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## Knockdown of Long Noncoding RNA NR\_026689 Inhibits Proliferation and Invasion and Increases Apoptosis in Ovarian Carcinoma HO-8910PM Cells

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Ovarian cancer is one of the leading causes of gynecological cancer-related deaths worldwide. We investigated the role of a newly discovered long noncoding RNA, NR\_026689, in cell proliferation, metastasis, and apoptosis in ovarian cancer cells. Our results showed that NR\_026689 was overexpressed in both clinical ovarian cancer patients and cultured ovarian cancer cells. Knockdown of NR\_026689 in HO-8910PM cells significantly decreased the cell proliferative rate and the ability to form colonies. Transwell assays revealed that depletion of NR\_026689 suppressed cell migration ability by 68% and cell invasive capacity by 71% in HO-8910PM cells. Moreover, specific shRNAs against NR\_026689 notably promoted cell apoptosis in HO-8910PM cells by upregulating the expression of proapoptotic proteins, including caspase 3, caspase 9, cytochrome C, and PARP. Our study suggests an oncogenic potential of NR\_026689 in ovarian cancer and might provide novel clues for the diagnosis and treatment of ovarian cancer in the clinic.

**Key words:** NR\_026689; Ovarian cancer; Proliferation; Metastasis; Apoptosis

### INTRODUCTION

Ovarian cancer is among the most common causes of cancer-related deaths for females around the world. More than 230,000 new ovarian cancer cases and approximately 152,000 deaths were reported in 2012<sup>1</sup>. Although great efforts have been made to improve the diagnosis and treatment of ovarian cancer, more than 75% of ovarian cancer patients are diagnosed at a very late stage<sup>2</sup>. Clinical treatment for these patients at an advanced stage is cytoreductive surgery combined with chemotherapy<sup>3</sup>, which is only effective in the initial period. A majority of these patients will relapse with a median progression-free survival (PFS) of 18 months and eventually die from ovarian cancer<sup>4</sup>. Thus, accumulative studies have been focusing on targeted therapies and proposing the application of biomarkers in the diagnosis and treatment of ovarian cancer at a relatively early stage<sup>5,6</sup>. However, no reliable molecular biomarkers have been discovered that are capable of identifying ovarian cancer before there is radiographic or biochemical evidence of progression.

Long noncoding RNAs (lncRNAs) are a category of RNAs that have a length of more than 200 nucleotides and are incapable of encoding proteins because of a lack of open reading frames<sup>7</sup>. According to their genomic locations,

lncRNAs are classified into four categories, namely, intergenic, intronic, antisense, and enhancer lncRNAs<sup>8</sup>. Recently, the role of lncRNAs has been increasingly recognized in tumorigenesis. For instance, it was found that lncRNA MALAT-1 was highly expressed in patients with ovarian cancer, and a high expression of MALAT-1 promoted cell proliferation and cell metastasis<sup>9</sup>. lncRNA ANRIL also showed its potential for promoting cell proliferation and cell cycle progression and suppressing cell apoptosis and senescence in ovarian cancer<sup>10</sup>. However, the detailed mechanism of how these lncRNAs regulated tumorigenesis in ovarian cancer is still an enigma for researchers. Furthermore, the effects of other lncRNAs on ovarian cancer also need to be uncovered.

lncRNA NR\_026689 is a newly discovered lncRNA that could potentially serve as a biomarker for lung carcinogenesis<sup>11</sup>. Researchers identified this lncRNA in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-injected rats and analyzed this with the Arraystar rat lncRNA array. Upon NNK injection, NR\_026689 was significantly upregulated in lung cancer tissues and rat whole blood. However, a more detailed knowledge of the involvement of NR\_026689 in the carcinogenesis of the lung and other organs remains largely unclear.

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In our study, the expression of NR\_026689 in ovarian cancer was explored *in vivo* and *in vitro*. Detailed roles of NR\_026689 in cell proliferation and metastasis were also assessed in ovarian cancer HO-8910PM cells. Furthermore, the effects of NR\_026689 on cell apoptosis and its preliminary mechanism were examined by knockdown of NR\_026689 with specific shRNAs in HO-8910PM cells. Our study indicated the oncogenic potential of NR\_026689 in ovarian cancer and might provide novel clues for the diagnosis and treatment of ovarian cancer in the clinic.

## MATERIALS AND METHODS

### *Human Tissues*

This study was approved by the ethics committee of Xi'an Jiaotong University. A total of 70 ovarian cancer patients gave their full intention to participate in our study, and written consent from each patient was obtained. These patients underwent clinical surgeries in The Second Affiliated Hospital of Xi'an Jiaotong University. No chemotherapy or radiotherapy preceded these surgeries. The tumor tissues and their adjacent noncancerous counterparts were dissected and frozen in liquid nitrogen immediately and subjected to RNA extraction.

### *Cell Culture and Transfection*

Human normal ovarian cell line HUM-CELL-0088 and ovarian cancer cell line OVCAR-3 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Three other ovarian cancer cell lines—HO-8910, HO-8910PM, and SK-OV-3—were commercially purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, P.R. China). Cells were cultured within the recommended medium, supplied with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and kept in a 5% CO<sub>2</sub> atmosphere at 37°C. Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocols.

### *Real-Time Polymerase Chain Reaction (RT-PCR)*

Total RNAs from both clinical tissues and cultured cells were extracted by TRIzol reagents (TaKaRa, Dalian, P.R. China) at a concentration of 1 ml/well in six-well plates. NanoDrop 2000 was used to quantify the extracted RNAs by collecting absorbance of 280 and 260 nm. cDNAs were reverse transcribed by PrimeScript RT Master Mix Reagent Kit (TaKaRa), and 10 ng from each sample was subjected to PCR analysis. The following primers were used: NR\_026689, 5'-TCATCCATCACCTTCCAACA-3' (forward) and 5'-ACCGCTCGCTTCTTAGCAAT-3' (reverse); GAPDH, 5'-ACAGCAACAGG GTGGTGGAC-3' (forward) and 5'-TTTGAGGGTGCA GCGAACTT-3' (reverse). Briefly, the protocol for the PCR assay was 95°C for 30 s followed by 40 cycles at

95°C for 5 s and 60°C for 1 min. GAPDH was included here as an internal control. The cycle threshold values were calculated with the ABI7900 software (ABI; Applied Biosystems, Foster City, CA, USA).

### *Cell Proliferation*

HO-8910PM cells were transfected with scramble or specific shRNAs against NR\_026689 for 48 h and then seeded into 96-well plates (3,000 cells/well) and allowed to grow overnight. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide methyl thiazolyl tetrazolium (MTT) solution (2 mg/ml) was mixed into each experimental well. After incubation for 4 h at 37°C, culture medium was removed, and 200 µl of DMSO was added into cells in each group. The plate was shaken for 5 min, and the optical density was obtained at 570 nm. Cell proliferation assay was performed for 5 consecutive days with each group repeated in triplicate.

### *Colony Formation Assay*

Human ovarian cancer HO-8910PM cells were cultured in 10-cm plates and treated with shRNAs targeting scramble or NR\_026689 sequences. After 48 h of incubation, infected cells were washed with PBS, reseeded in new plates (800 cells/well), and allowed to grow naturally. After 2 weeks of incubation, treated HO-8910PM cells were washed twice with PBS, fixed with paraformaldehyde, and then stained with crystal violet (1%) for 10 min. Afterward, colonies were washed with ddH<sub>2</sub>O and photographed under an inverted microscope with five random fields in each experimental group. Colonies that contained more than 50 cells were manually counted and calculated.

### *Transwell Assay*

HO-8910PM cells were cultured in 24-well plates and infected with specific shRNAs. After 48 h, cells were harvested with serum-free medium, and 200 µl of cell suspension (approximately 3 × 10<sup>4</sup> cells) was added into the upper chamber (Corning, NY, USA). The lower chamber was filled with 600 µl of culture medium supplied with 10% FBS. For the invasion assay, the chamber was precoated with Matrigel (Corning) for 6 h at 37°C before seeding cells. After incubation in a 37°C incubator for an additional 12 h, cells were fixed with precold methanol for 5 min and then stained with crystal violet (1%) for 5 min. The images were then captured under a Nikon microscope with five random fields in each experimental group.

### *Cell Apoptosis Assay*

Morphological analysis was performed to evaluate the apoptotic cells using Hoechst 33258 staining. HO-8910PM cells were treated with distinct shRNAs for 48 h and then washed with cold PBS and fixed in methanol/acetic acid (3:1) for 15 min at 37°C. Fixed cells were then stained

with 5  $\mu\text{g/ml}$  Hoechst 33258 for 10 min. Morphological changes in the nuclei of cells were imaged using a fluorescence microscope (Leica, Germany), and cell apoptotic rate was calculated.

#### Determination of Caspase Activities

The relative activities of caspase 3, caspase 8, and caspase 9 were determined by the Caspase 3 Activity Kit, Caspase 8 Activity Kit, and Caspase 9 Activity Kit, respectively, following the instructions from Beyotime (Nantong, P.R. China). Briefly, cell lysates were collected by centrifuging after shRNA treatment. Aliquots of 10- $\mu\text{l}$  proteins from cell lysates were added into 96-well plates and coincubated with 80  $\mu\text{l}$  of reaction buffer containing caspase substrate (caspase 3, caspase 8, and caspase 9; 2 mM for each). After incubation for an additional 4 h at 37°C, caspase activities were obtained with a TECAN reader at an absorbance of 405 nm.

#### Western Blot Analysis

Proteins from HO-8910PM cells, which were transfected with shRNAs in the presence or absence of NR\_026689 depletion, were extracted for subsequent immunoblot assays when cell growth confluence was approximately 90%. Briefly, an equal amount of 50  $\mu\text{g}$  of protein from each group was loaded into each well in a 10% SDS-PAGE gel. Immunoreactivity was developed with enhanced chemoluminescence autoradiography (Thermo Scientific, Pittsburgh, PA, USA) by the Las3000 machine. Primary antibodies against caspase 3, caspase 9, and cytochrome C (cyt C) were purchased from Abcam (Cambridge, MA, USA). Primary antibody against PARP and GAPDH as well as the secondary antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH was included as an internal control.

#### Statistical Analysis

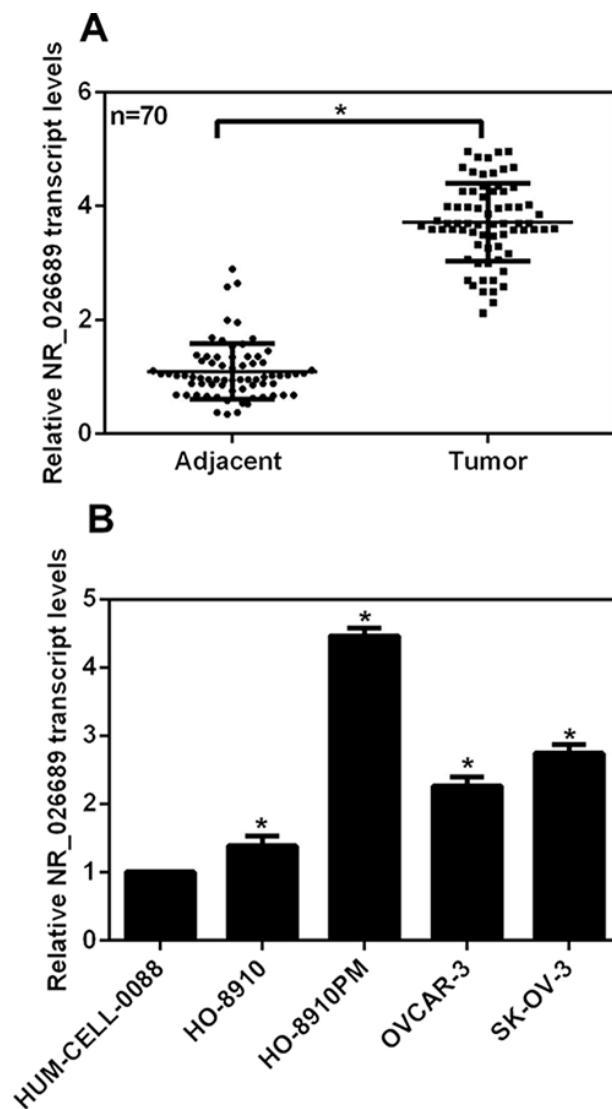
The results were presented as means  $\pm$  standard deviation (SD). Statistical analysis was carried out with the Student's *t*-test. Any difference with a value of  $p < 0.05$  was considered statistically significant. All experiments were repeated in triplicate.

## RESULTS

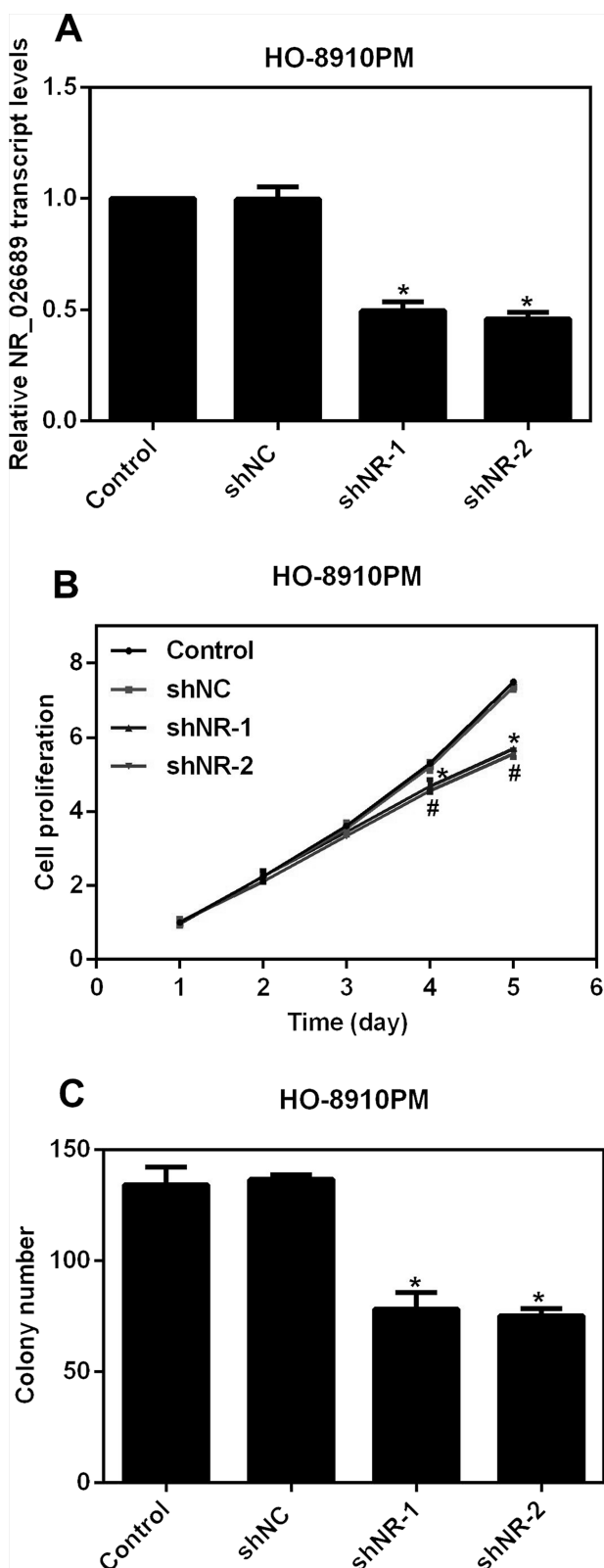
#### Long Noncoding RNA NR\_026689 Was Overexpressed in Human Ovarian Cancers

Cancer tissue and adjacent noncancerous tissue from a total of 70 ovarian cancer patients were collected and dissected for RT-PCR analysis. As shown in Figure 1A, the relative transcript level of NR\_026689 was notably increased by approximately 3.8-fold in tumor tissues compared with their control counterparts. Next, the transcript level of NR\_026689 was assessed in ovarian cancer cell lines. HUM-CELL-0088 is a normal ovarian cell line

and was also included, whereas HO-8910, HO-8910PM, OVCAR-3, and SK-OV-3 cell lines are derived from ovarian cancer patients, of which the HO-8910PM cells have the strongest migration potential. As shown in Figure 1B, compared with HUM-CELL-0088 cells, all of the ovarian cancer cell lines showed a higher expression of NR\_026689, which were increased by 1.5-fold



**Figure 1.** Long noncoding RNA NR\_026689 was overexpressed in human ovarian cancers. (A) A total of 70 ovarian cancer patients participated in our study, and their tumor tissues as well as their adjacent noncancerous tissues were obtained and subjected to RT-PCR analysis to detect the expression of long noncoding RNA NR\_026689.  $*p < 0.05$ , Tumor versus Adjacent. (B) Human normal ovarian cells (HUM-CELL-0088) and four ovarian cancer cell lines (HO-8910, HO-8910PM, OVCAR-3, and SK-OV-3) were included in the RT-PCR assays. The relative transcript level of NR\_026689 in each cell line was detected.  $*p < 0.05$  versus HUM-CELL-0088.



(HO-8910 cells), 4.5-fold (HO-8910PM), 2.2-fold (OVCAR-3), and 2.5-fold (SK-OV-3), respectively. Of note, the relative transcript level of NR\_026689 was the highest in HO-8910PM cells, indicating a potential association between the expression of NR\_026689 and cell metastasis. Our data suggested a higher expression of NR\_026689 in ovarian cancer in vivo and in vitro.

#### *Knockdown of NR\_026689 Inhibited Cell Proliferation and Colony Formation in Ovarian Cancer Cells*

To elucidate the detailed role of NR\_026689 in ovarian cancer, two specific shRNAs against NR\_026689 were designed and synthesized, shortly named shNR-1 and shNR-2. When HO-8910PM cells were transfected with shRNAs, the relative transcript level of NR\_026689 was significantly decreased by 50% and 45%, respectively, in shNR-1-treated cells and shNR-2-treated cells (Fig. 2A), demonstrating the high efficiency of designed shRNAs and transfection processes. Afterward, we explored the role of NR\_026689 in cell proliferation. As shown in Figure 2B, control shRNA (shNC) caused no notable difference in cell proliferation in HO-8910PM cells throughout the time period. However, transfection of shNR-1 in HO-8910PM cells inhibited the cell proliferative rate by 12% on the fourth day and 24% on the fifth day, while the inhibitory effects caused by NR-2 were somewhat stronger on the fourth and fifth days, accounting for 14% and 26%, respectively (Fig. 2B). Furthermore, more than 130 colonies were formed in control HO-8910PM cells after a 2-week incubation, whereas only 78 colonies were observed in shNR-1-treated cells, and 75 colonies were counted in shNR-2-infected cells (Fig. 2C). These data revealed that knockdown of NR\_026689 in HO-8910PM cells suppressed cell proliferation and colony formation.

#### *Knockdown of NR\_026689 Suppressed Cell Migration and Invasion in HO-8910PM Cells*

We hypothesized the association between the high expression of NR\_026689 and cell metastasis after the observations in Figure 1B. To this end, we performed

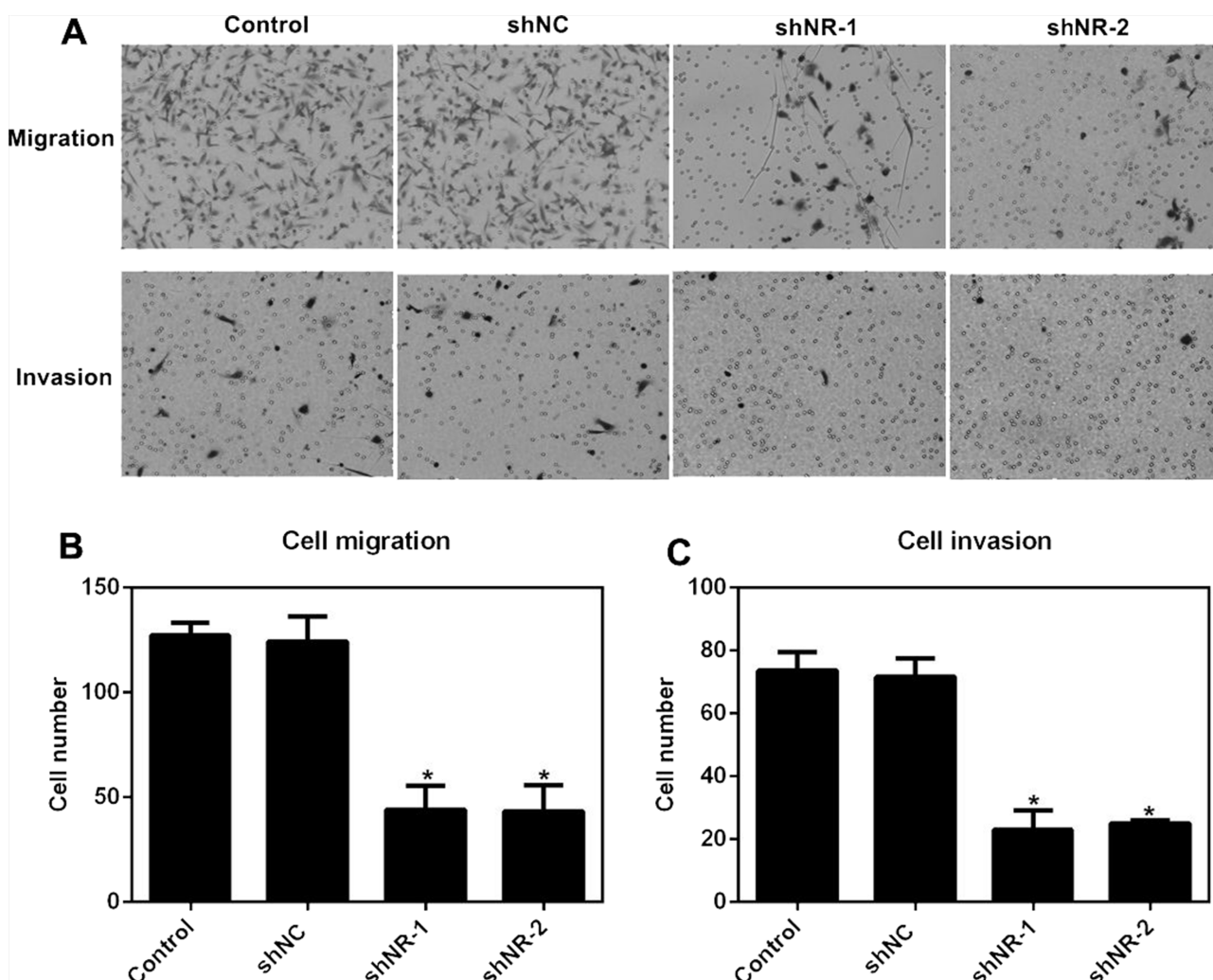
#### FACING COLUMN

**Figure 2.** Knockdown of NR\_026689 inhibited cell proliferation and colony formation in ovarian cancer cells. (A) Two specific shRNAs against NR\_026689 were designed and synthesized and transfected into HO-8910PM cells to knock down the expression of NR\_026689 for the subsequent analysis. \* $p < 0.05$  versus Control. (B) Cell proliferative rate was explored in 5 consecutive days upon the transfection of shRNAs in HO-8910PM cells. Knockdown of NR\_026689 significantly suppressed cell proliferation on the fourth day and fifth day. \* $p < 0.05$  versus Control by shNR-1. # $p < 0.05$  versus Control by shNR-2. (C) Colony formation assays were performed upon NR\_026689 depletion in HO-8910PM cells. \* $p < 0.05$  versus Control.

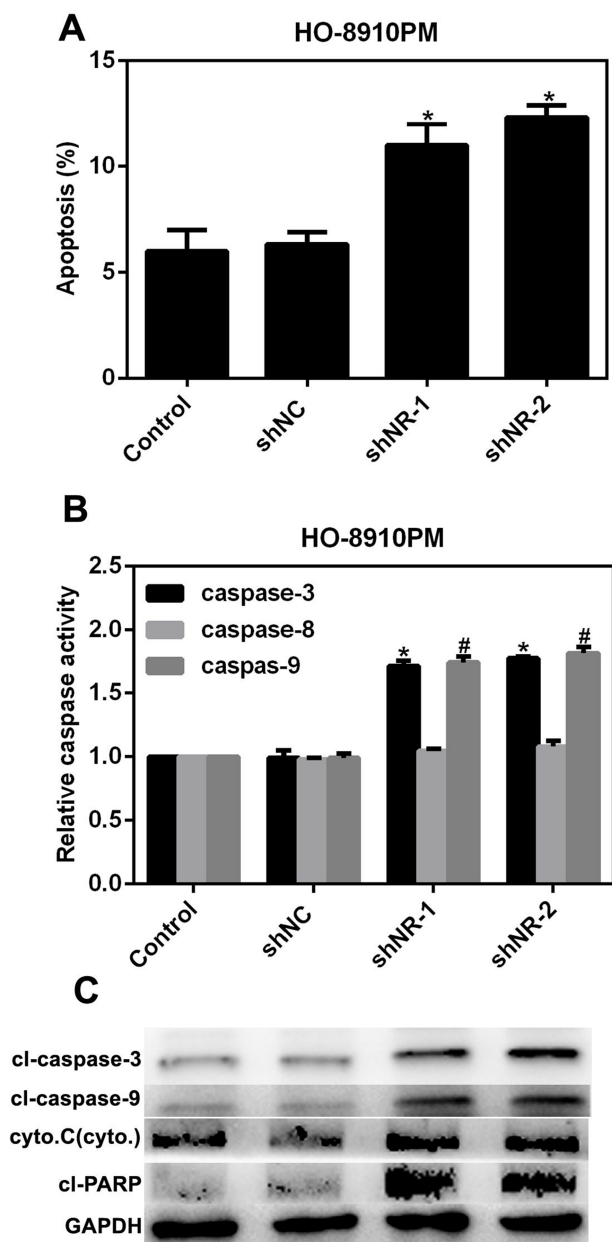
Transwell assays. Cells were transfected with shRNAs in the presence or absence of NR\_026689 knockdown. It was found that cells that migrated through the membrane and invaded to the lower surface of the chamber were remarkably decreased by either shNR-1 or shNR-2 transfection (Fig. 3A). Quantification of Transwell analysis revealed that the ability of cells to migrate was retarded by 58% by shNR-1 and 59% by shNR-2 in HO-8910PM cells (Fig. 3B). Moreover, the potential of cells to invade was suppressed by more than 70% by both shRNAs against NR\_026689 in HO-8910PM cells (Fig. 3C). These results verified our prior assumption that upregulated NR\_026689 in ovarian cancer was positively related to cell metastasis.

*NR\_026689 Depletion in HO-8910PM Cells Promoted Cell Apoptosis and the Activities of Related Caspases*

Inhibition of cell apoptosis is a good basis for the aggressive activities of cancer cells. Thus, we further explored the effects of depletion of NR\_026689 on cell apoptosis in ovarian cancer HO-8910PM cells. As shown in Figure 4A, the apoptotic rate of cells was prominently increased by 5% in shNR-1-treated HO-8910PM cells and by 6% in shNR-2-transfected cells, respectively. Next, we examined the relative activities of the key molecules in cell apoptotic signaling pathways. It was revealed that upon depletion of NR\_026689 in HO-8910PM cells, the relative activities of caspase 3 and caspase 9 were remarkably increased by 1.5-fold compared to the control.



**Figure 3.** Knockdown of NR\_026689 suppressed cell migration and invasion in HO-8910PM cells. (A) Representative images of cell migration and cell invasion assays are shown. Five random sights were photographed and quantified after cells were stained with crystal violet. (B) Quantification of cell migration assay revealed that knockdown of NR\_026689 in HO-8910PM cells inhibited cell migration abilities. (C) Quantification of cell invasion assay revealed that NR\_026689 depletion in HO-8910PM cells suppressed cell-invasive potentials. \* $p < 0.05$  versus Control.



**Figure 4.** NR\_026689 depletion in HO-8910PM cells promoted cell apoptosis and the activities of related caspases. (A) Knockdown of NR\_026689 by each shRNA against NR\_026689 consistently promoted cell apoptosis in HO-8910 PM cells. \* $p < 0.05$  versus Control. (B) The relative activities of caspase 3, caspase 8, and caspase 9 were determined in HO-8910PM cells upon the transfection of NR\_026689 shRNAs. \* $p < 0.05$  versus Control by shNR-1. # $p < 0.05$  versus Control by shNR-2. (C) Western blot analysis also revealed that the protein levels of caspase 3, caspase 9, cytochrome C, and PARP were upregulated in ovarian cancer HO-8910PM cells treated with NR\_026689 shRNAs.

However, the activity of caspase 8 remained unchanged. These data indicated that NR\_026689 might affect cell apoptosis through the intrinsic signaling pathway (Fig. 4B). Furthermore, the protein levels of caspase 3, caspase 9, cyt C, and PARP were significantly increased in shNR-treated HO-8910PM cells (Fig. 4C). These data suggested that depletion of NR\_026689 increased cell apoptosis by promoting the activities of key molecules in the intrinsic apoptotic signaling pathway.

## DISCUSSION

Since 1996, the standard regimen after surgery for ovarian cancer has been a systemic platinum/taxane-based method<sup>12</sup>. Unfortunately, most ovarian cancer patients experience recurrence and require further therapy despite high initial response rates. Thus, it is a high priority to find new clues for the diagnosis and treatment of ovarian cancer. lncRNAs have been reported to play a significant role in the tumorigenesis of many kinds of cancers, including ovarian cancer. As a result, we sought to explore the detailed role of lncRNA NR\_026689 in ovarian cancer progression.

NR\_026689 is a new lncRNA identified by Wu et al. in 2016<sup>11</sup>. However, in their article, they only elucidated that NR\_026689 was overexpressed in rat lung cancer induced by NNK, and the detailed role of NR\_026689 in tumor progression was not shown. Cell proliferation and metastasis are two main manifestations of malignant tumors and are studied widely<sup>13-15</sup>. Therefore, we explored the effects of NR\_026689 on ovarian cancer cells by cell proliferation assays, colony formation assays, and Transwell assays. Afterward, we probed the role of NR\_026689 in cell apoptosis. We found that NR\_026689 was significantly upregulated in ovarian cancer tissues and highly invasive ovarian cancer cells. Depletion of NR\_026689 inhibited cell proliferation and metastasis, and promoted cell apoptosis in HO-8910PM cells. These observations show conclusively that NR\_026689 exerts an oncogenic potential in ovarian cancer.

Cell apoptosis is a process of programmed cell death that occurs in multicellular organisms and various organs. There are two signaling pathways involved in the apoptotic process: intrinsic pathway and extrinsic pathway, depending on the apoptotic signal origin. The intrinsic pathway, namely, the mitochondrial pathway, is activated by intracellular signals generated when cells are stressed and depends on the release of proteins from the intermembrane space of the mitochondria<sup>16,17</sup>. cyt C is also released from the mitochondria in the intrinsic pathway and serves a regulatory function as it precedes morphological change associated with apoptosis<sup>18</sup>. Once cyt C is released, it can form a complex with apoptotic protease-activating factor 1 (Apaf-1) and ATP<sup>19,20</sup>, which binds to procaspase 9 to

create a new protein complex, named apoptosome. The apoptosome cleaves the procaspase to its active form of caspase 9, which in turn activates the effector caspase 3<sup>21</sup>. However, caspase 8 is a typical indicator of extrinsic pathway<sup>22</sup>. In our study, we demonstrated the upregulated expression of intrinsic pathway proteins including caspase 3, caspase 9, and cyt C and that the relative activity of caspase 8 remained stable upon NR\_026689 depletion in HO-8910PM cells, indicating that NR\_026689 might promote cell apoptosis via the intrinsic pathway.

Our study is the first that elucidated the detailed role of the newly identified lncRNA NR\_026689 in ovarian cancer. Knockdown of NR\_026689 in ovarian cancer cells inhibited cell proliferation and metastasis and also promoted cell apoptosis via the intrinsic pathway. This oncogenic potential of NR\_026689 might pave the way for novel clues to the diagnosis and treatment of ovarian cancer in the clinic.

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