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ORIGINAL ARTICLE

Knockdown of mutated ARID1A inhibited endometrial cancer cell proliferation and stimulated cell apoptosis

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Summary

Purpose: *Mutations in the gene encoding the AT-rich interacting domain containing protein 1A (ARID1A) are frequently observed in endometrial cancer, although the molecular mechanisms linking the genetic changes remain poorly understood. This study aimed to elucidate the influence of ARID1A mutations on endometrial cancer cells and tissues.*

Methods: *Reverse transcription quantitative polymerase chain reaction (RT-qPCR), western blot and immunohistochemistry (IHC) were used to investigate the expression of ARID1A in endometrial cancer cells and tissues. Lentiviral vector (LV)-shARID1A was constructed and transfected into HEC-1-A cells. The efficiency of mutated ARID1A knockdown was determined by RT-qPCR and western blotting. The proliferation and apoptosis capacity was examined by colony formation, MTT proliferation, Annexin V-APC cell apoptosis and cell cycle analysis.*

Results: *ARID1A was lower in endometrial cancer cells and endometrial carcinoma tissue than in normal endometrial cells and benign endometrium ($p<0.05$). Small interfering RNA (siRNA)-ARID1A were transfected into HEC-1-A cells and we observed reduced cell growth and proliferation, increased apoptosis, and a significantly increased proportion of cells in the G2/M phase.*

Conclusion: *Our results suggest that ARID1A mutation in endometrial cancer helped cell proliferation and inhibited cell apoptosis and also caused cell cycle arrest at the G2/M phase.*

Key words: *apoptosis, ARID1A, cell cycle, endometrial cancer, proliferation*

Introduction

Endometrial carcinoma is the most common invasive gynecologic carcinoma in developed countries worldwide. Although overall prognosis of endometrial cancer is relatively good, some patients present with recurrent disease shortly after the completion of therapy (surgery and radiotherapy) Thus, it is urgent to develop novel therapeutic agents to effectively treat this disease.

ARID1A (AT-rich interactive domain 1A; also known as BAF250) is located at Ch1p36.11, a region frequently deleted in human cancers. The relative molecular weight of ARID1A is 250 kD and encodes a nuclear protein involved in chromatin remodeling. The ARID1A gene is approximately 86 kb in length, including 32 exons, and the cDNA length is 8 kb. It is post-translationally modified and potentially regulating protein expression or protein-protein interactions [1].

As a member of SWI/SNF (mating type switching/sucrose non-fermenting) complexes, ARID1A is thought to contribute to specific recruitment of its chromatin remodeling activity by binding transcription factors and transcriptional complexes [2]. It has been hypothesized that ARID1A could play a significant role as a tumor suppressor to regulate cellular apoptosis and proliferation. ARID1A

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expression is induced in various types of cancer compared with healthy tissues, and its expression decreases with malignancy [3,4]. Recent genome-sequencing efforts support a strong genetic contribution to uterine carcinoma, including uterine clear-cell and endometrioid carcinomas, and uterine carcinosarcoma, based on the discovery of high-frequency (up to 41.6%) ARID1A mutations [5-7]. Mutations in the ARID1A gene usually lead to a loss of expression of the ARID1A encoded protein. ARID1A mutation may have many effects on SWI/SNF complexes that lead to transcriptional dysfunction, including disruption of nucleosome sliding activity, assembly of variant SWI/ SNF complexes, and targeting to specific genomic loci, and which may be further correlated with tumor progression in endometrial carcinomas [4]. Silencing mutated ARID1A may represent an approach to the prevention or treatment of malignant tumors.

However, previous studies have primarily provided insights into the function of wild-type ARID1A and focused on loss of ARID1A expression by immunohistochemistry or gene sequencing of mutations in endometrial cancer; to date, a comprehensive view of the cell function of ARID1A mutations in endometrial cancer pathophysiology and the mechanism by which its inactivation promotes tumorigenesis has not been achieved. Therefore, in our study, we sought to elucidate mechanistic details.

Methods

Tissue samples and cell culture

40 endometrial carcinoma tissues and 10 benign endometrial tissues were collected from the Department of Obstetrics and Gynecology, Shanghai General Hospital of Shanghai Jiao Tong University University (Shanghai, China). Use of these specimens was approved by the Ethics Committee of the Medical College, Shanghai Jiao Tong University, China. Human endometrial cancer cell lines (RL-95-2, HEC-1-A and Ishikawa) were purchased from Shanghai Genechem Co. Ltd. The cells were cultured with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. For transfections, cells were seeded in a 6-well plate 24 hrs before the experiment.

Real-time quantitative PCR (RT-qPCR)

To measure the abundance of both mutated and wild-type ARID1A mRNA, primers were chosen at codon 5022-5181 as described below and tested with different primer concentrations. Total RNA was extracted from endometrial cancer cells using the Trizol method. A reverse transcription kit was used to reverse transcribe the RNA into cDNA. RT-qPCR was performed using with the following gene-specific primers: ARID1A (F) 5-GTCCCTCAAGTCTGGTCTCC-3, ARID1A (R) 5-GATCTCAATCAGGCATCGTC-3. The relative gene expression levels were calculated using the $2^{-\Delta\Delta ct}$ method and normalized to the expression of GAPDH.

Western blot assays

The total protein was extracted from the purified or cultured cells in RIPA lysis buffer and washed twice with PBS. The primary antibodies used included rabbit anti-ARID1A (1:500, Abcam, San Francisco, USA) for 30 min. Synthetic peptide of rabbit anti-ARID1A corresponded to ARID1A aa 2199-2213 (C terminal). The data were automatically saved and normalized to β-actin expression by densitometry. Western blot automated quantitative analysis system Compass software (WES) was used for analyzing the grade test results. Quantitative results including molecular weight, signal intensity (area), % area, and signal-to-noise ratio for each immunodetected protein were obtained.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumor tissues were collected from 40 endometrial carcinoma tissues and 10 benign endometrial tissues. All benign endometrium specimens were at the secretory phase. Two independent investigators examined randomly all tumor slides. Paraffin-embedded tissue sections (5 μm) were deparaffinized by xylene and rehydrated in a graded alcohol series (100, 95, 80 and 70%, 5 min each). Then, sections were subjected to antigen retrieval by boiling in citrate buffer (pH 6.0), incubated for 30 min with 0.01% Trixon, and then incubated for 20 min with 5% bovine serum albumin. The sections were incubated overnight with rabbit anti-human ARID1A (1:200, Abcam, San Francisco, USA) primary antibodies at 4°C in a humid chamber, followed by 50-min incubation with biotinylated secondary antibody. Only the nuclear staining was considered positive and therefore scored for ARID1A (BAF250a) protein. The immunohistochemical staining was scored for both the percentage of positive cells (0=0%; 1=≤10%; 2=11–50%; 3=51–80% and 4≥80%) and staining intensity (0=negative; 1=weak; 2=moderate; and 3=strong), as previously described. Complete loss and weak expression of ARID1A was defined as negative expression, while moderate and strong expression of ARID1A was defined as positive expression.

Table 1. The sequences of synthesized oligonucleotides

NO.	*5'*	*STEM*	*Loop*	*STEM*	*3'*
ARID1A-RNAi(24485-1)-a	Ccgg	ccGTTGATGAACTCATTGGTT	CTCGAG	AACCAATGAGTTCATCAACGG	TTTTTg
ARID1A-RNAi(24485-1)-b	Aattcaaaaa	ccGTTGATGAACTCATTGGTT	CTCGAG	AACCAATGAGTTCATCAACGG	

Lentiviral vector production

The shRNA sequences were designed on codon 6795-6813 using the on-line siRNA software. The short hairpin RNA (shRNA) expression cassettes were designed according to the siRNA sequences, and the synthesized sequences are listed in Table 1. The shRNA vectors were generated by inserting annealed oligo sequences into the digested GV248 vectors (GeneChem Co., Ltd, Shanghai, China) between the Phu6 and Pubi sites. HEC-1-A cells were transfected with lentiviral vector GV248 (mock) or ARID1A-specific shRNA lentiviral particles in 6-well plates in the presence of polybrene (6 mg/ml) and then treated with puromycin (2 mg/ml) to generate stable clones, and an empty vector was used as control. ARID1A gene expression and protein levels were confirmed by qRT-PCR.

Colony formation assay

Forty-eight hrs after transfection, shRNA ARID1A and control cells were plated into 6-cm cell culture dishes (400-1,000 cells per dish) and incubated for 14 days. Cells were then stained with Giemsa, and the number of colonies with more than 50 cells was counted. The experiments were performed in triplicate. Colonies were manually counted, and the experiment was repeated in triplicate.

MTT proliferation assay

Cell proliferation was monitored with MTT assay. Twenty-four hrs after transfection, cells were plated in 96-well plates in medium containing 10% FBS at approximately 2,000 cells per well. For quantitation of cell viability, 20 µl of 5 mg/ml MTT (3–4,5-dimethylthiazol-2-yl-2,5- diphenyltetrazolium bromide) (Genview, USA) solution was added to each well and incubated for 4 hrs at 37°C. The medium was removed from each well, and the resulting MTT formazan was solubilized in 100 µl DMSO. Each solution was measured spectrophotometrically at 490 nm. Each experiment was performed in triplicate.

Cell apoptosis detection

Cell apoptosis detection was performed with Annexin V-APC Single staining. Briefly, 48 hrs after transfection, cells were harvested by 0.25% trypsin and washed twice with chilled PBS, followed by resuspension in 250 µl binding buffer. Staining solution containing Annexin V-APC and propidium iodide (PI) was added to the cell suspension. After incubation in the dark for 10-15 min, the cells were analyzed using a FACS Calibur flow cytometer (Olympus, Japan).

Cell cycle analysis

For cell cycle analysis, cells were seeded in 6-well plates, serum-starved for 24 hrs and then incubated for 24 hrs. Cells were then centrifuged and washed twice in cold PBS. The resulting cell pellet was stained with PI solution (PI 2 mg/ml and RNase 10 mg/ml in PBS) for 20 min at 4°C. The cell cycle was analyzed using flow cytometry and FACSDiva software. The experiment was repeated three times.

Statistics

Statistical analyses were performed with SPSS 22.0 (SPSS, Chicago, ILL, USA). Comparisons between two groups were performed by Student's t-test, one-way ANOVA test and non-parametric Mann-Whitney U-test. A two-sided p-value less than 0.05 or 0.01 was considered statistically significant. The data are presented as mean±standard deviation.

Results

The expression of ARID1A in endometrial carcinoma cells and tissues

To analyze ARID1A gene transcript levels, RT-qPCR was performed in three endometrial cancer cell lines and compared with normal endometrial cells cultured from non-transformed primary uterine epithelial cells. The relative expression levels of ARID1A mRNA in RL95-2 (p=0.02), HEC-1-A (p=0.018) and Ishikawa (p=0.005) cells were significantly lower than the levels in normal endometrial cells (Figure 1A). We confirmed that ARID1A was reduced in HEC-1-A cells compared with normal endometrial cells by Western blot automated quantitative analysis system Compass software (WES) (Figures 1B,C). Moreover, ARID1A in middle-grade and high-grade endometrial endometrioid carcinoma (EEC) tissues was lower than in matched benign endometrium (Figures 1B,D). We then detected ARID1A expression using immunohistochemistry in 40 EEC specimens and 10 corresponding benign endometrial tissues. Complete loss and weak expression of ARID1A was defined as negative expression, while moderate and strong expression of ARID1A was defined as positive expression. Negative expression of ARID1A was observed in 77.5% of endometrial carcinoma cases (31/40), and 22.5% (n=9) showed positive expression. Among the 10 benign endometrial specimens, 30% (3/10) showed negative expression of ARID1A and 70% (7/10) showed positive expression (Figure 1E). A significant difference in ARID1A staining was found between EEC cases and benign endometrial cases (p=0.007) (Figure 1F). The protein levels of ARID1A were consistent with the mRNA levels.

Silencing of the mutated ARID1A gene decreased proliferation in endometrial carcinoma cells

To investigate the role of mutated ARID1A in the biological behavior of endometrial carcinoma, shRNA targeting mutated ARID1A(LV-ARID1A-RNAi-24485-1) as KD group, one negative control pair (NC), and one blank control (MOCK: empty

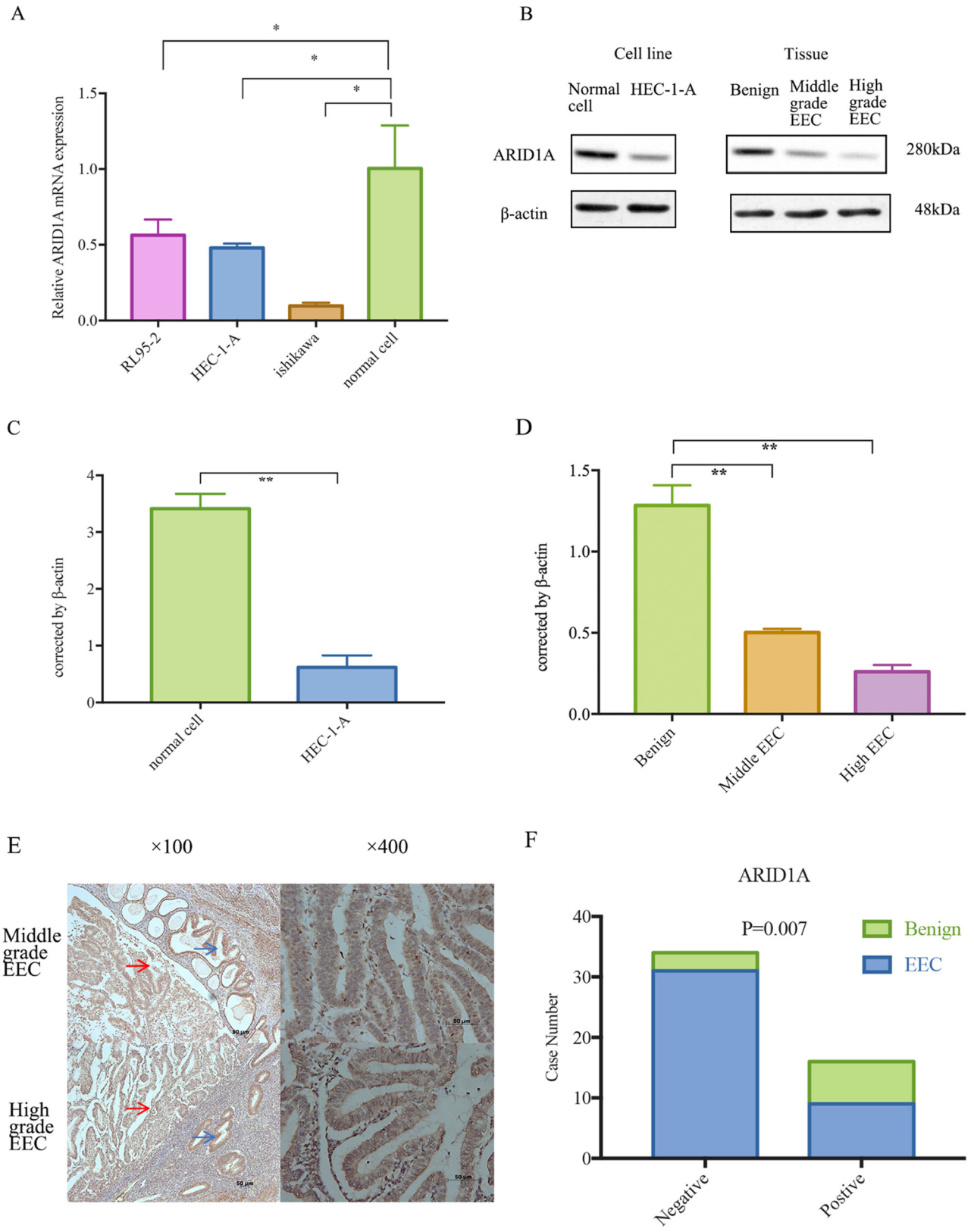

Figure 1. A: ARID1A mRNA in three endometrial carcinoma (EC) cell lines were significantly lower compared with normal endometrial by RT-qPCR($p<0.05$). **B:** ARID1A in HEC-1-A cells **(C)** and EEC tissues **(D)** was lower than that in normal endometrial cells and benign endometrium tissues by Western blot (WES), and quantified by peak area of triplicate experiments. β-actin was included as an internal control. **E:** EEC cases with coexisting moderate-grade and normal components; red arrows show weak ARID1A staining of adenocarcinoma cells as compared with blue arrows showing moderate ARID1A staining in normal glandular cells by immunohistochemistry. **F:** Siginificant difference in staining was found between EEC cases and benign endometrial cases ($p<0.05$). Data are presented as mean ± SD.*$p<0.05$, ** $p<0.01$.

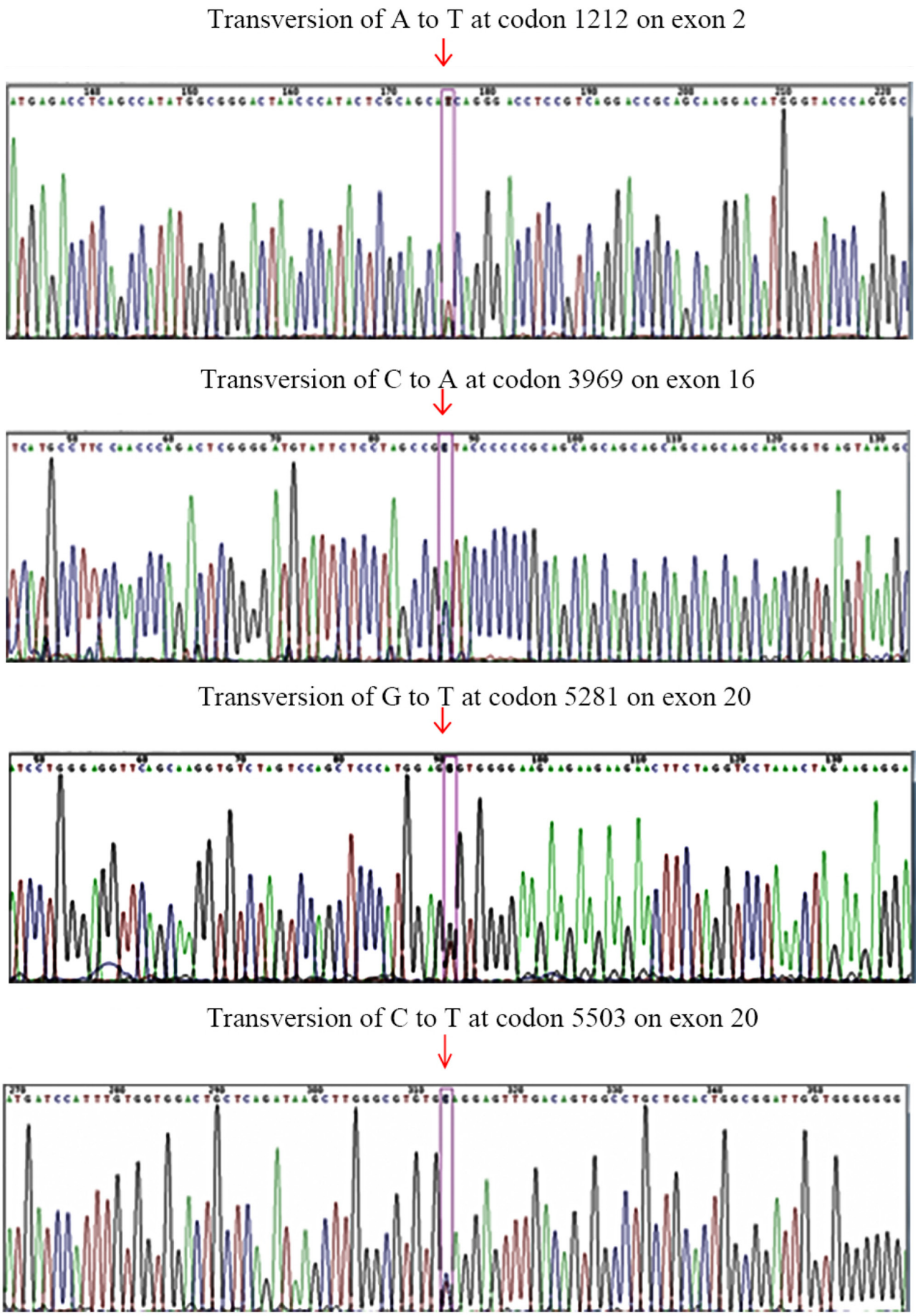

Figure S1. ARID1A mutation in HEC-1-A cell line verified by Sanger sequencing.

vector-transfected cells) were designed and cloned into HEC-1-A cell line. As the shRNA-ARID1A sequences were designed on codon 6795-6813, the shRNA-ARID1A did not fall on the mutation position, as described in Figure S1, therefore mutated ARID1A could be depleted by shARID1A knockdown plasmids in the KD group. ARID1A mRNA level was reduced by 78.8% when infected with ARID1A shRNA lentivirus (RNAi), compared with the control group (p=0.0042) as detected by RT-qPCR (Figure 2A). Western blot showed that ARID1A protein was decreased in the KD group compared to the NC group (Figure 2B).

Next, colony formation assays were performed to investigate the effect of mutated ARID1A on endometrial carcinoma cell proliferation. Upon mutated ARID1A knockdown, the colony formation ability of the shARID1A-HEC-1-A cells was significantly decreased compared with controls (p=0.00009) (Figures 2B,C).

Then, the proliferative activity of the three groups (MOCK, NC and KD) after transfection (24, 48, 72, 96 and 120 hrs) was assessed by MTT assay. Each solution was measured spectrophotometrically at 490 nm. Mutated ARID1A knockdown in HEC-1-A cells induced decreased proliferation compared to control cells in a time-dependent manner at 72 hrs (p=8.95E-06) and 120 hrs (p=1.29E-06; Figure 2D). OD490/fold indicates the OD490-fold of day 1 to day 5 relative to the value of day 1, representing the fold increase on each day. Similar trends were observed spectrophotometrically by the OD490/fold between the KD and control group (p<0.01; Figure 2E).

Silencing of the mutated ARID1A gene promoted apoptosis and caused cell cycle arrest at the G2/M phase in endometrial carcinoma cells

Considering that silencing of mutated ARID1A significantly slowed cell proliferation, we further investigated apoptosis in the shARID1A-HEC-1-A cell. Downregulation of mutated ARID1A in HEC-1-A cells increased the rate of cell apoptosis compared with controls after 120 hrs (p=1.46E-08; Figures 3A-C), suggesting enhanced cell death in mutated ARID1A knockdown cells.

Furthermore, we performed a cell cycle analysis to determine whether mutated ARID1A knockdown inhibits cell growth via alteration of the cell cycle. Reduction of mutated ARID1A in the HEC-1-A cell line altered the cell cycle by promoting the G0/G1 to G2/M phase transition. Knockdown of mutated ARID1A in the HEC-1-A cell line significantly reduced the percentage of cells in the G1 phase (p=9.23E-05) and increased the percentage of cells in the G2/M phase (p=0.00012) compared with that in the parental and mock controls. However, there was no significant difference in the percentage of cells in the S phase (p=0.789475), suggesting that depletion of mutated ARID1A caused cell cycle arrest at the G2/M phase in endometrial carcinoma cells and induced cell apoptosis (Figures 3D,E).

Discussion

It is now widely accepted that proliferation and apoptosis are multistep processes involving complex and highly coordinated interactions between tumor cells. ARID1A could regulate protein expression and contribute to specific recruitment of SWI/SNF complexes, and chromatin remodeling activity by binding transcription factors and transcriptional coactivator complexes [1]. The SWI/SNF complex is involved in the induction of cellular senescence and apoptosis, as well as in oncogenesis, and is required for transcriptional activation or repression of genes by chromatin-remodeling [8]. Studies have suggested roles for ARID1A in processes relevant to tumor proliferation, differentiation and apoptosis. Restoration of wild-type ARID1A expression in one clear cell carcinoma-derived cell line with bi-allelic ARID1A inactivation suppressed the growth of the cells *in vitro* [9]. Guan reported that restoring wild-type ARID1A expression in ovarian cancer cells that harbor ARID1A mutations was sufficient to suppress cell proliferation and tumor growth in mice. RNAi-mediated silencing of wild-type ARID1A expression in non-transformed epithelial cells was sufficient to enhance cellular proliferation and tumorigenicity [10-12].

However, all these studies only focused on knockdown of wild-type ARID1A in an ARID1A mutation cell line. Wild-type ARID1A is a tumor suppressor gene, while ARID1A mutation could cause loss of its expression and loss of the tumor suppressing function. Collectively, there are two types of ARID1A mutations. The first is the mutations around the nuclear export signal sequence resulting in reduced nuclear export of ARID1A, and the second is the mutations affecting interactions between ARID1A and the other SWI/SNF subunits disturbing the stability of the whole protein complex [13]. Again, mutations affecting ARID1A expression may occur in non-coding regions of the genome not assayed by Exon sequencing techniques and epigenetic silencing might affect the whole complex. Jones has reported that both wild-type and mutated alleles in 30% of the ovarian clear cell carcinomas with ARID1A mutations

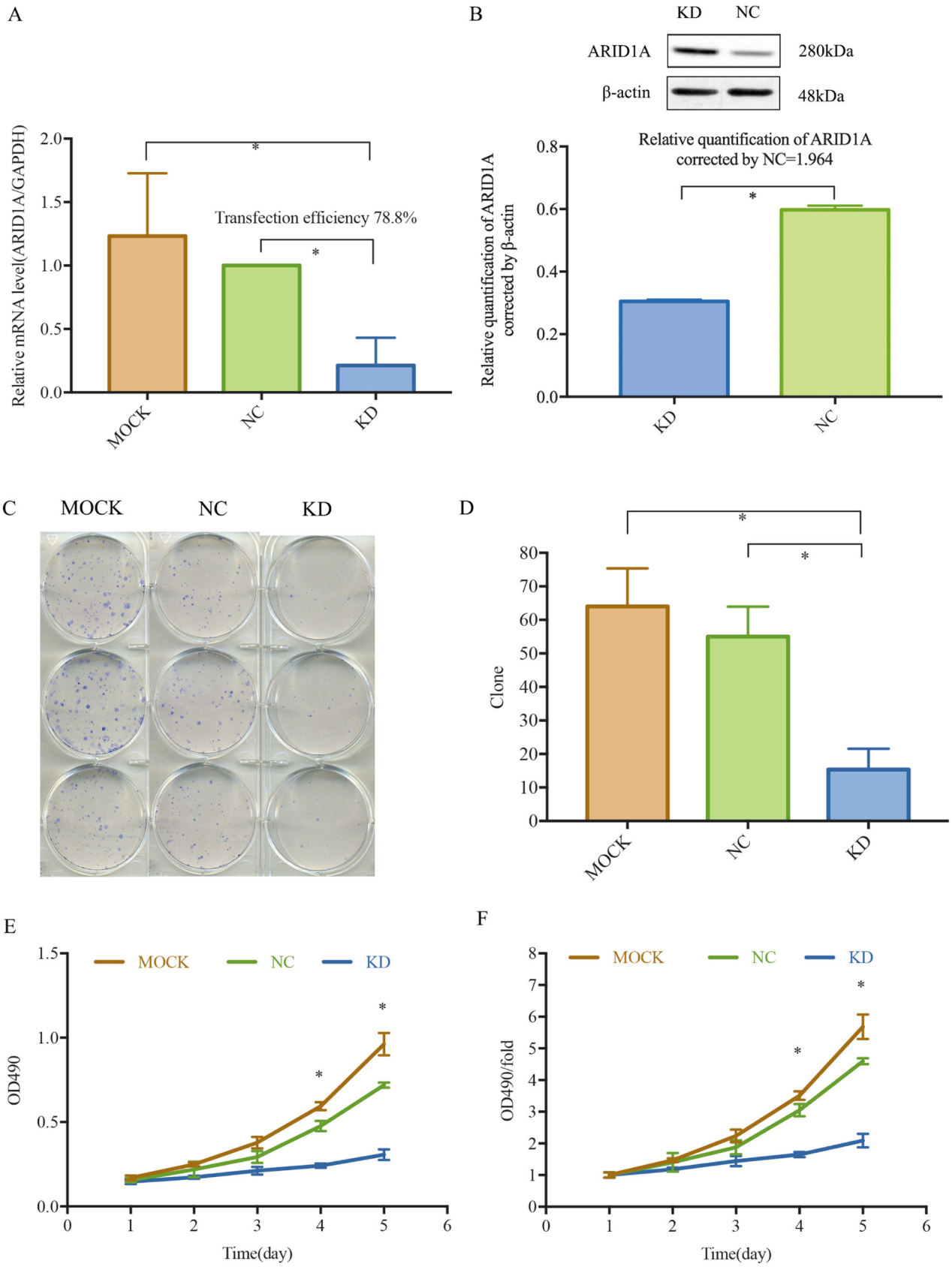

Figure 2. A: Knockdown effect (78.8%) of lentiviral shRNA vectors in HEC-1-A cell line was examined by RT-qPCR. **B:** Western blot showed that ARID1A protein was decreased in the KD group compared to the NC group. **C:** Figure of colony formation in KD, MOCK and NC group (magnification, ×10). **D:** A marked decrease in colony formation was seen in ARID1A depletion group. **E,F:** Note a time dependent decrease of proliferation in ARID1A- depleted HEC-1-A cells at 72 h and 120 h measured spectrophotometrically at 490 nm **(E)** and OD490/fold **(F)** values. Data are presented as mean ± SD. *p<0.01

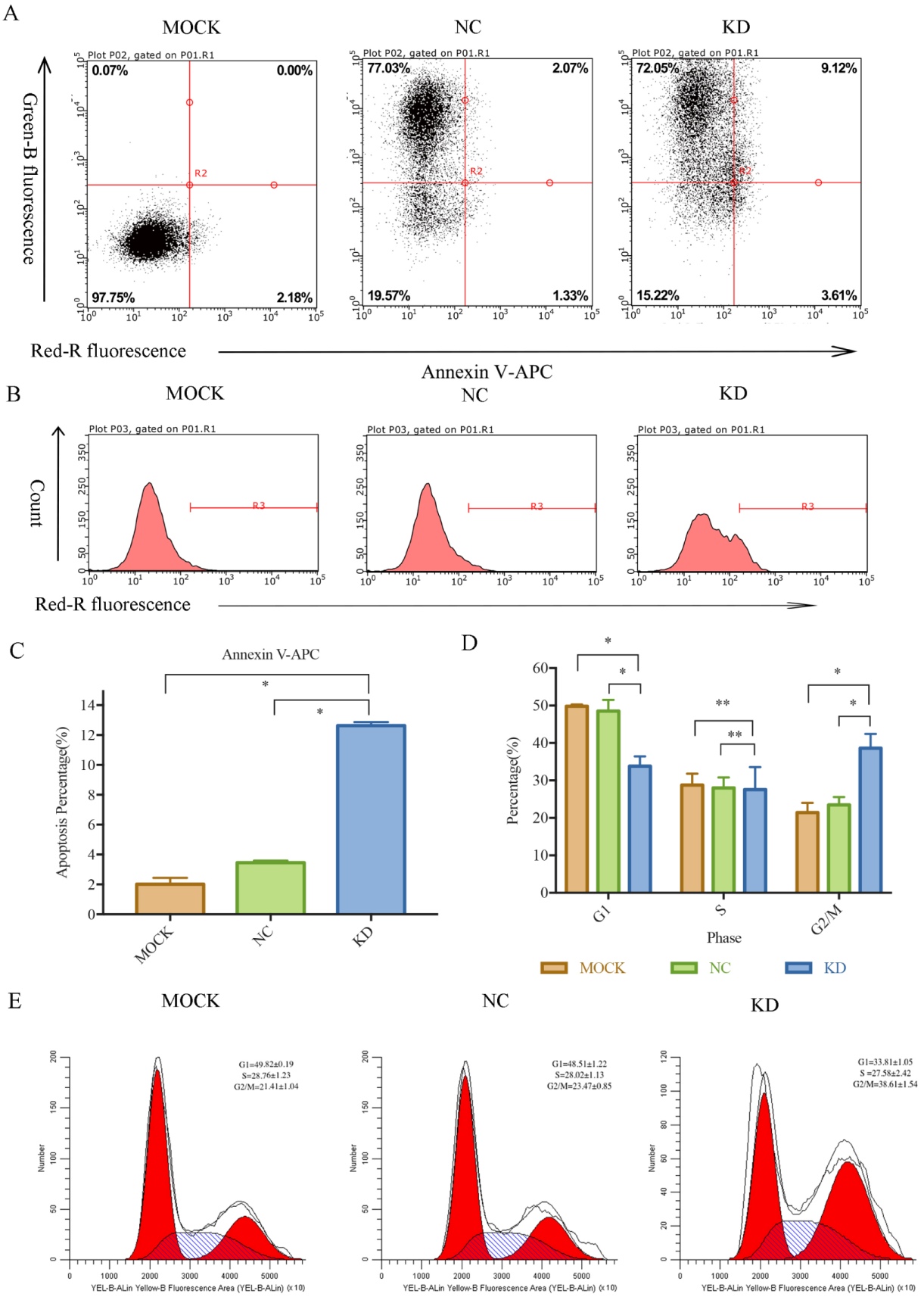

Figure 3. A: The upper left quadrant represented the cells infected by Green-B fluorescence. The right quadrant represented apoptotic cells. **B:** X-axis represented cell infected by Red-R fluorescence. **C:** The rate of apoptosis was markedly increased in ARID1A- depleted HEC-1-A cells in comparison to the control cells. **D,E:** Compared with MOCK and NC group after a day, knock-down of ARID1A in HEC-1-A cell line significantly increased the percentage of cell number in the G2-M phase, reduced in the G1 phase ($p<0.01$). Data are presented as mean ± SD. *$p<0.01$, **$p>0.05$.

had both alleles affected, thus postulated that post-transcriptional mechanisms account for loss of ARID1A protein in ovarian clear cell carcinomas harboring heterozygous mutations without loss of heterozygosity [14,15]. By immunohistochemistry, 27% of the ARID1A heterozygous ovarian clear cell carcinomas retain detectable protein expression, as did 5% of tumors not found to have coding mutations [14]. Again, 25% of gastric cancer harboring heterozygous mutations retained ARID1A expression by immunohistochemistry [16].

However, different mutation patterns among cancer subtypes imply tissue-specific mutational effects, which may limit the extent to which observations in one cancer type or cell line may be applied to other model systems. Therefore, we sought to elucidate mechanistic details of ARID1A mutations on type I endometrial cancer pathophysiology and tumorigenesis by performing cell function studies. We selected the HEC-1-A cell line to exclude the effects of PTEN mutation and of estrogen receptors (ER) in this cell line, as this cell line expresses negative or low ER and carries wild-type PTEN [17]. The vast majority of cancer-associated mutations in ARID1A (>97%) are inactivating, with nonsense or frameshift rather than silent or missense mutations detected throughout the gene [15]. In our study, missense, synonymous and nonsense heterozygous mutations of ARID1A in HEC-1-A cell line were verified by sanger sequencing as described in Figure S1. Missense mutation could make the polypeptide chain lose its original function. Nonsense mutation could inactivate the protein of the mutant gene and affect the expression of the other gene adjacent to the mutant gene, thus resulting in reducing the expression of the neighboring gene. Only synonymous mutation does not affect the structure and function of the protein it translates. As ARID1A is post-translationally modified, mutation on ARID1A gene resulted in decrease or loss of protein expression, therefore we observed that ARID1A was lower in endometrial carcinoma cells and endometrial carcinoma tissue than in normal endometrial cells and benign endometrium ($p<0.05$). ARID1A is recurrently and specifically mutated on one allele but expressed from the other allele, and this has raised the possibility that reduced levels of ARID1A may mediate a haploinsufficient effect in cancer. We used shARID1A knockdown plasmids to transfect the HEC-1-A cell line to deplete mutated ARID1A, confirming thus that silencing ARID1A gene in an ARID1A mutation cell line induced decreased proliferation and increased apoptosis, in contrast to knockdown of wild-type ARID1A in ARID1A mutation cell line described before, since ARID1A mutation caused loss of the tumor suppressive function and may have many effects on SWI/SNF complexes that lead to transcriptional dysfunction and are correlated with tumor progression in endometrial carcinoma [18]. Thus, downregulation of mutated ARID1A might influence the chromatin-remodeling processes, resulting in altered transcription and DNA synthesis rates. ARID1A expression varies during the cell cycle, being highest during G0/G1 and significantly diminished in S and G2/M phases [19]. Knockdown of ARID1A inhibited cell cycle arrest in murine preosteoblasts [20,21]. The G2-M DNA damage checkpoint is an important cell cycle checkpoint which ensures that cells won't initiate mitosis before they have a chance to repair damaged DNA after replication. Cells that have a defective G2-M checkpoint enter mitosis before repairing their DNA, leading to death after cell division [22]. In our study, we observed a blockage that occurred at the G2/M phase ($p<0.01$) in ARID1A-depleted HEC-1-A cell line, which suggested that ARID1A mutation altered the cell cycle by inducing G0/G1 to G2/M phase transition, and thus resulting in induction of apoptosis.

Conclusions

In summary, our observations demonstrated for the first time that ARID1A mutations may exert anti-apoptotic effects, cause acceleration of the cell cycle transition and cell transformation, and stimulate or be a consequence of endometrial carcinoma progression, thus inducing tumor cell proliferation and invasion in endometrial carcinoma development. These molecular insights provide us with an opportunity not only to understand its carcinogenesis more clearly, but also to validate new therapeutic targets to prevent endometrial cancer progression.

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Conflict of interests

The authors declare no conflict of interests.

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