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CRISPR/Cas9-Mediated Gene Knockout of ARID1A Promotes Primary Progesterone Resistance by Downregulating Progesterone Receptor B in Endometrial Cancer Cells

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Medroxyprogesterone (MPA) is used for the conservative treatment of endometrial cancer. Unfortunately, progesterone resistance seriously affects its therapeutic effect. The purpose of the current study was to investigate the influence of deletion of AT-rich interactive domain 1A (ARID1A) in progesterone resistance in Ishikawa cells. Ablation of ARID1A was conducted through the CRISPR/Cas9 technology. Acquired progesterone-resistant Ishikawa (Ishikawa-PR) cells were generated by chronic exposure of Ishikawa cells to MPA. The sensitivity of the parental Ishikawa, Ishikawa-PR, and ARID1A-deficient cells to MPA and/or LY294002 was determined using the Cell Counting Kit-8 (CCK-8) assay and flow cytometry analysis. In addition, Western blot analysis and reverse transcription-polymerase chain reaction was performed to evaluate the mRNA and protein expression levels of ARID1A, progesterone receptor B (PRB), and P-AKT. Both Ishikawa-PR and ARID1A knockout cells showed insensitivity to MPA, downregulation of PRB, and hyperphosphorylation of AKT compared to the parental Ishikawa cells. Pretreatment with LY294002 significantly enhanced the ability of MPA to suppress proliferation and to induce apoptosis in the parental and Ishikawa-PR cells via the inhibition of AKT activation and upregulation of PRB transcriptional activity. However, the PRB transcriptional activity and insensitivity to MPA were irreversible by LY294002 in ARID1A-deficient cells. Ablation of ARID1A is associated with low PRB expression, which serves an important role in primary progesterone resistance. Akt inhibition cannot rescue PRB or sensitize to MPA in ARID1A knockout cells. These findings suggest that ARID1A may act as a reliable biomarker to predict the response for the combination of AKT inhibitor and MPA treatment.

Key words: Endometrial cancer; Progesterone resistance; AT-rich interactive domain 1A (ARID1A); Progesterone receptor B (PRB); PI3K/AKT pathway

INTRODUCTION

Endometrial cancer (EC) is one of the most common gynecologic malignancies worldwide, and approximately 80% of cases are endometrioid adenocarcinoma (type I endometrial cancer)[1]. Type I endometrial carcinomas are related to chronic estrogen exposure without progesterone antagonism. Surgery is considered the usual treatment for type I endometrial carcinomas. However, progesterone-based pharmacotherapy is commonly prescribed to reproductive age patients as a conservative endocrine treatment[2,3]. Currently, approximately 30% of endometrioid adenocarcinomas are resistant to progesterone treatment[4,5]. It is clear that improvements are needed in the treatment of progesterone.

Progesterone mediates its inhibitory effects primarily by binding to the reflection element (PRE) on the intronuclear progesterone receptor (PR) and initiating transcription. In addition, progesterone can bind to the PR on the cell membrane, thereby activating the phosphoinositide 3 kinase/protein kinase B (PI3K/AKT) signaling pathway to exert nontranscriptional effects[6–8]. PR has two main isoforms, PRA and PRB. Data show that PRB may be the predominant isoform responsible for the antitumor effect of progesterone in the endometrium. Inadequate PRB expression and abnormal regulation of

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signaling pathways are closely related to the effect of progesterone treatment[9,10]. Recent progress in restoring PRB function and activity has raised extensive concerns, including the application of new sensitizing drugs for targeted agents.

Endometrial cancer displays a variety of gene mutations, which may serve as new therapeutic targets or as marker molecules for targeted therapy[11,12]. AT-rich interactive domain 1A (ARID1A), which is one of the members of Switch/Sucrose nonfermentable (SWI/SNF) chromatin remodeling family, is frequently mutated in endometrial hyperplasias and endometrial cancers (26%–40%)[13–15]. Depletion of ARID1A significantly activates the PI3K/AKT signaling pathway, and inappropriately elevated expression of AKT phosphorylation is related to downregulation of PRB expression[16,17]. However, the relationship among ARID1A, PRB expression, and the PI3K/AKT signaling pathway remains unclear.

Most studies in the field have only focused on acquired progesterone resistance. This research is aiming to fill the gap of primary drug resistance. In this study, we knocked out the ARID1A gene using CRISPR/Cas9 genome editing technology to establish an ARID1A-deficient Ishikawa cell line and investigated the effect of ARID1A deficiency on the regulation of PRB; furthermore, we explored the possible underlying mechanisms. In addition, progesterone-resistant Ishikawa cell lines (Ishikawa-PR) were generated by long-term exposure to medroxyprogesterone (MPA), and the potential role of ARID1A in progesterone resistance was examined. We hypothesized that ARID1A could act as a potential molecular marker method for conservative treatment of endometrial carcinoma in the future.

MATERIALS AND METHODS

Cell Culture

The progesterone receptor-positive (PGR^+) endometrial cancer cell line Ishikawa was obtained from Enzyme Research Biotechnology Co., LTD. (Shanghai, P.R. China). These cells were maintained in DMEM/high glucose (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA) at 37°C in a 5% CO_2 humidified incubator.

Reagents and Antibodies

Medroxyprogesterone acetate (MPA), LY294002 (LY), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-AKT Ser473 (P-AKT, rabbit monoclonal) and PRB (rabbit polyclonal) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against ARID1A (rabbit polyclonal) was obtained from Abcam (Cambridge, MA, USA). Antibody against actin was purchased from Beyotime Biotechnology (Shanghai, P.R. China). IRDye® 800CW goat anti-rabbit IgG (H + L) and IRDye® 680CW goat anti-mouse IgG (H + L; LI-COR Biosciences, Lincoln, NE, USA) were used as secondary antibodies.

Generation of the ARID1A Deletion Using the CRISPR/Cas9 System

The CRISPR single-guide RNA (sgRNA) was designed using the CRISPR design tool (CRISPR Design, http://crispr.mit.edu) as follows: 5′-CACCGAAAGCG AGGGCCCCGCCGT-3′ (forward) and 5′-AAACAC GGCGGGGCCCTCGCTTTC-3′ (reverse). The two complementary oligos were denatured at 95°C for 5 min, and then cooled to 25°C over a period of 45 min to allow for annealing. The complementary oligonucleotides were cloned into the *Bbs*I-linearized pSpCas9(BB)-2A-Puro (PX459 version 2.0; (#62988; Addgene, Cambridge, MA, USA). Parental Ishikawa cells were plated in six-well plates and transfected with the pX459/sgRNA-expressing plasmid (2 μg for each) using Fugene 6, according to the manufacturer's instructions. The cells were placed in medium containing puromycin (1 μg/ml) 24 h after transfection. Forty-eight hours later, the culture medium was replaced with a new one containing puromycin (2 μg/ml). Four days after transfection, the cells were digested to extract DNA (18 μl direct PCR, 2 μl proteinase K), and the DNA was amplified by PCR using the following primers: 5′-GGGGGAGAAGACGAAGACAG-3′ (forward) and 5′-CTGGACTTCTTGAGCTTGCC-3′ (reverse). The PCR amplification products were collected for sequencing.

Induction of Progesterone-Resistant Endometrial Cancer Cell Lines

A progesterone-resistant Ishikawa cell line (Ishikawa-PR cells) was obtained from parental Ishikawa cells via continuous exposure to increasing amounts of MPA dissolved in DMSO[18]. Under these conditions, the proliferation of cells was the same as parental Ishikawa cells, indicating an acquisition of resistance to the growth inhibitory effect of MPA. Monoclonal-resistant cell lines were obtained from a pool of resistant cells by serial dilutions. The Cell Counting Kit-8 (CCK-8) assays (Dojindo, Kumamoto, Japan) were used to verify the establishment of the Ishikawa-PR cell line. Parental and Ishikawa-PR cells were seeded in a 96-well plate at a density of 3,000 cells/well and cultured for 1–7 days. CCK-8 assays were added to each well every 24 h, and the cells were cultured for an additional 1 h. The optical density (OD) value for each well was read at 450 nm using an automated microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were repeated three times.

Cell Growth Assay

Cell viability was measured using the CCK-8 assay according to the manufacturer's protocol. Parental Ishikawa and ARID1A-deficient cells (clone 1, clone 2, and clone 3) were plated in different 96-well plates at a concentration of 3,000 cells/well. After 24 h of incubation, the cells were incubated in DMEM/high-glucose medium containing CCK-8 for another 1 h at 37°C. The optical density (OD) value for each well was read at 450 nm using an automated microplate reader. The experiments were repeated three times.

Colony Formation Assay

Parental Ishikawa cells and ARID1A-deficient cells (clone 1, clone 2, and clone 3) were cultured in 3.5-cm petri dishes (200 cells/dish) for 2 weeks. The resulting cell clones were fixed and stained with 2% Giemsa after washing with PBS.

Cell Proliferation

To study the cytotoxic effect of the drugs, cells were plated in different 96-well plates at a concentration of 5,000 cells/well and grown for 24 h, and then treated with different concentrations of MPA (5, 10, 20, 40, 80, and 160 μmol/L) or LY294002 (1.25, 2.5, 5, 10, 20, and 40 μmol/L). Control columns contained cells with 0.1% DMSO, and blank columns contained medium alone. After 24 h of incubation, the drug-containing medium was replaced with DMEM/high-glucose medium containing 10% CCK-8 for another 1 h at 37°C. The OD value for each well was read at 450 nm using an automated microplate reader.

Apoptosis Assay

Parental Ishikawa, Ishikawa-PR, and clone 2 cells were incubated in six-well plates at a concentration of 1×10^5/well. The cells were allowed to attach overnight, and the next day they were treated with 40 μM MPA or/and 10 μM LY294002 for 24 h. The cells were harvested and resuspended in binding buffer containing annexin V-PE, and propidium iodide (PI) was then added using Annexin-V/PI apoptosis kit (Solarbio, Beijing, P.R. China). Flow cytometry analysis (FACScan, Beckman Coulter, Brea, CA, USA) was conducted to detect apoptosis.

Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the mRNA level of molecules in different cells. Total RNA was extracted using TRIzol reagent (Sigma-Aldrich), and then the RNA purity was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA samples were reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). Real-time PCR was then performed using PowerUp™ SYBR™ Green Master Mix and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The cDNAs of three transcripts were amplified for 40 cycles (15 s at 94°C, 15 s at 60°C, and 60 s at 72°C). The PCR primers (ARID1A, PRB, -actin) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, P.R. China) as follows: ARID1A 5′-GGGGGAGAAGACGAAGACAG-3′ (forward) and 5′-CTGGACTTCTTGAGCTTGCC-3′ (reverse); PRB TCGGACACCTTGCCTGAAGT (forward) PRB CAGGGCCGAGGGAAGAGTAG (reverse); and -actin 5′-CTCACCATGGATGATGATATCGC-3′ (forward) and 5′-AGGAATCCTTCTGACCCATGC-3′ (reverse). Fold change values were calculated using the Ct method.

Western Blot

Protein expression was assessed by immunoblot analysis of cell lysates in RIPA buffer supplemented with protease and phosphatase inhibitors (Beyotime Biotechnology). The lysates were denatured in SDS loading buffer. Next, the mixtures were separated on SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membranes. The membranes were then incubated with a primary antibody against ARID1A (1:1,000), p-AKT (1:1,000), PRB (1:1,000), or -actin (1:1,000). IRDye® 800CW goat anti-rabbit IgG (H + L) and IRDye® 680CW goat anti-mouse IgG (H + L) (1:7,500) were used as secondary antibodies. Western blot lanes were quantified using the Odyssey infrared imaging system (LI-COR Bioscience) and analyzed using Image Studio Lite 5.2 (LI-COR Bioscience).

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Differences between two groups were examined using Student's *t*-test, and one-way ANOVA was performed when there were more than two groups with SPSS version 17.0 software. The histograms and line charts were produced using GraphPad Prism 5.0 software. Differences with a value of $p < 0.05$ were considered to indicate a significant difference. All experiments were repeated at least three times.

RESULTS

Establishment of ARID1A Knockout Ishikawa Cell Lines Using the CRISPR/Cas9 System

To introduce the ARID1A knockout by gene editing with the CRISPR/Cas9 system, two CRISPR guide sequences close to the target site were designed. The Cas9 cleavage site was separated from the target site in forward

and reverse sgRNAs (Fig. 1A). PCR amplification was used to screen for monoclonal mutant cells, and PCR products from three monoclones were sequenced to validate the gene knockout. The sequencing results revealed that a frame shift mutation occurred in the expected cleavage point of the ARID1A gene (Fig. 1B). The expression levels of ARID1A mRNA and protein were significantly decreased in the three clone cells (clone 1, clone 2, and clone 3) compared with the levels in the parental Ishikawa cells ($p < 0.01$) (Fig. 2A and B). These results indicated that ARID1A-knockout Ishikawa cell lines were successfully constructed.

Detection of Cell Proliferation and Colony Formation in ARID1A-Deficient and Parental Ishikawa Cells

The impact of ARID1A deficiency on the growth of the Ishikawa cells was assessed, and the results showed that the ARID1A knockout cells had higher proliferation capacity than the parental subline ($p < 0.05$ or $p < 0.01$) (Fig. 3A). In addition, colony formation assays confirmed that the ARID1A knockout cells formed significantly more colonies than the parental Ishikawa cells (Fig. 3B). Based on the identification results, we selected the clone 2 cell line that was subjected to ARID1A dual allelic inactivation for further functional studies.

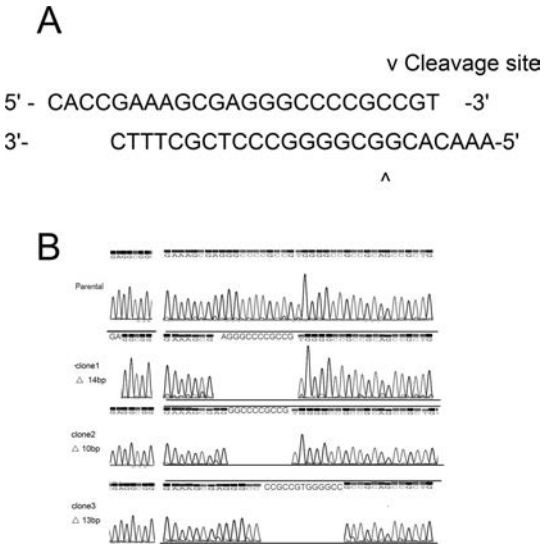

Figure 1. Establishment of AT-rich interactive domain 1A (ARID1A)-knockout Ishikawa cell lines using the CRISPR/Cas9 system. (A) Schematic diagram of the CRISPR/Cas9 system. Two (forward and reverse) single-guide RNA (sgRNA) sequences are indicated above and below the wild-type sequence. The arrow indicates the expected Cas9 cleavage site. (B) PCR identification and Sanger sequencing analysis of wild-type and clone cell lines. Clones 1, 2, and 3 showed 14 bp, 10 bp, and 13 bp deletions, respectively, and were selected to establish the ARID1A-deficient cell lines.

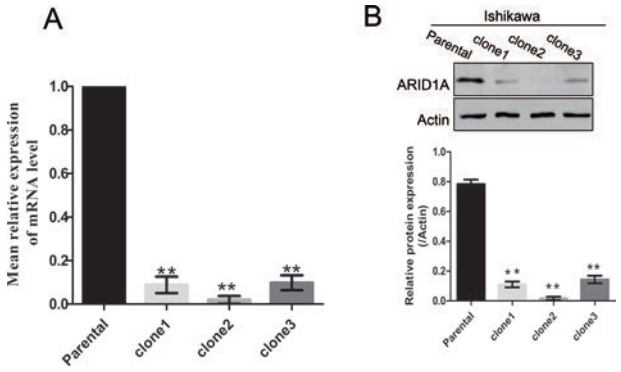

Figure 2. The expression levels of ARID1A in parental and ARID1A-deficient cells (clones 1, 2, and 3). (A) Histogram showing the average relative expression levels of ARID1A mRNA in control and ARID1A-deficient cells. (B) Western blot analysis. Electrophoretograms showing the protein expression level of ARID1A and actin. $^{**}p < 0.01$ versus parental Ishikawa cells.

Different Levels of Medoxyprogesterone Acetate (MPA) Sensitivity in the Parental Ishikawa, Ishikawa-PR, and ARID1A-Deficient Cell Lines

Through long-term (7 months) exposure to increasing concentrations of MPA, parental Ishikawa cells were successfully transformed into an Ishikawa-PR cell line. We then characterized the Ishikawa-PR cells by examining cell growth and cell viability. The CCK-8 results showed that there was no significant difference between the parental Ishikawa and Ishikawa-PR cells (Fig. 4A). MPA at various doses (0, 5, 10, 20, 40, 80, and 160 μmol/L) was administered to three cells lines for 24 h. The inhibition rate was markedly decreased

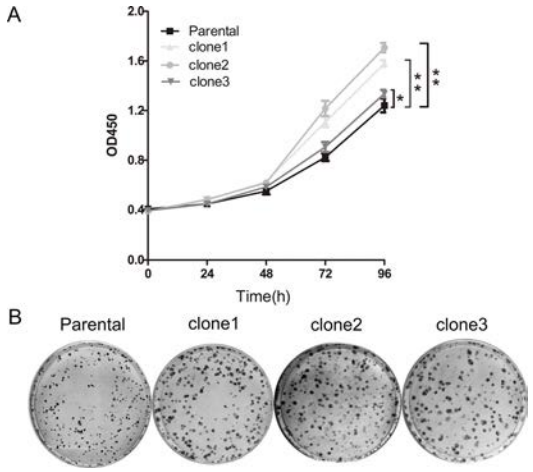

Figure 3. Effects of ARID1A deficiency on cell growth curves and colony formation rate. (A) Knockout of ARID1A promoted cell proliferation as assessed by Cell Counting Kit-8 (CCK-8) assays. (B) Colony formation rate in ARID1A-deficient and parental cells. $^{*}p < 0.05$, $^{**}p < 0.01$ versus parental Ishikawa cells.

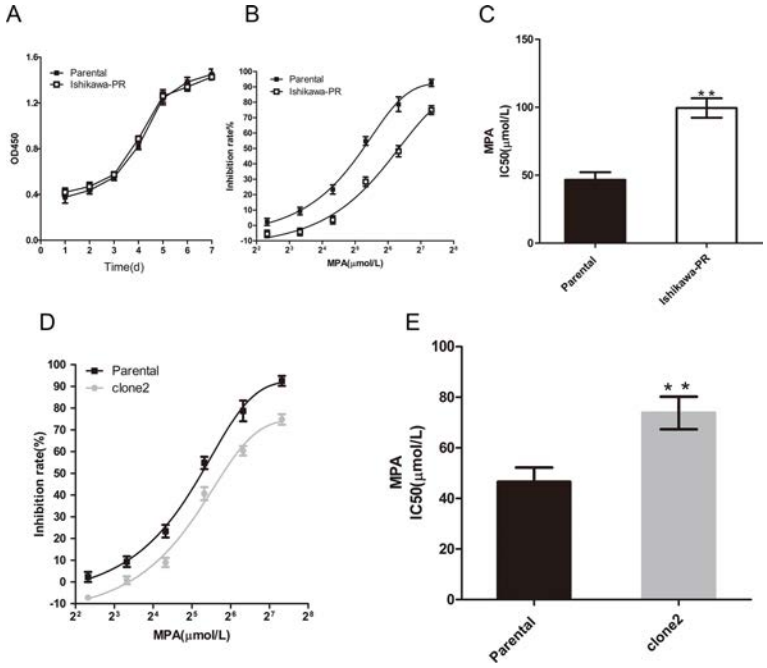

Figure 4. Effects of medroxyprogesterone (MPA) on parental Ishikawa, Ishikawa-PR, and ARID1A-deficient cells. (A) Growth curves of parental Ishikawa and Ishikawa-PR cells. (B) Parental Ishikawa and Ishikawa-PR cells were cultured with different concentrations of MPA (0–160 μmol/L), and cell viability was determined using the CCK-8 assay. (C) IC_{50}s of the parental and resistant cells following MPA treatment. (D) The parental Ishikawa and clone 2 cell lines were cultured with different concentrations of MPA (0–160 μmol/L), and cell viability was determined using the CCK-8 assay. (E) The IC_{50}s of parental and ARID1A-deficient cells following MPA treatment. $^{**}p < 0.01$ versus parental Ishikawa cells.

in a dose-dependent manner. In addition, both of the Ishikawa-PR and ARID1A-deficient Ishikawa cell lines were less sensitive to MPA treatment compared with the parental cells (Fig. 4B and D). The half-maximum inhibitory concentration (IC_{50}) of the Ishikawa-PR and ARID1A-deficient cells increased remarkably compared with the parental Ishikawa cells ($p < 0.01$) (Fig. 4C and E).

Association Between ARID1A and PRB in Ishikawa-PR and ARID1A-Deficient Cells

PRB is a strong predictor of progestin sensitivity in Ishikawa cells. Therefore, Western blot analysis and RT-PCR were performed to evaluate PRB expression. Both Ishikawa-PR cells and clone 2 cells were found to have significantly lower PRB mRNA ($p < 0.01$) (Fig. 5A and B) and protein ($p < 0.01$) (Fig. 5C and D) levels compared with the parental Ishikawa cells. However, the results of the RT-PCR and Western blot showed that there was no significant difference in ARID1A mRNA and protein expression between the parental Ishikawa and Ishikawa-PR cells ($p > 0.05$) (Fig. 5A and C).

Hyperactivation of Akt Phosphorylation in Ishikawa-PR and ARID1A-Deficient Cell Lines

To better understand the molecular mechanisms associated with the depletion of ARID1A, the activation of the PI3K/Akt signaling pathway was studied. The results showed that the expression of p-AKT protein significantly increased in Ishikawa-PR and ARID1A-deficient cells compared with the parental Ishikawa cells ($p < 0.01$) (Fig. 6A and B).

The Effects of LY294002/MPA Combination Treatment on the Growth of the Parental Ishikawa, Ishikawa-PR, and ARID1A-Deficient Cells

To confirm the specific concentration of LY294002 required for inhibiting P-AKT in Ishikawa cells, LY294002 in various doses (0, 1.25, 2.5, 5, 10, 20, and 40 μmol/L) were administered to three cell lines for 24 h. The inhibition rate was markedly decreased in a concentration-dependent manner in the three cell lines. In addition, the IC_{50} values of LY294002 in ARID1A-deficient and Ishikawa-PR cells were distinctly reduced compared with the parental Ishikawa cells ($p < 0.01$) (Fig. 7A). Ishikawa cells were treated with different concentrations (0, 2.5, 5, and 10 μmol/L) for 1 h, and the P-AKT levels were measured. The results showed that the expression of P-AKT was decreased in a dose-dependent manner ($p < 0.01$) (Fig. 7B). Combined with these results, the proper concentration of LY294002 was determined to be 10 μmol/L. Parental Ishikawa, Ishikawa-PR, and clone 2 cells were treated with various

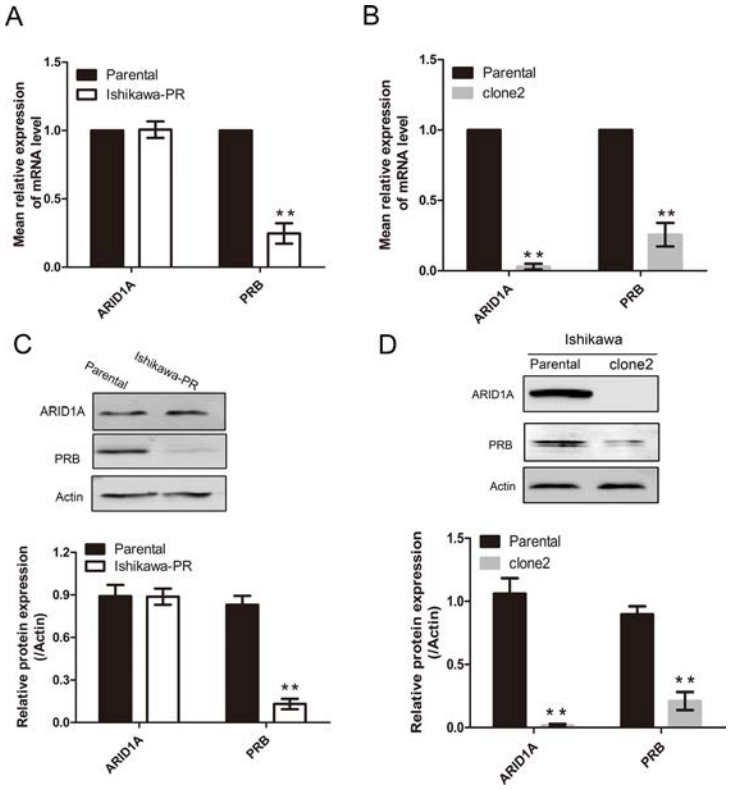

Figure 5. Expression levels of progesterone receptor B (PRB) and ARID1A in parental Ishikawa, Ishikawa-PR, and ARID1A-deficient cell lines. (A) The mRNA expression levels of ARID1A and PRB in parental Ishikawa and Ishikawa-PR cells were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) analysis. (B) The mRNA expression levels of ARID1A and PRB in parental Ishikawa and ARID1A-deficient cells were evaluated by RT-PCR analysis. (C) Western blot analysis of ARID1A and PRB in parental Ishikawa and Ishikawa-PR cells. (D) Western blot analysis of ARID1A and PRB in parental Ishikawa and ARID1A-deficient cells. $^{**}p < 0.01$ versus parental Ishikawa cells.

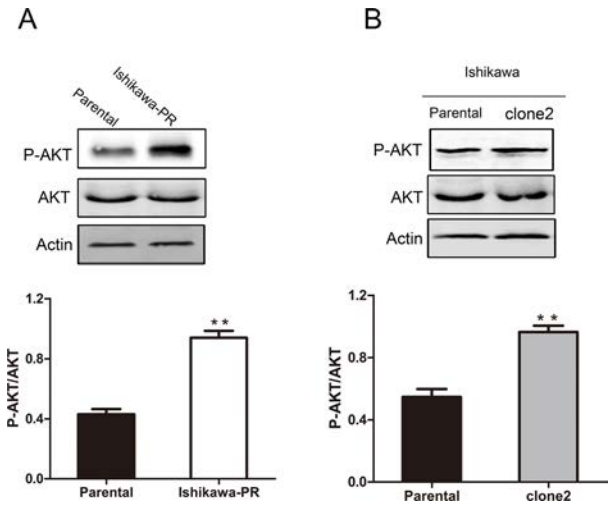

Figure 6. Hyperactivation of Akt phosphorylation in Ishikawa-PR and ARID1A-deficient cell lines. (A) Western blot analysis of P-AKT in Ishikawa-PR and parental cells. (B) Western blot analysis of P-AKT in Ishikawa and ARID1A-deficient cells. $^{**}p < 0.01$ versus parental Ishikawa cells.

doses of MPA (0, 5, 10, 20, 40, 80, and 160 μmol/L) for 24 h with or without pretreatment with LY294002 (10 μmol/L), and cell growth inhibition was evaluated using CCK-8 assays. The results showed that LY294002 significantly increased MPA-induced growth inhibition in the parental Ishikawa and Ishikawa-PR cells (Fig. 7C and D) but not the clone 2 cells (Fig. 7E).

Measurement of Cell Apoptosis in the Parental Ishikawa, Ishikawa-PR, and Clone 2 Cells Using an Annexin-V and Propidium Iodide Binding Assay via FACS

Apoptosis was evaluated in parental Ishikawa, Ishikawa-PR, and ARID1A-deficient cells. The three different cell lines were treated with vehicle (V), 10 μmol/L of LY294002 (LY), 40 μmol/L of MPA, or a combination of LY294002 + MPA (LY + MPA), and the results showed that LY294002 significantly increased MPA-induced apoptosis in parental Ishikawa and Ishikawa-PR cells ($p < 0.01$) (Fig. 8). However, we found that the apoptosis rate of MPA-combined LY294002 was essentially the same as that of MPA alone in the ARID1A-deficient cells ($p > 0.05$).

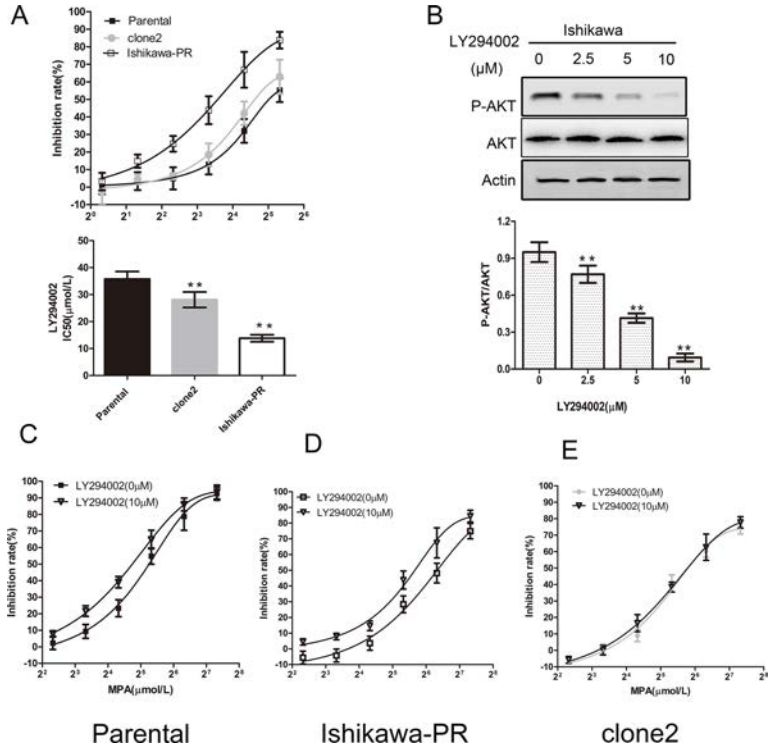

Figure 7. LY294002 increased MPA-induced cell proliferation in parental Ishikawa and Ishikawa-PR cells but not clone 2 cells. (A) Parental Ishikawa, Ishikawa-PR, and clone 2 cells were cultured with different concentrations of LY294002 (0–40 μmol/L), and cell viability was determined using the CCK-8 assay. (B) Western blotting was performed to examine the expression levels of P-AKT in parental Ishikawa cells that were treated with LY294002 at various doses. Parental Ishikawa, Ishikawa-PR, and clone 2 cells were treated with 0 or 10 μmol/L of LY294002 for 1 h, and the protein levels of P-AKT were measured by Western blot. (C–E) Parental Ishikawa, Ishikawa-PR, and clone 2 cells were treated with various doses of MPA (0–160 μmol/L) for 24 h with or without LY294002 pretreatment (10 μmol/L). $^{**}p < 0.01$ versus parental Ishikawa cells.

The Expression of PRB Cannot be Successfully Upregulated by Inhibition of P-AKT in ARID1A-Deficient Cells

To explore whether inhibition of the PI3K/AKT signaling pathway upregulated PRB expression, parental Ishikawa, Ishikawa-PR, and clone 2 cells were treated with MPA in the presence or absence of LY294002, and the PRB mRNA and protein levels and p (Ser473)-AKT and PRB were measured by RT-PCR and Western blot. As depicted in Figure 9A, the PRB mRNA level was significantly upregulated by LY294002 in parental Ishikawa and Ishikawa-PR cells ($p < 0.05$ or $p < 0.01$). In addition, LY294002 treatment could decrease the levels of P-AKT, both in the presence and absence of MPA in the different cell lines ($p < 0.01$) (Fig. 9B and C). However, the expression of PRB mRNA and protein cannot be successfully upregulated via inhibition of P-AKT in ARID1A-deficient cells ($p > 0.05$) (Fig. 9A and D). Taken together, ARID1A deficiency negatively regulates PRB expression at the transcriptional level and cannot be reversed by AKT pathway inhibitors.

DISCUSSION

Progesterone therapy is regarded as the primary treatment for reproductive age women with early stage, well-differentiated endometrial cancer. However, progesterone resistance has become a serious obstacle to conservative treatment. It has been reported that the effects of progestins are mediated primarily through their receptor, progesterone receptor (PR). The expression and function of PR, especially the B-subunits (PRB), play key roles in progesterone resistance[9,19]. However, the molecular mechanisms underlying progesterone resistance in endometrial cancer are poorly understood, and methods for reversing the down-regulation of PRB must be studied. Previous evidence indicated that progesterone resistance is closely related to the degree of excessive activation of the PI3K/AKT signaling pathway in endometrial cancer. Consistently, AKT hyperphosphorylation was shown in long-term induction of progesterone-resistant endometrial cancer cells, and inhibition of the PI3K/AKT/mTOR pathway could sensitize endometrial cancer cells to progestin[8,10].

Previous research has shown that ARID1A plays a key role in the drug sensitivity of various cancer cells. For

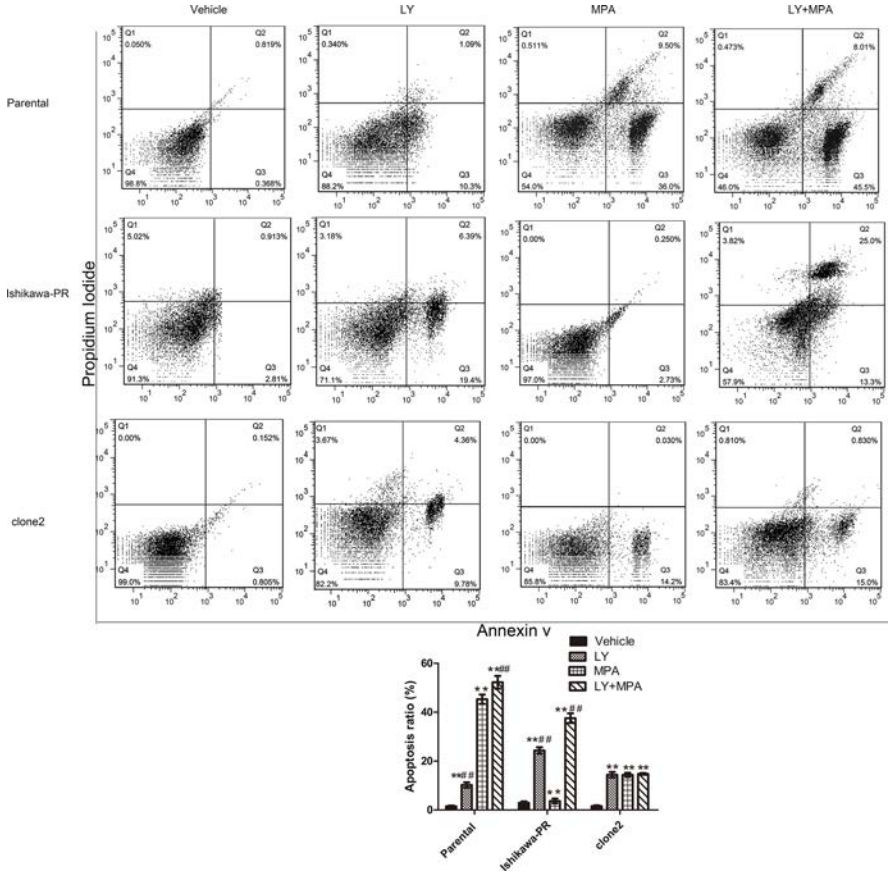

Figure 8. LY294002 increased MPA-induced cell apoptosis in parental Ishikawa and Ishikawa-PR cells, but not clone 2 cells. Sensitivity to MPA cannot be effectively restored by inhibiting the PI3K/AKT pathway in ARID1A-deficient Ishikawa cells. $^{**}p < 0.01$ versus vehicle. $^{\#\#}p < 0.01$ versus MPA group.

example, ARID1A gene silencing reduced the sensitivity of ovarian clear cell carcinoma to cisplatin and enhanced the radioresistance of pancreatic cancer cells, which is associated with abnormal activation of the PI3K/AKT signaling pathway[20,21]. Despite these findings, to the best of our knowledge, no further reports regarding the role of ARID1A in progesterone resistance in endometrial cancer have been published. In the present study, we established an ARID1A-deficient Ishikawa cell line using CRISPR/Cas9 gene editing and investigated the effects of the deficiency on the PRB expression and progesterone therapy in Ishikawa cells.

The CRISPR/Cas9 genome editing technology is based on RNA-guided nuclease Cas9 and guide RNAs, which enable rapid, specific, and efficient engineering of target genes in many cell types[22]. To our knowledge, the ARID1A gene knockout in Ishikawa cells has not been reported. In this study, we successfully inactivated ARID1A in Ishikawa cells using CRISPR/Cas9 technology. To ensure an effective outcome, two (forward and reverse) sgRNA sequences were designed, and three mutant monoclones were isolated as ARID1A-deficient candidates based on PCR product sequencing. All of the three clone cells showed that knockout of ARID1A significantly promoted the growth and colony formation of Ishikawa cells. However, the proliferation was greater in one clone cell line compared with the others. Clone 2 cells were chosen to act as the biallelic ARID1A knockout Ishikawa cells for the following research.

In addition, progesterone-resistant cells were acquired through long-term exposure to MPA, a simulating clinical cause of disease. ARID1A knockout enhanced MPA resistance by downregulating the expression of the progesterone receptor B subunit and excessively activating the PI3K/AKT signaling pathway, and these results were consistent in Ishikawa-PR cells. However, there was no difference in the ARID1A expression between progesterone-sensitive and -resistant cells. These results demonstrate the importance of ARID1A for the downregulation of PRB in Ishikawa cells.

LY294002 has been shown to inhibit cell proliferation and to induce apoptosis by downregulating AKT/protein kinase B activation[23]. Our current experiments revealed the role of the PI3K/AKT signaling pathway in ARID1A-deficient Ishikawa cells and Ishikawa-PR cells. The results showed that the antitumor activity of LY294002 was

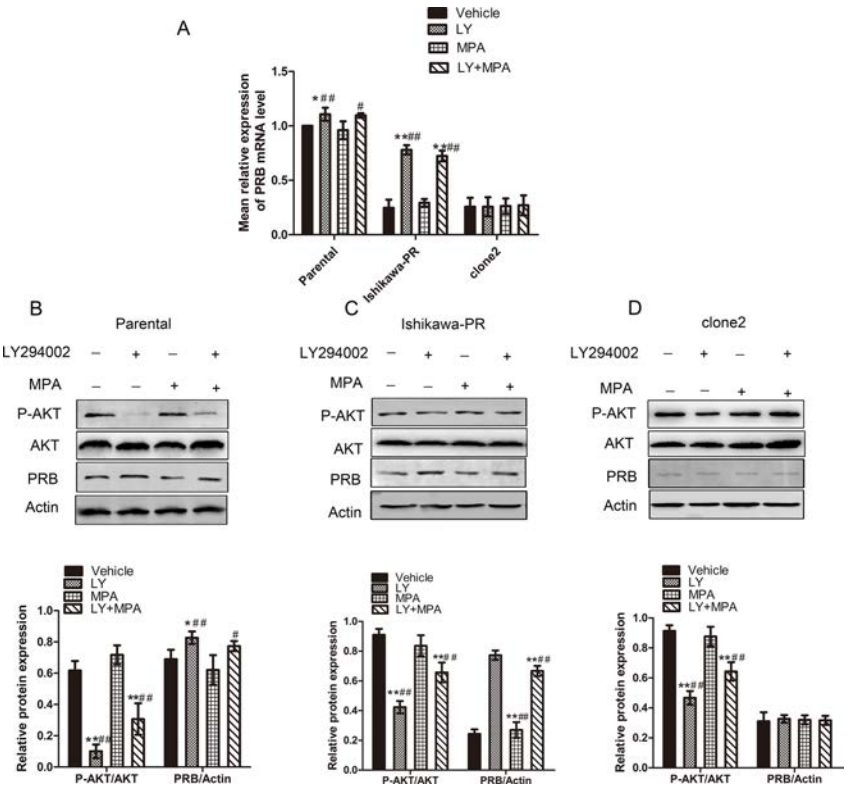

Figure 9. The expression of PRB cannot be successfully upregulated by inhibiting P-AKT in ARID1A-deficient cells. (A) Parental Ishikawa, Ishikawa-PR, and clone 2 cells were treated with vehicle, LY294002, MPA, or LY294002 + MPA, and the PRB mRNA levels were measured by RT-PCR. (B) Parental Ishikawa cells were treated with vehicle, LY294002, MPA, or LY294002 + MPA, and the protein levels of P-AKT and PRB were measured by Western blot. (C) Ishikawa-PR cells were treated with vehicle, LY294002, MPA, or LY294002 + MPA, and the protein levels of P-AKT and PRB were measured by Western blot. (D) Clone 2 cells were treated with vehicle, LY294002, MPA, or LY294002 + MPA, and protein levels of P-AKT and PRB were measured by Western blot. $*p < 0.05$, $**p < 0.01$ versus vehicle. $\#p < 0.05$, $\#\#p < 0.01$ versus MPA group.

more significant in Ishikawa-PR and ARID1A-deficient cells than in the parental cells. In addition, a combination with LY294002 and MPA could induce MPA sensitivity in Ishikawa-PR cells. However, the sensitivity of ARID1A-deficient cells was not changed by LY294002 treatment. Previous evidence indicated that inhibition of PI3K/AKT can reverse progestin resistance through the attenuation of nongenomic activation of the PI3K/AKT pathway or by directly modulating PRB transcriptional activity[8,24]. In contrast to what was previously shown, in this study PRB activity cannot be upregulated via inhibition of LY294002 in ARID1A-deficient Ishikawa cells. These results indicated that Akt inhibition could not directly upregulate PRB transcriptional activity and that other molecules might be involved in PRB activity and functional damage.

ARID1A encodes the ARID1A/BAF250a subunit of the Switch/Sucrose nonfermentable (SWI/SNF) chromatin remodeling complex. In the process of gene expression, the SWI/SNF complex regulates gene expression by exposing the functional sites on DNA and by promoting the binding of DNA to transcription factors and other key proteins[25,26]. In this study, our results demonstrate that ARID1A knockout decreases PRB expression and reduces the sensitivity of Ishikawa cells to MPA. The transcription activity and protein expression of PRB could not be restored by LY294002. Thus, it appears that ARID1A may act as a required mediator in the AKT-dependent regulation of PRB transcriptional activity. However, it remains to be determined whether ARID1A acts as a direct transcriptional coactivator of PRB at the level of chromatin.

In conclusion, the present study shows the ARID1A deficiency could cause primary MPA resistance in Ishikawa cells by downregulation of PRB expression. Moreover, unlike acquired progesterone resistance, inhibition of hyperactive Akt signaling cannot upregulate PRB transcriptional function (Fig. 10). The results provide new insight into the molecular mechanism by which ARID1A knockout can contribute to primary progesterone resistance in endometrial cancer. Taken together, our results suggest that a combination treatment with Akt inhibitor and MPA may be more effective for the treatment of ARID1A-proficient than for ARID1A-deficient endometrial cancer.

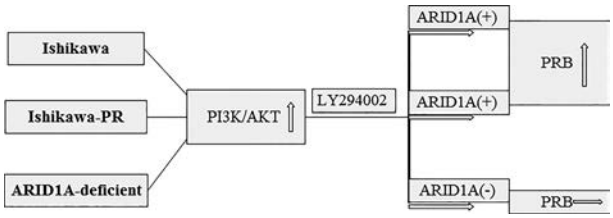

Figure 10. A diagram of the PRB/Akt/ARID1A pathway in parental Ishikawa, Ishikawa-PR, and ARID1A-deficient cells.

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