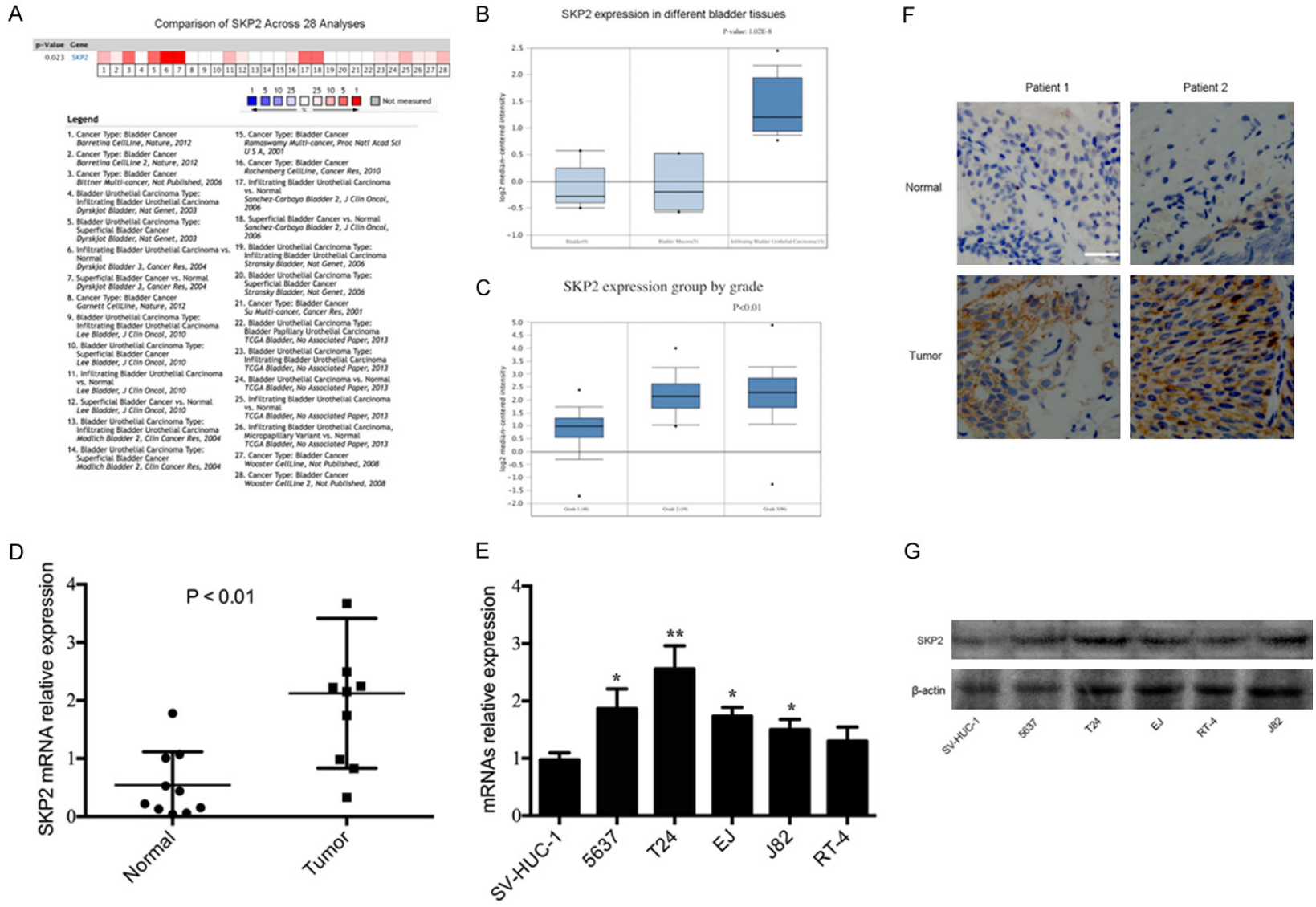

Figure 1. Skp2 is over-expressed in BCa samples and cell lines. (A) Comparison of Skp2 expression across 28 studies using original published data from Oncomine (www.oncomine.org) (P = 0.023). (B) The expression levels of Skp2 were higher in BCa samples than in normal bladder mucosa (P = 1.02E-8). (C) Skp2 overexpression was associated with higher tumor grade (P<0.01). (D) Skp2 mRNA expression was increased in human BCa tissues compared with adjacent normal tissues (P<0.01). The results were normalized to GAPDH. (F) Skp2 protein expression in bladder tissues was detected by IHC. (E) Skp2 mRNA and protein (G) expression

was increased in 5637, T24, EJ and J82 cell lines compared with primary normal human bladder epithelial cells (SV-HUC-1), (*P<0.05, **P<0.01). GAPDH levels were used as an internal control.

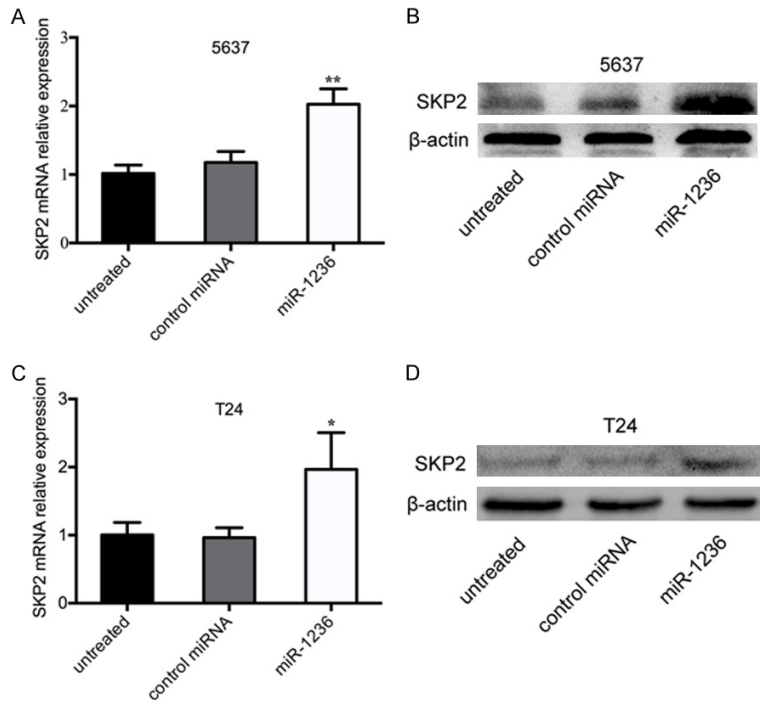

Figure 2. miR-1236 effectively up-regulated Skp2 expression both in 5637 and T24 cells. 5637 and T24 cells were transfected with 50 nM of the indicated miRNA for 72 h. A and C. Skp2 mRNA expression was detected by qRT-PCR in both 5637 and T24 cells. The results were normalized to GAPDH. *P<0.05, **P<0.01 compared with untreated or control miRNA groups. B and D. Skp2 protein expression was observed by Western blot analysis. β-actin levels were used as an internal control.

ing formula: V = length × width^2 × 0.5. Finally, animals were sacrificed 28 days after injection, and tumors were weighed.

Statistical analysis

Statistical analyses were performed in the SPSS17.0 software (SPSS Inc., Chicago, IL, USA). All data are shown as the mean ± standard deviation (SD) for the three independent experiments. Differences between groups were analyzed with Student's t-tests. Statistical significance between three or more groups was analyzed by one-way ANOVA. P<0.05 was considered significant.

Results

Skp2 is overexpressed in BCa samples and cell lines

We compared the expression levels of Skp2 across 28 studies using original published data available from Oncomine (www.oncomine.org). We observed that Skp2 was significantly overexpressed in BCa samples in comparison with normal bladder samples (**Figure 1A** and **1B**). Moreover, overexpressed Skp2 was associated with higher tumor grades (**Figure 1C**). To investigate the potential significance of Skp2 in BCa, we first examined its expression in tissues and cells. Skp2 expression levels were significantly increased in 4 BCa cell lines, including 5637, EJ, T24 and J82, compared with the control SV-HUC-1 cells (**Figure 1E**). Protein analysis by immunoblotting further demonstrated that Skp2 is overexpressed in BCa cells (**Figure 1G**). We then evaluated the expression levels of Skp2 in a cohort of 10 pairs of BCa samples and the corresponding adjacent normal tissues. The average Skp2 mRNA expression level was significantly higher in tumor tissues than in adjacent normal tissues (**Figure 1D**). Moreover, we evaluated the Skp2 protein levels in BCa tissues, and we found that Skp2 was more highly expressed in BCa tissues than in the corresponding adjacent controls (**Figure 1F**).

MiR-1236-3p up-regulates Skp2 expression in human BCa cells

After transfection of miR-1236-3p into BCa cells, gene chip analysis was performed. The results showed that Skp2 expression was increased in the miR-1236-3p group. To further evaluate the effect of miR-1236-3p on Skp2 in human BCa cells, we examined the expression of Skp2 in 5637 and T24 cells after transfection with miR-1236-3p. As shown in **Figure 2A** and **2C**, Skp2 mRNA expression was significantly enhanced by miR-1236-3p in T24 and 5637 cells, which was consistent with the microarray results. This result was further confirmed by immunoblotting, where the elevated

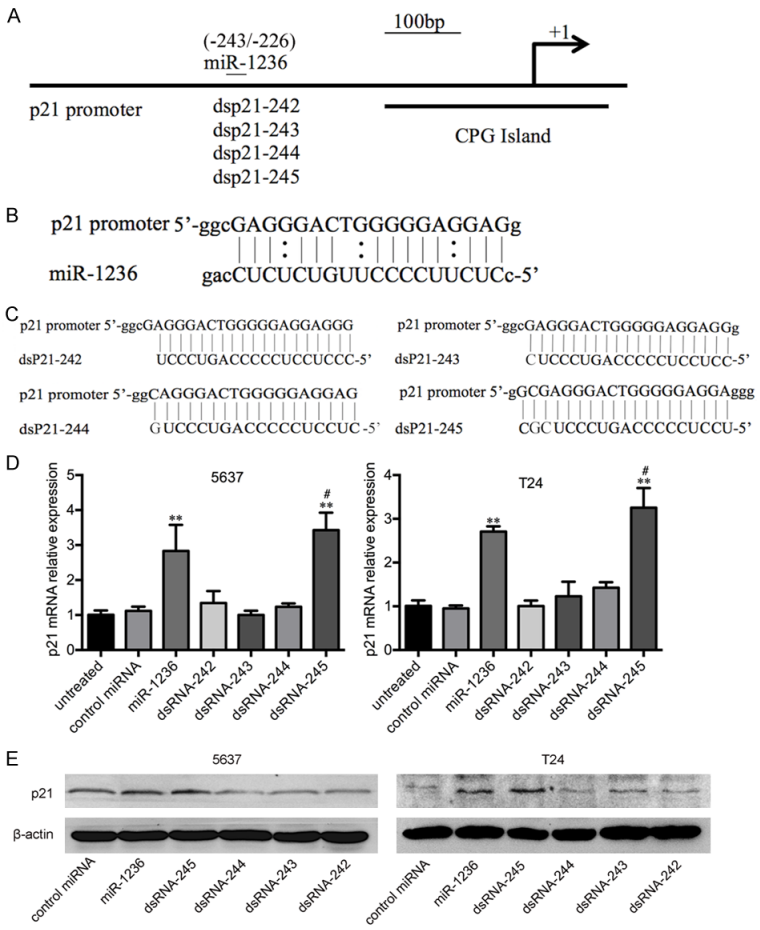

Figure 3. Expression of p21 was induced by miR-1236 and corresponding dsRNAs through promoter targeting BCa cells. A. Schematic representation of the p21 promoter and corresponding dsRNA target sites. B. The miR-1236 seed sequence is complementary to the p21 promoter. C. The four dsRNAs are fully complementary to the putative miR-1236 target site. D. p21 mRNA levels were assessed by qRT-PCR. GAPDH was used as an internal control. **P<0.01 compared to untreated or control miRNA group; #P<0.05 compared to miR-1236 group. E. Expression of p21 protein was examined by Western blot analysis. β-actin level was used as an internal control.

Skp2 protein levels were strongly consistent with the mRNA trend (**Figure 2B** and **2D**).

Activation of p21 by miR-1236-3p and its corresponding dsRNAs

Previously, we have demonstrated that miR-1236-3p is robustly able to induce p21 expression by targeting the promoter sequences at -243/-226 relative to the transcription start site (**Figure 3A** and **3B**) [14]. To evaluate whether sequence homologous dsRNAs fully complementary to the putative miR-1236-3p target site could induce p21 expression and up-regulate the expression of Skp2, we designed four different dsRNAs through an Excel macro template and obtained detailed scores according to the design rules (**Figure 3C** and **Table 3**) [16]. Each duplex was named according to its target within the p21 promoter.

Next, we transiently transfected the four dsRNAs (dsRNA-242, dsRNA-243, dsRNA-244 and dsRNA-245) and miR-1236-3p mimics into T24 and 5637 cells for 72 h. As seen in **Figure 3D**, the p21 mRNA expression was significantly enhanced by miR-1236-3p and dsRNA-245 in T24 and 5637 cells. Additionally, the expression of dsRNA-245 caused higher p21 mRNA expression levels than the expression of miR-1236-3p in T24 and 5637 cells. However, other dsRNAs failed to significantly induce p21 mRNA expression in the two cell lines. The gene activation effect was further confirmed by immunoblot analysis (**Figure 3E**).

miR-1236-3p modulation of Skp2 expression was irrelevant to p21 activation

To further investigate the mechanism of Skp2 up-regulation by miR-1236-3p, we transiently transfected the four synthetic dsRNAs and miR-1236-3p mimics into BCa cells for 72 h. As seen in **Figure 4A**, Skp2 mRNA expression was significantly enhanced by miR-1236-3p but was not influenced by the four synthetic dsRNAs in T24 and 5637 cells. Skp2 expression changes were further confirmed by immunoblot analysis, which showed that Skp2 protein levels followed a similar trend to its mRNA levels (**Figure 4B**). Then, we co-transfected sip21 with miR-1236-3p mimic or dsRNA-245 into BCa cells and found that p21 expression was dramatically abrogated. However, Skp2 expression also increased in the miR-1236+sip21 group and was unchanged in the dsRNA-245+sip21 group compared with the control

Table 3. The sequences of the four synthetic dsRNAs and the detailed scores

	Sense (5'-3')	Antisense (5'-3')	Score
dsRNA-242	AGGGACUGGGGGAGGAGGG	CCCUCCUCCCCCAGUCCCU	-0.5
dsRNA-243	GAGGGACUGGGGGAGGAGG	CCUCCUCCCCCAGUCCCUC	-0.5
dsRNA-244	CGAGGGACUGGGGGAGGAG	CUCCUCCCCCAGUCCCUCG	-1
dsRNA-245	GCGAGGGACUGGGGGAGGA	UCCUCCCCCAGUCCCUCGC	0.5

miRNA group (**Figure 4C**). Immunoblotting further demonstrated the changes in p21 and Skp2 expression following co-transfection with sip21 (**Figure 4D**).

Overexpression of miR-1236-3p influenced the expression of Skp2 target genes

We further identified the effect of miR-1236-3p and dsRNA-245 on the expression of genes downstream of Skp2 in two BCa cell lines. In the dsRNA-245 group, the expression of p27 and CDK1 did not change obviously, while the transfection of the miR-1236-3p mimic caused a significant decrease in p27 mRNA expression and an increase in CDK1 expression in the tested cell lines (**Figure 4E**). The effect of dsRNA-245 and miR-1236-3p on p27 and CDK1 protein expression levels was further confirmed by Western blotting (**Figure 4F**).

Likewise, we detected CDK1 and p27 expression after co-transfection with miR-1236-3p mimics and siSkp2 to verify whether Skp2 plays a key role in miR-1236-3p regulation of cell proliferation-related genes. After co-transfection with miR-1236-3p mimics and siSkp2, we observed a significant decrease in Skp2 expression by both qRT-PCR and Western blot. Our results also showed that depletion of Skp2 expression dramatically reversed the miR-1236-3p-mediated changes in CDK1 and p27 mRNA (**Figure 4G**) and protein (**Figure 4H**) expression in both 5637 and T24 cell lines.

MiR-1236-3p and dsRNA-245 had different inhibitory effects on BCa cells proliferation in vitro and in vivo

To elucidate the inhibitory effects of miR-1236-3p and dsRNA-245 in BCa cells, we overexpressed miR-1236-3p and dsRNA-245 in 5637 and T24 cells via transient transfection. Compared to the control miRNA group, both tested cell lines exhibited progressively retarded growth starting at 48 h following transfection of miR-1236-3p and dsRNA-245, as measured by MTS assay. Then, 72 h later, the growth inhibition caused by dsRNA-245 was noticeably greater than that caused by miR-1236-3p (**Figure 5A**). Then, we verified whether depletion of Skp2 could affect the inhibitory function of miR-1236-3p in 5637 and T24 cells. We found that knockdown of Skp2 clearly enhanced the miR-1236-3p-mediated anti-proliferative effect in both cell lines (**Figure 5B**). Surprisingly, there was no significant difference between the dsRNA-245 group and the miR-1236-3p+siSkp2 group (**Figure 5B**). Furthermore, we performed colony formation assays and found that cells formed significantly fewer and smaller colonies in the miR-1236-3p, dsRNA-245 and miR-1236-3p+siSkp2 groups compared to the control miRNA group (**Figure 5C**). In addition, colony formation rates in the miR-1236-3p, dsRNA-245 and miR-1236-3p+siSkp2 transfected cells were remarkably lower than in the control miRNA-treated cells. Further, the colony formation rate in the dsRNA-245 group was noticeably lower than that in the miR-1236-3p group. There was no obvious difference between the dsRNA-245 group and the miR-1236-3p+siSkp2 group (**Figure 5D**). Next, 5637 cells stably expressing miR-1236-3p, dsRNA-245 or the control miRNA were used to generate xenografts in nude mice. As shown in **Figure 5E**, Lenti-miR-1236 and Lenti-dsRNA-245 significantly reduced xenograft tumor growth. In addition, Lenti-dsRNA-245 more strongly su-ppressed tumor growth than Lenti-miR-1236. Furthermore, the average tumor volume and weight in the Lenti-miR-1236 and Lenti-dsRNA-245 groups was significantly decreased compared with the Lenti-dsControl group at 4 weeks post-injection. Similarly, tumors from the Lenti-dsRNA-245 group were smaller and lighter than those in the Lenti-miR-1236 group (**Figure 5F** and **5G**). Together, these data imply that miR-1236-3p and dsRNA-245 can inhibit the proliferation and colony formation of BCa cells. Notably, the inhibition caused by miR-1236-3p was weaker than that caused by dsRNA-245, mainly because miR-1236-3p induced the expression of Skp2.

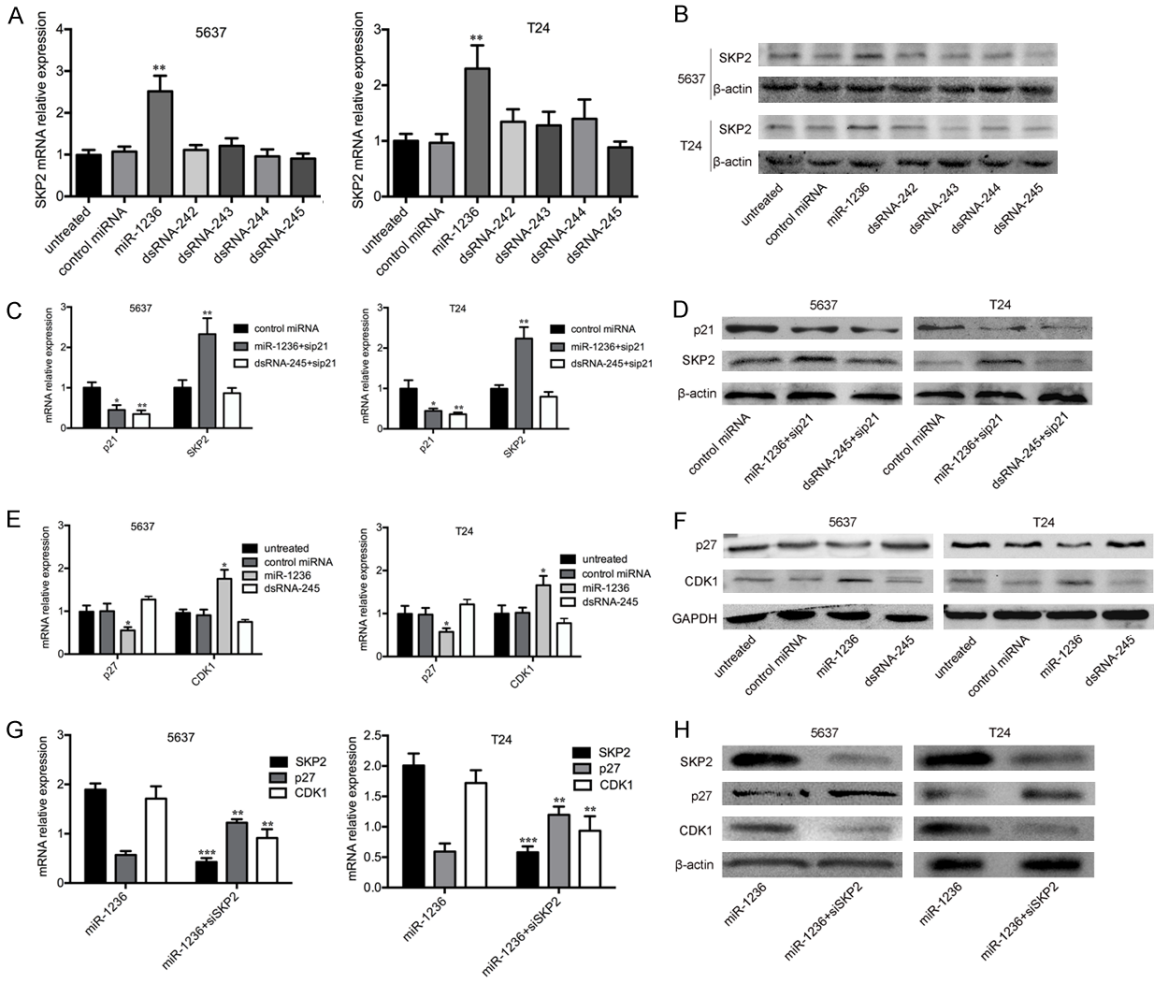

Figure 4. miR-1236 induced Skp2 expression independent of p21 activation and influenced downstream Skp2 gene expression. A. Skp2 mRNA levels were evaluated by qRT-PCR. **P<0.01 compared to control miRNA group. B. Expression of Skp2 protein was further detected by Western blot analysis. Only miR-1236 up-regulated Skp2 expression. C. Expression of p21 and Skp2 mRNA in BCa cells was detected by qRT-PCR. *P<0.05, **P<0.01 compared to control miRNA group. D. Western blotting was conducted to detect the expression of p21 and Skp2 protein in BCa cells. Knockdown with sip21 had no influence on the up-regulation of Skp2 by miR-1236. E. mRNA expression levels of p27 and CDK1 were assessed by qRT-PCR. *P<0.05 compared to the control miRNA group. F. P27 and CDK1 expression levels were detected by Western blot in 5637 and T24 cells. miR-1236 decreased p27 expression and up-regulated CDK1 expression. dsRNA-245 had no significant effect on p27 and CDK1. G. Expression of Skp2, p27 and CDK1 was detected by qRT-PCR. GAPDH served as an internal control. **P<0.01, ***P<0.001 compared with the miR-1236 group. H. Skp2, p27 and CDK1 protein expression levels were detected by Western blot. β-actin levels were used as an internal control. miR-1236 influenced p27 and CDK1 expression through up-regulation of Skp2.

MiR-1236-3p and dsRNA-245 had different inhibitory effects on the proliferation and cell cycle distribution of BCa cells mainly because of differences in Skp2 expression

To further detect the changes in cell proliferation caused by miR-1236-3p and dsRNA-245, an EdU assay was conducted. The results showed that overexpression of miR-1236-3p and dsRNA-245 inhibited the proliferation of both 5637 and T24 cells. Further, dsRNA-245 demonstrated a more potent inhibitory effect than miR-1236-3p. In cells co-treated with miR-1236-3p and siSkp2, the inhibitory effect was significantly enhanced and was similar to that caused by dsRNA-245 (**Figure 6A**). Additionally, we quantified cell proliferation based on the number of positive cells (**Figure 6B**). Furthermore, we conducted flow cytometry to measure cell cycle distribution. When 5637 and T24 cells were transfected with miR-1236-3p or dsRNA-245 for 72 h, cells in the G0/G1 phase increased, and the S phase population decreased compared with the control miRNA gr-

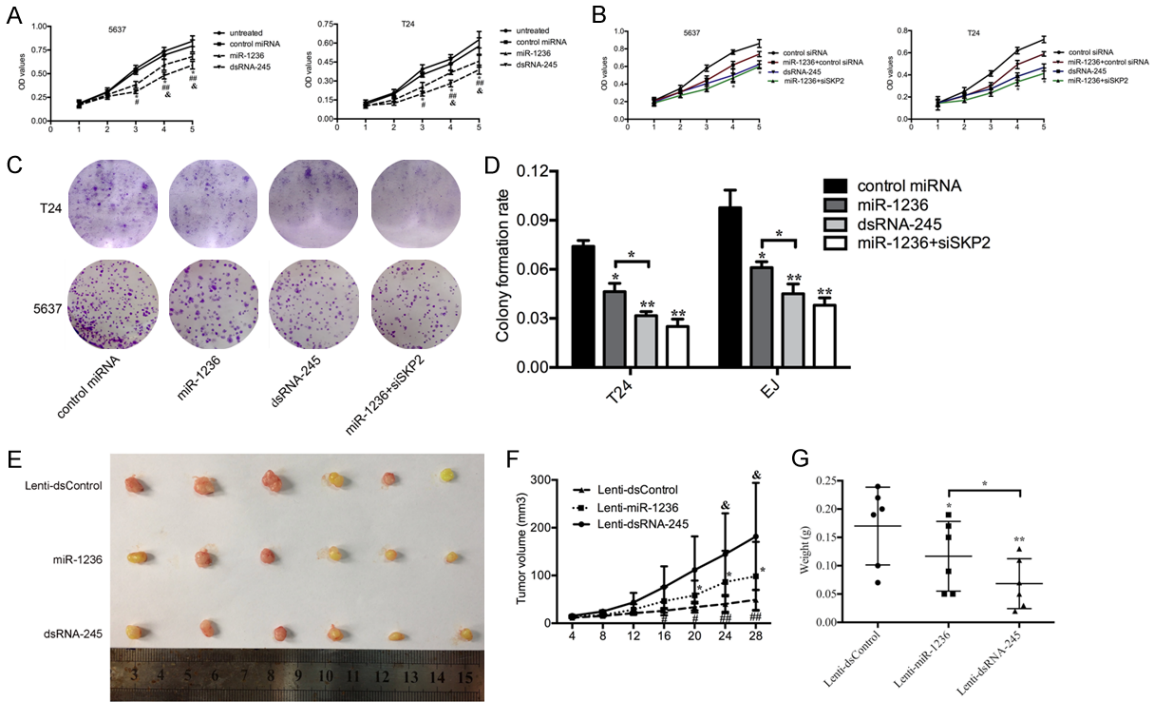

Figure 5. Overexpression of miR-1236 and dsRNA-245 exerted different inhibitory effects on BCa cell growth in vitro and in vivo. Viable cells were measured using the MTS assay kit on days 1 to 5 following transfection with the indicated miRNAs/dsRNAs. Results are plotted as OD values. A. *P<0.05 reveals that the miR-1236 group significantly inhibited cell growth compared to untreated or control groups at the same time point. Similarly, #P<0.05, ##P<0.01 reveal that the dsRNA-245 group significantly decreased cell growth compared to the untreated or control groups at the same time point. &P<0.05 reveals that the miR-1236 group attenuated cell proliferation compared with the dsRNA-245 group at the same time point. B. After co-transfection with miR-1236 and siSkp2, cell proliferation was gradually inhibited compared with the miR-1236+control siRNA group. *P<0.05. C. Representative photographs of the colony formation assay. D. Quantification of colony formation. Overexpression of miR-1236 and dsRNA-245 significantly inhibited 5637 and T24 cell proliferation compared with the control miRNA group. The inhibitory effect of dsRNA-245 was stronger than that of miR-1236. There was no significant difference in the dsRNA-245 and miR-1236+siSkp2 groups. *P<0.05, **P<0.01. E. Photographs of tumors excised 28 days after inoculation of stably transfected cells 5637 into nude mice. F. Mean tumor volume was measured by caliper every 3 days. *P<0.05, #P<0.05, and ##P<0.01 represent that the miR-1236 and dsRNA-245 group obviously suppressed bladder cancer cell growth in vivo compared with Lenti-dsControl group at the same time point. &P<0.05 reveals that the growth inhibition in the dsRNA-245 group was more pronounced than that in the miR-1236 group at the same time point. G. Weight of tumors from each nude mouse at the end of 28 days. *P<0.05, **P<0.01.

oup. However, the reduction in S phase cells was not significant in the miR-1236-3p group. Additionally, transfection of 5637 and T24 cells with dsRNA-245 significantly decreased S phase cells compared with miR-1236-3p, mainly due to differences in Skp2 expression (**Figure 6C** and **6D**). Taken together, these results showed that miR-1236-3p could induce Skp2 expression and thus attenuate the inhibition of BCa cell growth. Moreover, Skp2 could alter the cell cycle distribution and prompt an increase in S phase cells. Therefore, Skp2 weakened the inhibitory effects of miR-1236-3p in BCa cells (**Figure 7**) (Original western images for all relevant western blots as shown in Supplementary Figure 1).

Discussion

In the present study, we identified that the endogenous miR-1236-3p and an exogenous synthetic dsRNA (dsRNA-245, which is fully complementary to the putative miR-1236-3p target site within the p21 gene promoter) boasted the considerable ability to induce p21 expression in T24 and 5637 BCa cells. In accordance with our microarray results, miR-1236-3p increased the abnormal expression of Skp2 while activating p21, whereas dsRNA-245 had no significant effect on the expression of Skp2. Moreover, there were obvious differences between the ability of miR-1236-3p and dsRNA-245 to inhibit BCa cell growth

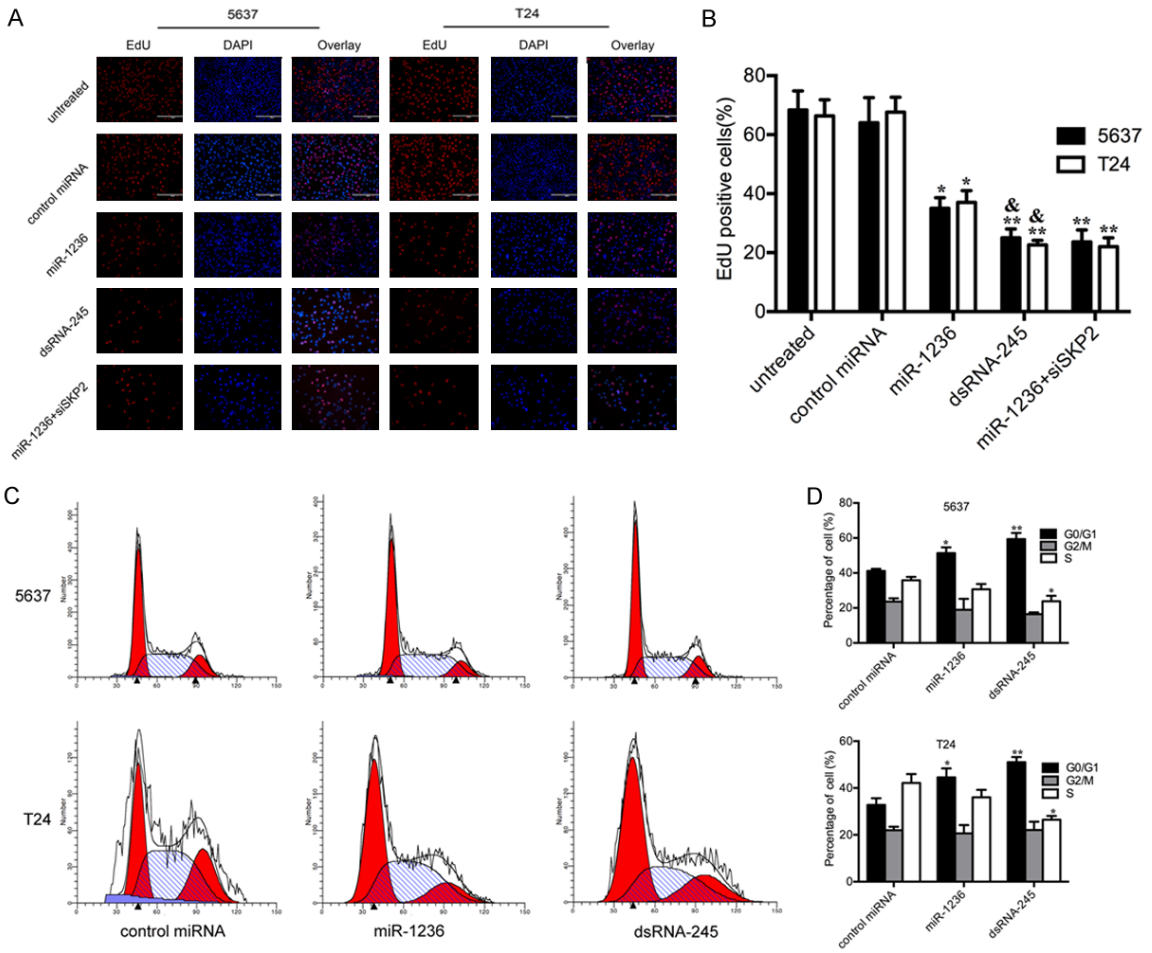

Figure 6. The differences in BCa cell proliferation inhibition and cell cycle distribution changes caused by miR-1236 and dsRNA-245 are mainly related to Skp2 expression. A. Representative micrographs of EdU-positive cells (red). The nucleus was stained with DAPI (blue). The EdU-positive cells from the miR-1236 and dsRNA-245 groups are fewer than in the control miRNA group or the untreated group. Compared with the miR-1236 group, there were fewer positive cells in the dsRNA-245 group. B. Quantification of EdU-positive cells. *P<0.05, **P<0.01 compared with untreated or control miRNA groups. &P<0.05 compared with miR-1236 group. C. Representative photographs of cell cycle analysis. D. Quantification of cell cycle distribution. Up-regulation of miR-1236 and dsRNA-245 significantly increased the proportion of G0/G1 phase cells. Overexpression of dsRNA-245 could reduce the proportion of S phase cells. *P<0.05, **P<0.01.

in vitro and in vivo, mainly due to differences in Skp2 expression in T24 and 5637 cell lines.

RNAa is a recently discovered gene transcriptional activation phenomenon induced by small dsRNAs [12]. RNAa has been reported to activate transcription by targeting specific sequences within gene promoters and gene antisense transcripts, leading to changes in chromatin structure [17]. Although the exact mechanism of RNAa remains unclear, these observations indicate that further study of gene regulation may provide promising new therapeutic strategies for the treatment of tumors.

As RNAa requires sequence complementarity between the dsRNA and the promoter of its target gene, it may induce target gene expression in a manner similar to miRNAs. Briefly, sequence complementarity is one of the prerequisites for miRNA suppression or activation; miRNAs may function through targeting various sites such as the 3'/5'-UTR, coding sequence or promoter [18, 19]. Nevertheless, while perfect complementarity usually occurs in plants, imperfect binding of the miRNA to the target site appears to be the dominant process in mammals [20]. Studies have shown that even stringent complementarity to

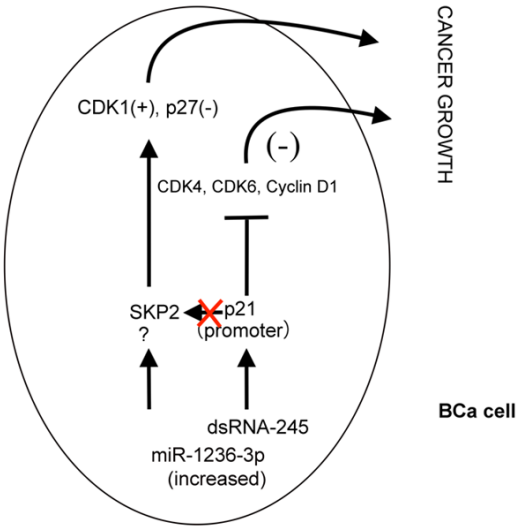

Figure 7. A schematic model. MiR-1236 and dsRNA-245 activate p21 expression by targeting a site within the p21 gene promoter, thereby inhibiting BCa cell proliferation. However, miR-1236 also induces Skp2 expression, which weakens the ability of miR-1236 to inhibit BCa cell proliferation.

a target site may not always reveal bona fide miRNAs without experimental validation [21]. Therefore, imperfect binding leads to difficulties in predicting the roles of miRNAs in the regulation of cell signaling pathways. miRNAs have been reported to play essential roles in carcinogenesis and the regulation of signal transduction pathways in BCa [20].

Skp2 is known to target more than 20 molecules for degradation through its ubiquitin ligase activity [22]. Further, Skp2 has been shown to be overexpressed in a variety of cancer types, where it contributes to malignant progression [23, 24]. We have reported that miR-1236-3p could directly activate p21 gene expression in BCa cells by targeting predicted sites in the p21 promoter. However, in the present study, we found that miR-1236-3p up-regulated the expression of the oncogene Skp2 while activating the tumor suppressor gene p21. Even when we silenced p21 expression, miR-1236-3p was still able to increase Skp2 expression, indicating that the miR-1236-3p-mediated increase in Skp2 expression did not depend on p21 activation. Further, we utilized an endogenous miRNA partly complementary to a specific sequence in the p21 promoter and designed four exogenous dsRNAs completely complementary to the similar p21 promoter region. Using these miRNA/dsRNAs, we found that only miR-1236-3p and dsRNA-245 stably enhanced target gene expression. However, dsRNA-245 did not affect Skp2 expression. Our data also showed that both miR-1236-3p and dsRNA-245 could inhibit cell proliferation and clonegenesis and alter cell cycle distribution. We were surprised to discover that the inhibition of cell proliferation caused by miR-1236-3p was considerably weaker than that caused by dsRNA-245 and that when Skp2 was knocked out, the inhibitory effect of miR-1236-3p was similar to that of dsRNA-245. These results indicated that miR-1236-3p induces Skp2 expression, thereby weakening its antitumor effect.

It has been reported that Skp2 plays a role in the ubiquitin-mediated degradation of several cyclin-dependent kinase (CDK) inhibitors, such as $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$ [25-28]. The expression of Skp2 is minimal in G0/G1 phase but increases in S phase. The overexpression of Skp2 in the G0/G1 phase could result in the dysregulation of the G1/S checkpoint and disruption of both cell proliferation and differentiation [25, 26]. Our data showed that CDK1 expression was higher and p27 expression was lower in the miR-1236-3p group compared with the dsRNA-245 group. Moreover, in both BCa cell lines, transfection with miR-1236-3p and dsRNA-245 led to a radical increase in the G0/G1 population compared with the control miRNA group. Further, dsRNA-245 could considerably decrease the number of S phase cells. Thus, dsRNA-245 was more effective than miR-1236-3p in inhibiting BCa cell growth.

In summary, we have provided evidence that the constant region of p21 promoters can be directly targeted and activated by endogenous miR-1236-3p and exogenous dsRNA-245 in BCa cell lines. Moreover, miR-1236-3p induced Skp2 expression while activating p21. We speculate that stimulation of miRNA expression (via stress response, drug treatment, etc.) may lead to the activation of some unknown genes and downstream target genes. Therefore, identifying more natural targets induced by miRNAs may provide additional insights into the gene expression profiles associated with certain miRNAs.

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Disclosure of conflict of interest

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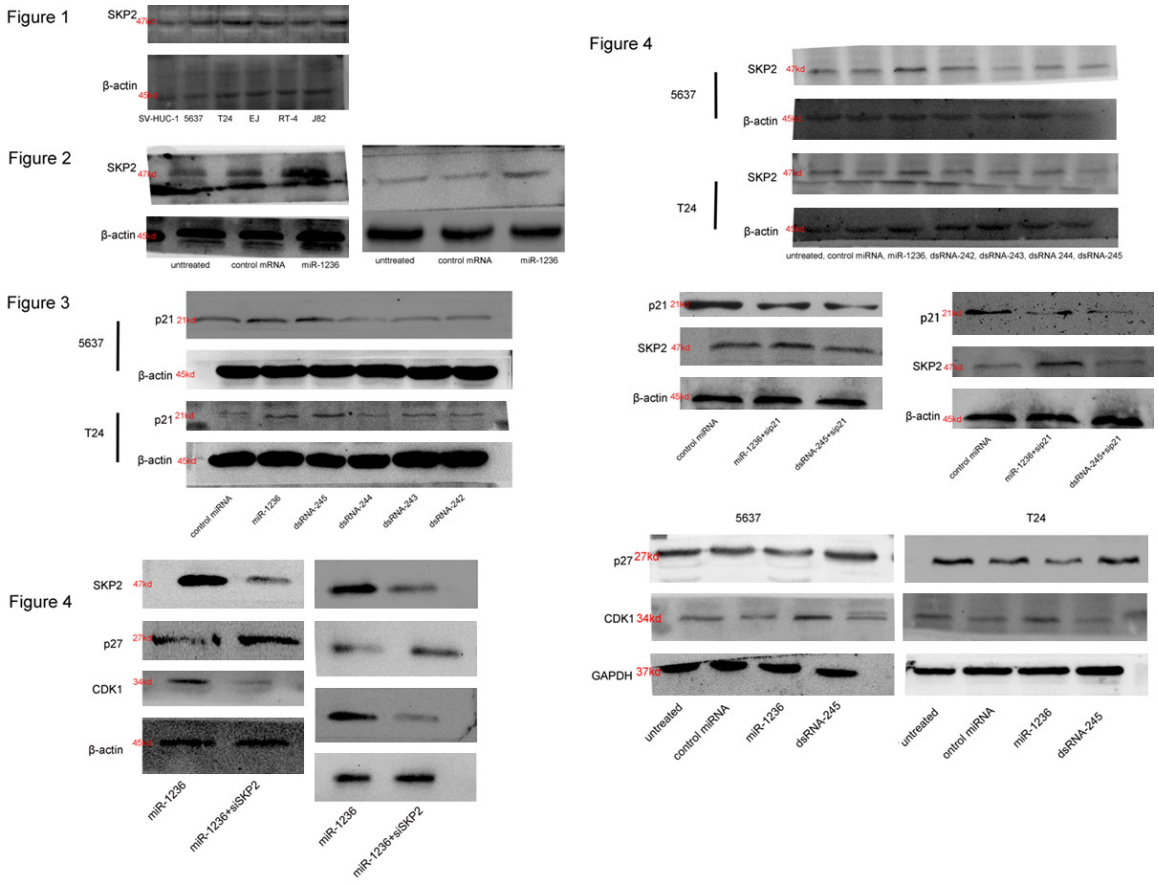

Supplementary Figure 1. Original western images for all relevant western blots.