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# Silencing nc886, a Non-Coding RNA, Induces Apoptosis of Human Endometrial Cancer Cells-1A *In Vitro*

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Data Interpretation D  
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**Background:** The role that nc886, a non-coding microRNA, plays in human endometrial cancer is unknown. The present study aimed to describe the functional role of nc886 in human endometrial cancer-1A (HEC-1A) cell line, which may provide another target for human endometrial cancer treatment.


**Material/Methods:** The expression levels of nc886 in normal human endometrial tissue and the early phase and late phase of human endometrial cancer tissues were determined and compared by fluorescence *in situ* hybridization (FISH). Small interference RNA (siRNA) was used to inhibit nc886, and cell proliferation was evaluated with the MTT test. mRNA levels of PKR, NF-κB, vascular endothelial growth factor (VEGF), and caspase-3 were determined against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) between the HEC-1A control group and the silenced group (nc886 silenced with siRNA) by real-time reverse transcription polymerase chain reaction (RT-PCR). The protein levels of PKR (total and phosphorylated form), NF-κB, VEGF, and caspase-3 were determined against GAPDH by Western blotting, and cell apoptosis was determined by flow cytometry.

**Results:** Our results indicated that a higher level of nc886 was expressed in the late phase of human endometrial cancer tissue, less than in the early phase but still higher than in normal human endometrial tissue. After nc886 was silenced, protein levels of p-PKR (phosphorylated PKR) and caspase-3 were increased, whereas NF-κB and VEGF were decreased.

**Conclusions:** The rate of apoptosis in the silenced group was increased and the rate of cell proliferation was slower in comparison to the control.

**MeSH Keywords:** **Caspase 3 • Endometrial Stromal Tumors • Uterine Diseases**

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## Background

MicroRNA (miRNA), a class of non-coding nucleotides, does not encode any genes, but rather regulates gene functions at either the post-transcriptional or translational level. miRNA regulates gene expression by directly cleaving the targeted mRNA, or interacting with mRNA (miRNA: mRNA), usually targeting the 3' untranslated regions (UTRs) of mRNA to cause dysfunctional translation. Alternatively, miRNA may physically repress PKR (protein kinase RNA-activated), an interferon-inducible and double-stranded RNA (dsRNA)-dependent kinase, in order to perform their regulatory functions through PKR [1–3].

The origin of miRNA stems from the nucleus; it is first processed by RNAase III Drosha, associated with a double-stranded (ds) RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8), and then assembled as the microprocessor complex to eventually generate -70 nucleotides precursor miRNA products, which is translocated to the cytoplasm via the Ran-GTP-dependent transporter-Exportin 5. In the cytoplasm, the cropping process is performed by the RNAse III enzyme Dicer associated to TRBP (TAR RNA-binding protein) or PKR and Argonaute (AGO1-4), thereby cleaving the miRNA precursor hairpin to produce the transitory miRNA/miRNA duplex, including mature single-stranded miRNA [4].

Nc886 is a 101-nucleotide (nt)-long, double-stranded, non-coding RNA, but it physically interacts with PKR for tumor surveillance. Nc886 was first described and named by Kunkeaw et al. in 2013 [5] in a report showing that nc886, which is suppressed by siRNA in cholangiocarcinoma (CCA) cells, is able to activate the canonical PKR/eIF2 $\alpha$  cell death pathway with elevated PKR. In addition, nc886 is a tumor suppressor in esophageal squamous cell carcinoma (ESCC). Nc886 was found to be hypermethylated in ESCC patients with poor survival, and artificially knocked-down nc886 can activate oncogene expression [6].

Different molecules may serve as an oncogene or a tumor suppressor in different cancers at different developmental stages. The role of nc886 remains unclear. The present study indicated that nc886 is up-regulated and over-expressed in late-phase human endometrial cancer tissue, and a higher level of nc886 is expressed in the HEC-1A cell line compared to the HeLa cell line (data not shown). Nevertheless, when nc886 is silenced with siRNA, phosphorylated PKR and caspase 3 are increased, whereas NF $\kappa$ B and VEGF are decreased. The profiles of both mRNA and protein expressions of the above are likely to be the same. The rate of apoptosis is increased in HEC-1A cells, whereas the rate of cell proliferation is decreased.

## Material and Methods

### Reagents

Human endometrial cancer cell line (HEC-1A) was purchased from the American Type Culture Collection (ATCC HTB-112). Fetal bovine serum (FBS) was purchased from Gibco Company. siRNA was synthesized and purchased from Guangzhou RiboBio Technology Company. DMEM and other cell culture media were bought from Hyclone (Thermo Scientific Company). Lipofectamine 2000 Transfection Reagent was purchased from Life Technologies. The MTT ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) Cell Proliferation Assay Kit (KGA312) was purchased from Nanjing KeyGen Biotech Company. Q-RT-PCR reagents were purchased from Invitrogen and Qiagen companies. PrimeScript II 1<sup>st</sup> Strand cDNA Synthesis Kit (D6210A) and SYBR Premix Ex Taq™ II Reagent Kit were purchased from TaKaRa Bio-Tech Company. The Annexin V-FITC Apoptosis Detection Kit for flow cytometry was purchased from Beyotime Institute of Biotechnology. Diethyl pyrocarbonate (DEPC) and DAPI (2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride) staining kits were purchased from Guangzhou Gension Biotechnology Company. Polyvinylidene fluoride membrane (PVDF) was purchased from Emdmillipore Company. Antibodies PKR (Cat#ab32052) and pPKR(Cat#ab32036) were purchased from ABCAM company, and caspase-3 (Cat#sc1225), VEGF (Cat#sc507), and GAPDH (Cat#sc166574) were purchased from Santa Cruz Biotech Company, except for NF- $\kappa$ B (cat. no. 13681), which was from Cell Signaling Technology Company. The fluorescence *in situ* hybridization (FISH) reagent kit was purchased from Biosene Company (Guangzhou, China).

### siRNA transfection

The procedure of transfection was done as previously described [7,8]. According to the nc886 sequence, siRNA of GGGTCGGAGTTAGCTCAAGCGG was synthesized, and demonstrated to be effective after screening. Briefly, HEC-1A cells at  $2 \times 10^6$  cells/ml concentration were plated into 6-well plates at 2 ml per well. After cells grew in confluence up to about 70%, cells were ready for transfection. The final concentration of siRNA: siRNA was diluted to 50 mM with DMEM medium without serum and antibiotics. After gently shaking, 50  $\mu$ l was added and shaken again, and then incubated for 30 min with shaking every 15 min. The final mixture was added into the cell culture medium with swirling several times, and incubated for 24 h.

### MTT cell proliferation assay

The procedure was performed by following the manufacturer's instructions and a previous report [9]. Briefly, 100  $\mu$ l contained  $1 \times 10^4$  cells from the siRNA silenced group and the

control group were separately planted in a 96 well-plate, and cells were allowed to be continuously incubated for another 24 hours. Concentrated 5×MTT was diluted to 1×MTT with the dilution buffer. The diluted MTT 50 µl was added into each well of the 96 well-plate, and the incubation were allowed to continue for 4 hours, so that MTT was able to be reduced to its insoluble formazan. After centrifugation, the supernatant was discarded, but replaced with 150 µl of dimethyl sulfoxide (DMSO) to dissolve the cell layer, and then the suspension was taken to read at 490 nm on a Microplate Reader (Perkin Elmer VICTOR X5). At least 2 independent experiments were carried out as each sample was quadruplicated in each experiment.

### Real-Time RT-PCR

A series of procedures were performed as previously described [10–12]. After treatment, cell debris was ground in liquid nitrogen, and RNA was extracted according to the instructions from the Invitrogen and Qiagen kits. All reagents were prepared with DEPC-treated water. Full-length cDNA was synthesized using the PrimeScript II 1<sup>st</sup> Strand cDNA Synthesis Kit (TaKaRa). PCR primers for real-time PCR were (5'-----3'). Real-time PCR was completed by using SYBR Green incorporation following the instructions of the manufacture (TaKaRa). The final parameter was expressed as  $2^{-\Delta\Delta Ct}$ , and underwent statistical analysis. At least 2 independent experiments were carried out. Each sample was quadruplicated.

### Western blotting

Western blot analysis was performed as described by Xu et al. [13]. Briefly, after the culture medium was discarded, a cell layer was washed gently with 0.1 M phosphate-buffered saline (PBS, pH 7.4). Harvested cell debris was homogenized with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol-phosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin, and 1 mM phenylmethylsulphonyl fluoride). Protein concentration was determined using Lowry's method. We loaded 40–60 µg protein (upon richness of specific type of protein in this cell line) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 5% concentrated gel, and 15% separated gel). Protein was transferred to a PVDF membrane, and then incubated with primary antibodies overnight. After incubating with HRP-linked secondary antibodies (Boster company, BA#1054), the membrane was color-developed with Beyo ECL Plus. Integrated colorimetric density was calculated and statistically analyzed. At least 2 independent experiments were carried out.

### Flow cytometry

The procedure was performed as described in previous studies [14,15]. After cells were harvested, aliquots of 1×10<sup>6</sup> cells/100 µl were collected into FACS tubes. The cells were washed twice with 0.1 M PBS, and were re-suspended and sequentially stained with 5 µl annexin V-FITC and 10 µl propidium iodide staining solution. After mixing gently, the process of staining was completed by incubation at room temperature (20–25°C) in the dark for 20 min, and then preserved on wet ice. Fluorescence was captured on Flow Cytometry (Accuri C6) with propidium iodide (PI) at Ex 488 nm, Em 617 nm, and fluorescein isothiocyanate (FITC) at Ex 488 nm and Em 530 nm. At least 2 independent experiments were carried out and each sample was quadruplicated in each experiment.

### FISH

The procedure was performed as described in previous studies [16,17] and followed the protocol from the manufacture (Biosense, Guangzhou, China). Briefly, paraffin from slides was removed with absolute ethanol and air dried, and then underwent a series of steps, including RNase A treatment, post-fixation, dehydration, Proteinase K digestion, dehydration, denaturation, *in situ* hybridization, post-hybridization rinse, and fluorescent staining. The probe of cDNA for nc886 was synthesized and employed as (5'→3') AAAGGGTCAGTAAGCACCCGCGGTCTCGAACCCAGCACAGAGATGGACAGATAGAAAGTCCGGCATGAGGAGGTAACCGCTTGA GCTAACTCCGACCC. At least 2 independent experiments were carried out.

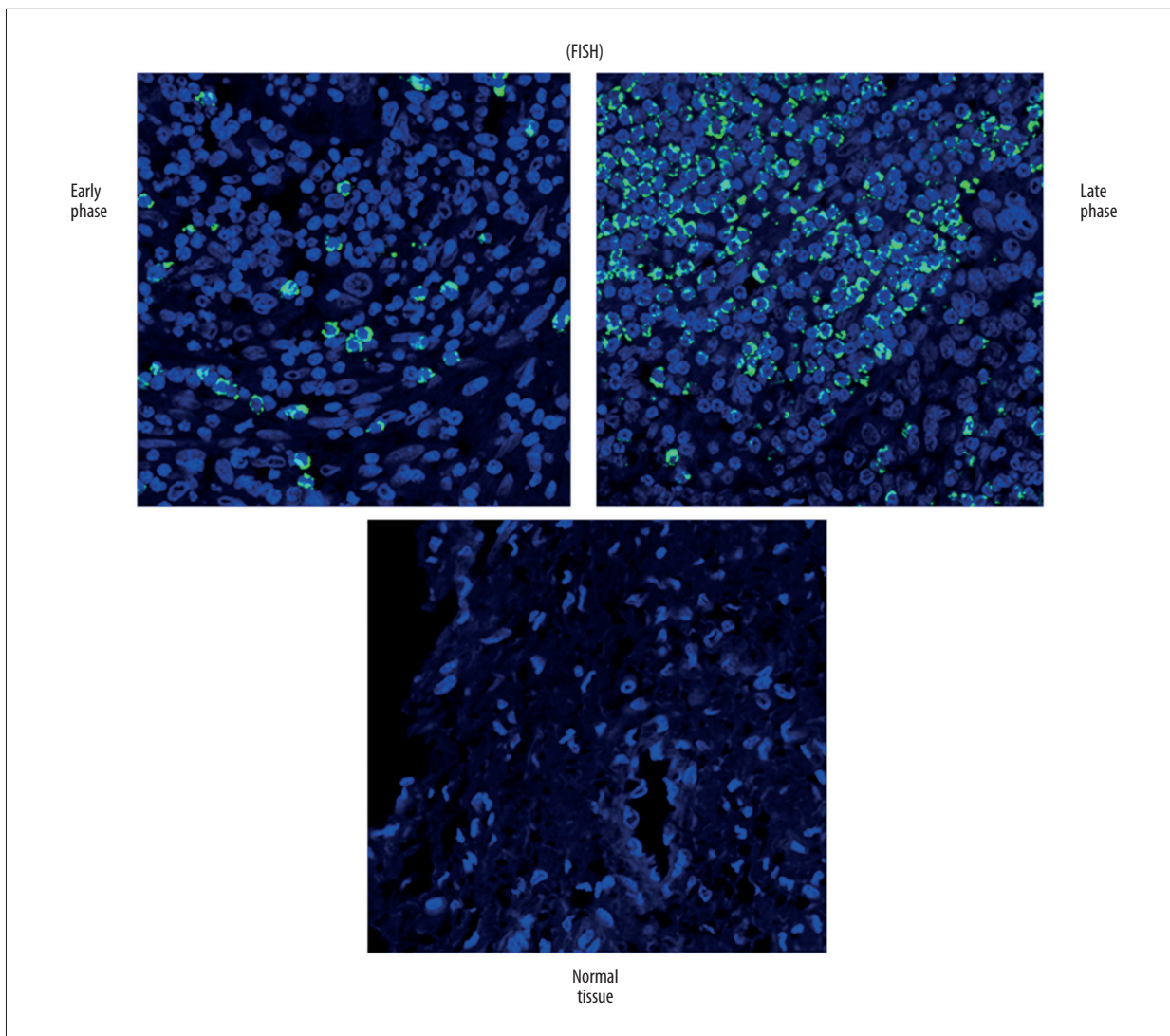
### Statistical analysis

All quantitative data are expressed as mean ±SD, and were analyzed using SigmaStat 12.5 statistical software from SYSTAT, Inc. One-way ANOVA program followed by Dunnett's test was used to compare treatment groups to a single control group, and the statistical significance was determined by P value.

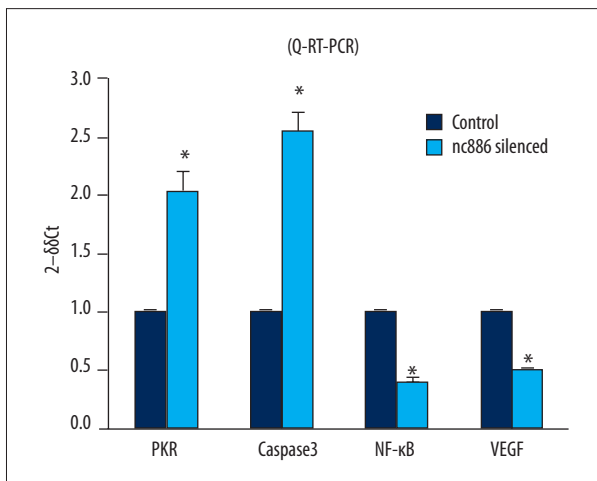
## Results

### Over-expression of nc886 in the late phase of human endometrial cancer tissue

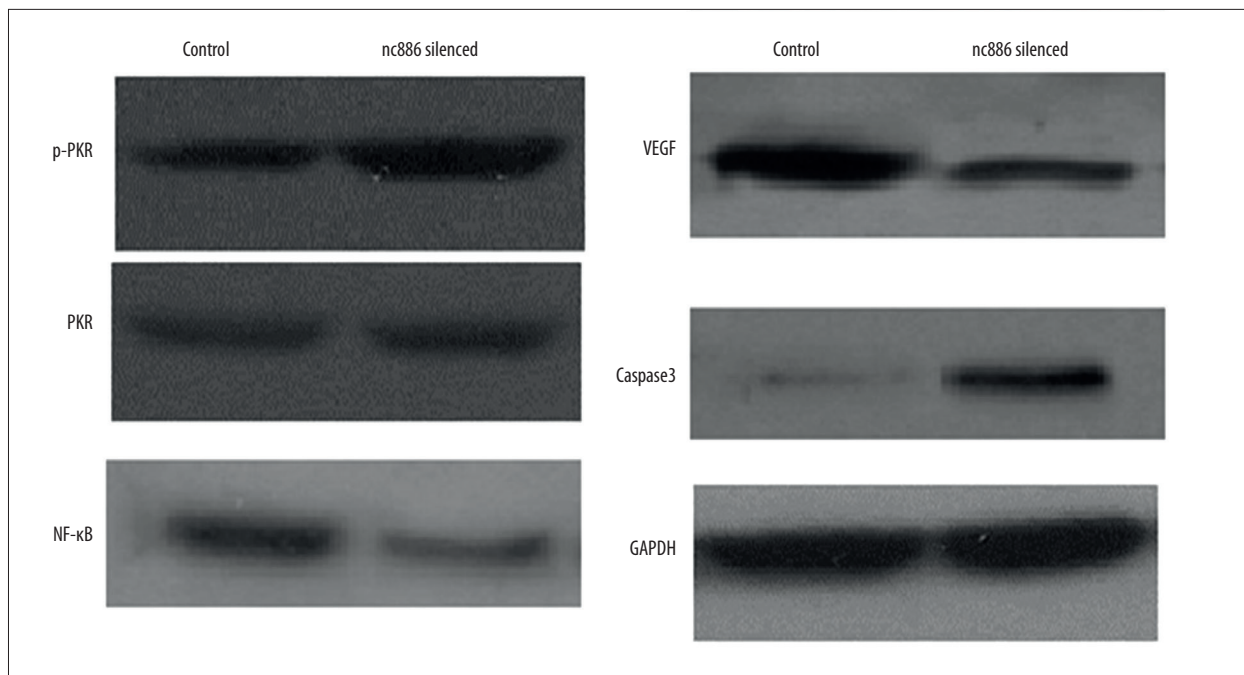
As seen in Figure 1, nc886 was abundantly expressed in cytoplasm. The amount of expression is displayed in dots, bands, or crescent moon shapes around the nuclei and such expressions were individually scattered at the early phase of human endometrial cancer (HEC) tissue. However, at the late phase of HEC, the amount of nc886 expression in individual cell was greatly increased and the numbers of nc886 over-expressed



**Figure 1.** Expression of nc886 is greatly increased from the early phase to the late phase of human endometrial cancer as shown by fluorescence *in situ* hybridization (FISH). Magnification  $\times 400$ .



**Figure 2.** Increases of PKR and caspase-3 but decreases of NF-κB and VEGF are induced by the silenced nc886 as compared to the control (\*  $P < 0.01$ ). Expression levels of PKR, caspase-3, -NF-κB, and VEGF were determined by real-time RT-PCR.



**Figure 3.** Phosphorylated PKR and caspase-3 are increased but NF-κB and VEGF are decreased at the protein level induced by the silenced nc886 as compared to the control. GAPGH protein was used as the control protein. All proteins were measured by Western blot analysis.

**Table 1.** The apoptotic rate of Human endometrial cancer cells (HEC-1A) is greatly induced by silenced nc886. Apoptosis was measured by flow cytometry.

Groups	UR (%)	UL (%)	LL (%)	LR (%)	UR+LR (%)	Mean ±SD	P Value
Control	2.21	1.01	96.61	0.17	2.38	3.163±1.37	<0.01
	3.46	1.24	95.04	0.26	2.36		
	2.11	0.69	94.56	2.64	4.75		
Nc886 silenced	3.49	2.90	75.10	18.51	22.00	25.95±4.31	<0.01
	5.31	8.62	60.84	25.23	30.54		
	8.44	5.12	69.56	16.88	25.32		

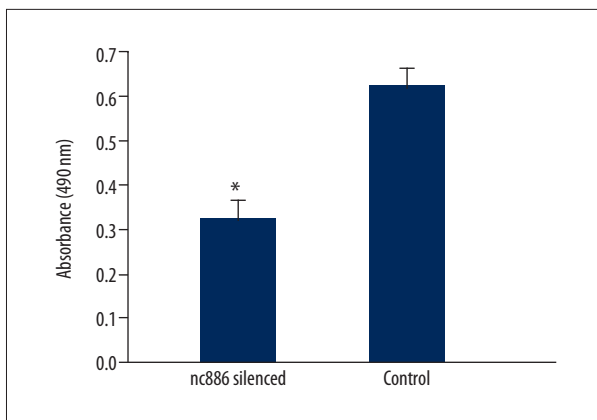
LR – Annexin V-FITC+PI– (X axis apoptotic cells at the early stage); UR+UL – Annexin V-FITC+PI+/- (Necrotic cells or dead cells); UR+LR – Annexin V-FITC+PI+ (necrotic cells & apoptotic cells at the early and late stages).

cells were also greatly increased, and these cells tended to converge together in higher density to form a potential neoplastic focus. In contrast, the expression of Nc886 in normal tissue was relatively lower, as indicated by the fluorescence intensity in the image.

### Silenced-nc886 resulting in increased PKR and caspase 3 but decreased NFκB and VEGF

Results of a pilot study showed that the level of nc886 was highly expressed in HEC-1A cells. Several pairs of siRNA aimed at different sites of nc886 sequence were constructed. The

percentages of knock-down were screened until one of the effective siRNAs (GGGTCGGAGTTAGCTCAAGCGG) was found to be able to successfully knock-down nc886 mRNA expression (data not shown). As shown in Figure 2, after normalization of mRNA, the silenced nc886 led to decreases of both NFκB and VEGF at mRNA levels but increases of both PKR and caspase 3 at mRNA levels. Nevertheless, the ranges of increases in PKR and caspase 3 were 2-fold higher, whereas the ranges of decreases in NFκB and VEGF were just 1-fold higher.



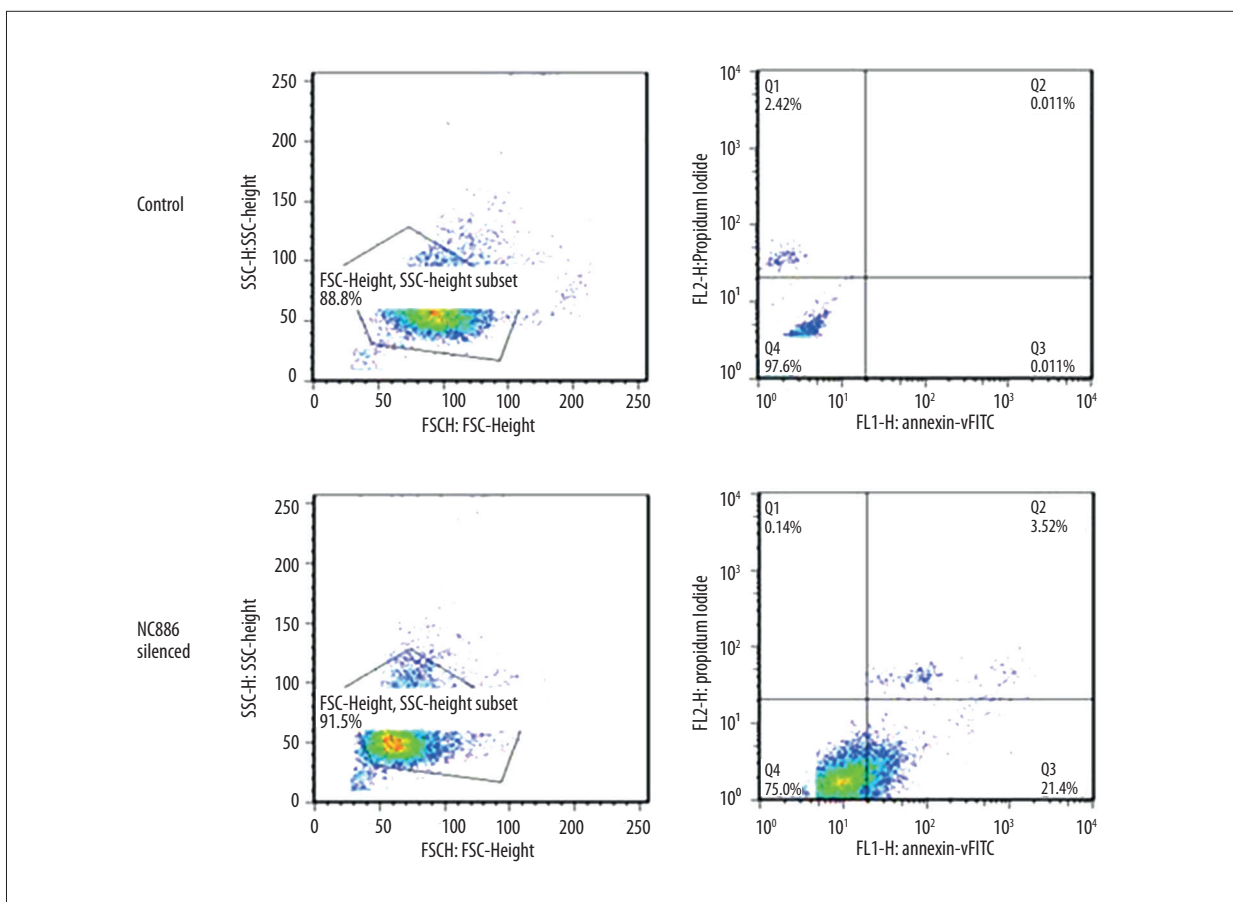
**Figure 4.** The proliferation rate of human endometrial cancer cells (HEC-1A) is decreased by silenced nc886 as compared to the control (\*  $P < 0.01$ ). The cell proliferation rate was measured by MTT test.

**Silenced nc886 resulted in increased PKR and caspase 3 but decreased NFκB and VEGF**

The profile of protein expression of phosphorylated PKR (pPKR), caspase3, NFκB, and VEGF displayed similar characteristics as found in mRNA. As shown in Figure 3, after nc886 was silenced, the pPKR level was higher than in the HEC-1A control cells. Caspase3 in the nc886-silenced cells was significantly higher than in the HEC-1A control cells. However, protein levels of NFκB and VEGF were greatly lower than in the HEC-1A control cells.

**Silenced-nc886 induced apoptosis**

After nc886 was silenced, an apoptotic rate of HEC-1A cells was significantly increased and was almost 10-fold higher than in the HEC-1A control cells. As shown in Table 1 and Figure 4, the proportion of apoptotic cells in the nc886-silenced group was much higher than in the HEC-1A control group ( $P < 0.01$ ), whereas the proportion of living cells in the nc886-silenced group was much lower than in the HEC-1A control group ( $P < 0.01$ ).



**Figure 5.** The apoptosis of human endometrial cancer cells (HEC-1A) is induced by silenced nc886 via analysis of flow cytometry.

## Silenced nc886 inhibited cell proliferation

As shown in Figure 5, after nc886 was silenced, the rate of cell proliferation assayed by the MTT test was slower in comparison with the HEC-1A control group. Quantitatively, the rate of cell proliferation in the nc886-silenced group was half as low as in the HEC-1A control group.

## Discussion

Our study shows that nc886 acts as an oncogene in human endometrial cancer, and is highly expressed in the late phase of HEC. When nc886 is silenced, PKR (especially pPKR) is released and starts to play its role in cell proliferation and apoptosis. Thus, VEGF and NF- $\kappa$ B are decreased, whereas caspase 3 is increased, thereby leading to increased HEC-1A cell apoptosis and inhibited cell proliferation. Our results suggest that nc886 is involved in initiation, propagation, and metastasis of human endometrial cancer.

Previous studies reported that nc886 was occasionally suppressed, or its CpG hypermethylated, in a variety of cancers. However, we cannot form a firm conclusion based on a few reports, because nc886 is complicated and incompletely understood, and the underlying mechanism of nc886 remains unclear. Theoretically, if nc886 was suppressed in cancers, PKR would be released for auto-phosphorylation and activated to provoke cellular apoptosis via phosphorylation of the  $\alpha$ -subunit of the eukaryotic translation initiation factor 2 (eIF2- $\alpha$ ) to block *de novo* protein synthesis or onset of the caspase system [3,5,11]. Conversely, if nc886 serves as an oncogene, it would be highly expressed in cancer. Our present study corroborates the hypothesis that nc886 is an oncogene in human endometrial cancer.

Although nc886 is universally found in human cells, and nc886 physically interacts with PKR via its nts 46–56 [18], the mechanism by which nc886 regulates PKR is unknown. Unlike nc886, PKR has been extensively investigated, and it has been demonstrated that PKR plays a major role in regulating apoptosis [19–21].

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PKR is considered to be a tumor suppressor, but its activation is caspase-dependent, which directly leads to apoptosis via blocking global protein synthesis through eIF2 $\alpha$  phosphorylation [19,20]. In neuroblastoma cells, tunicamycin, an inhibitor of protein glycosylation, provoked endoplasmic reticulum stress and triggered apoptosis through PKR based on caspase-3 activation [22]. PKR was also set up as a target for Bozepinib, a novel antitumor agent, through triggering apoptosis [23,24]. These findings strongly support our results showing that up-regulated p-PKR triggers HEC-1A cells apoptosis through elevated caspase-3 levels.

As a matter of fact, once PKR was knocked down with siRNA *in vitro* or PKR<sup>-/-</sup> mice *in vivo*, VEGF was significantly decreased by 54%, demonstrating that PKR mediates angiogenesis through the VEGF pathway, which may help develop treatments for peripheral artery disease (PAD). Our study results show that PKR inhibits HEC-1A cell proliferation, possibly due to decreased levels of VEGF and NF- $\kappa$ B, which may lead to new methods for preventing metastasis. In addition, a report indicated that micro-RNA340 inhibits tumor cell proliferation and induces apoptosis in endometrial carcinoma cell line RL-95-2 [25], suggesting that some correlations might be worth investigating further.

## Conclusions

Taken together, the evidence shows that nc886 serves as an oncogene with a possible role in human endometrial cancer, and plays an important role in mediating apoptosis through activating PKR, increasing caspase-3, and decreasing VEGF and NF- $\kappa$ B, which may provide a series of targets to for treating human endometrial cancer in the future.

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