Original Research

Megestrol acetate drives endometrial carcinoma cell senescence via interacting with progesterone receptor B/FOXO1 axis

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Impact statement

This study demonstrates that megestrol acetate could induce irreversible G1 arrest and cellular senescence, affect the survival and growth of endometrial cancer cells, thus exhibiting anticancer property in human endometrial cancer. Moreover, megestrol acetate exerts its anticancer roles in human endometrial cancer through PR-B/FOXO1. These findings provide a novel understanding for investigating the molecular mechanism of megestrol acetate in human endometrial cancer treatment.

Abstract

Megestrol acetate is a common and efficient anticancer progesterone. To explore the activity and the therapeutic mechanisms of megestrol acetate in endometrial cancer, human endometrial cancer cell lines Ishikawa and HHUA overexpressing progesterone receptor A (PR-A) and progesterone receptor B (PR-B) were treated with megestrol acetate. Cell viability, apoptosis, cycle arrest, and senescence, as well as the expressions of p21 and p16, two hallmarks of cellular senescence, were evaluated. Compared with the control, >10 nmol/L megestrol acetate treatment could significantly reduce endometrial cancer cell growth, and induce the irreversible G1 arrest and cell senescence. The expression of cyclin D1 in megestrol acetate treated cells was downregulated, while the expressions of p21 and p16 were upregulated via PR-B isoform. FOXO1 inhibitor AS1842856 could significantly abrogate megestrol acetate-induced cell senescence, suggesting that FOXO1 was involved in megestrol acetate/PR-B axis. These findings may provide a new understanding for the treatment of human endometrial cancer.

Keywords: Endometrial carcinoma, megestrol acetate, progesterone receptor B, senescence, Bcl-2

***Experimental Biology and Medicine* 2021; 246: 2307–2316. DOI: 10.1177/15353702211026566**

Introduction

Endometrial carcinoma (EC) is one of the most common malignant cancers among women worldwide, with increasing incidence and mortality rates.[1,2] There are two predominant types of EC, Type I and Type II. Type I EC, often arising from atypical hyperplasia/endometrioid intraepithelial neoplasia, accounts for approximately 70% of all patients with endometrial cancer.[1,3] Type I ECs are estrogen sensitive, and mostly demonstrate low histological grade (well or moderate differentiation), with a favorable prognosis.[4] Type II EC is more likely to be high grade with a poor prognosis and a high risk of relapse and metastasis, including serous, clear cell histologic type, undifferentiated carcinosarcoma, and other non-endometrioid histology.[5–7] Most patients with stage I and II EC will have a favorable prognosis, whereas patients with stage III or IV EC will have a worse likelihood of survival despite advanced development in surgery, radiotherapy, and chemotherapy. Hence, further understanding is needed to improve the therapeutic strategies and clinical prognoses in human EC.

Progestin therapy for the treatment of EC was proposed in 1960s, and numerous retrospective studies have been published examining the roles of hormonal therapy, including nomegestrol acetate, medroxyprogesterone acetate, megestrol acetate, levonorgestrel, cyproterone acetate, hydroxyprogesterone caproate, and other unspecified/miscellaneous progestins.[8,9] The majority of published studies reported treatments with either medroxyprogesterone acetate or megestrol acetate, with no consensus on the optimal dosage and duration. Megestrol acetate, a synthetic progestin, belongs to the 17 alpha-hydroxyprogesterone derivates, and is usually used as a short-acting contraceptive or an anticancer drug for the treatment of terminal

breast or endometrial cancer.[10] Megestrol acetate administration has been reported to be associated with reduced serum cortisol concentrations in patients with cancer or AIDS.[11–13] In older individuals, megestrol acetate administration could affect the secretion of several pituitary hormones and end-organ hormone synthesis.[14] Since the 1971 approval for the palliative treatment of advanced EC, megestrol acetate is frequently used in endometrial cancer patients, mostly in type I endometrial cancer. In gynecologic oncology group (GOG) study #121, high-dose megestrol acetate yields a response rate of 26%.[15] The effect of megestrol acetate on the endometrium has been studied *in vivo* and *in vitro*.[16–18]

The roles of megestrol acetate are mainly mediated by progesterone receptors (PRs) family members, progesterone receptor A (PR-A) and progesterone receptor B (PR-B).[19] Stable expression of PR-A and PR-B is crucial for normal physiological development, whereas dysregulation of PR-A and PR-B can initiate and exacerbate various types of gynecological cancers.[20] In endometriosis and other related diseases, altered expressions of PR-A and PR-B have been reported.[21,22] And overexpression of PR-B could suppress cell invasiveness and inhibit EC growth via affecting the expression of matrix metalloproteinases.[23]

Senescence is a genetically regulated mechanism that involves in the normal development, and is responsible for the ending of tumor cells after chemotherapy. In endometrial cancer, downregulation of Sushi domain containing 2 (SUSD2), an endometrial mesenchymal stem cell marker, induced senescence and death of endometrial cancer cells.[24] In human breast cancer cells, activation of PR-B by the specific ligand hydroxyprogesterone counteracted the senescence and autophagy.[25] In the present study, we aim to investigate whether megestrol acetate inhibits or suppresses the development of endometrial cancer cells by activating PR-B and, to further explore the protective roles of megestrol acetate in human endometrial cancer.

Materials and methods

Cell culture and treatment

Human endometrial cancer cell lines Ishikawa and HHUA were purchased from ATCC (Manassas, VA, USA), and cultured in the Dulbecco's Modified Eagles' medium containing 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin (Gibco), and streptomycin (Invitrogen) solution at 37°C in a 5% CO_2 atmosphere.

For megestrol acetate treatment, megestrol acetate (Adooq Bioscience, Shanghai, China) stock solution (10 mmol/L) was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in all experimental solutions was set below 0.25% (v/v). After washing with PBS, cultured cells were treated with different concentrations of megestrol acetate (1, 10, or 100 nmol/L) or the vehicle DMSO, or not (control) and incubated for 96 h. Then, cells were collected for subsequent analysis. For forkhead box protein O1 (FOXO1) inhibition, a selective inhibitor AS1842856 (EMD Millipore) was used. Cells were pretreated with AS1842856 for 1 h prior to the addition of megestrol acetate.

Western blotting

Total proteins were extracted from cultured cells and then separated by SDS-PAGE. After electrophoretic transfer to a polyvinylidene fluoride (PVDF) membrane, the blots were incubated with primary antibodies (rabbit antiprogesterone receptor, cat# ab177930; anti-Bcl-2, cat# ab32124, 1:1000 dilution, Abcam; mouse anti-β-actin, cat# ab20272, 1:3000 dilution, ABclonal) at 4°C overnight. Then, blots were incubated with specific secondary antibodies for 1 h at room temperature. Enhanced chemiluminescence (Amersham Pharmacia, NJ, USA) was performed to visualize the bands.

Cell transfection

PR-A or PR-B stable overexpression cell lines were generated by transfecting cells with 2.0 μg of PR-A or PR-B plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions, respectively. The small interfering RNA (siRNA) targeting PR-B, as well as negative control siRNA, was synthesized by Takara (Dalian, China). A blast search was performed to ensure the absence of homology in selected sequences with any other sequences. Specific PR-B and negative control siRNA were transfected into cultured cells using Lipofectamine 3000 according to the manufacturer's instructions, respectively. After 48 h, interference efficiency was detected by Western blotting. Transfected cells were conducted for subsequent experiments.

Cell growth assay

The CCK-8 kit (Bioroot, Shanghai, China) was used to analyze the growth of identical cells following the manufacturer's guidelines. Cells were cultured in 96-well plates (5×10^4 cells/mL) for 12, 24, 48, 72, and 96 h, respectively. Then, 10 μL of CCK-8 solution was added into the wells, followed by incubation for 4 h at 37°C. The absorbance of cells was tested at 450 nm.

Cell cycle analysis

Cell cycle analysis was performed using flow cytometry. In brief, cultured cells (5×10^6 cells/well) were harvested with 500 μL of ice-cold PBS, and fixed with 70% ethanol. Then, cells were re-suspended gently with binding buffer to obtain monodispersed cell suspension, and double stained with annexin V-FITC and propidium iodide (PI) at 37°C for 1 h, followed by analysis using FACS CaliburTM (Becton Dickinson).

Beta-galactosidase assay

Beta-galactosidase assay was performed using the senescence β-galactosidase staining kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, cultured cells were washed with ice-cold PBS and

fixed for 15 min at room temperature. Cell suspensions (5×10^6 cells/well) were stained with fresh β-galactosidase staining solution overnight at 37°C. Senescent cells were counted under microscopy, with four random fields for each sample.

Statistical analysis

The data were expressed as mean ± SD. The statistical analysis was conducted using the GraphPad Prism 6 (La Jolla, CA, USA) and SPSS 18.0. Comparisons between two groups were calculated using two independent sample *t*-tests, and comparisons among multiple groups were calculated using one-way ANOVAs and LSD tests. All experiments were performed independently at least three times.

Results

Megestrol acetate reduces cell growth in human endometrial cancer cells

To determine the role of megestrol acetate in endometrial cancer, we firstly investigated the effect of megestrol acetate on the growth of endometrial cancer cells *in vitro*. Human endometrial cancer cell lines Ishikawa and HHUA were treated with different concentrations of megestrol acetate (1, 10, or 100 nmol/L) for different times, respectively. Compared with untreated control, the growth of Ishikawa cells was significantly reduced in a time-dependent manner upon megestrol acetate treatment (Figure 1(a)). Similar results were found in HHUA cells (Figure 1(b)). To further

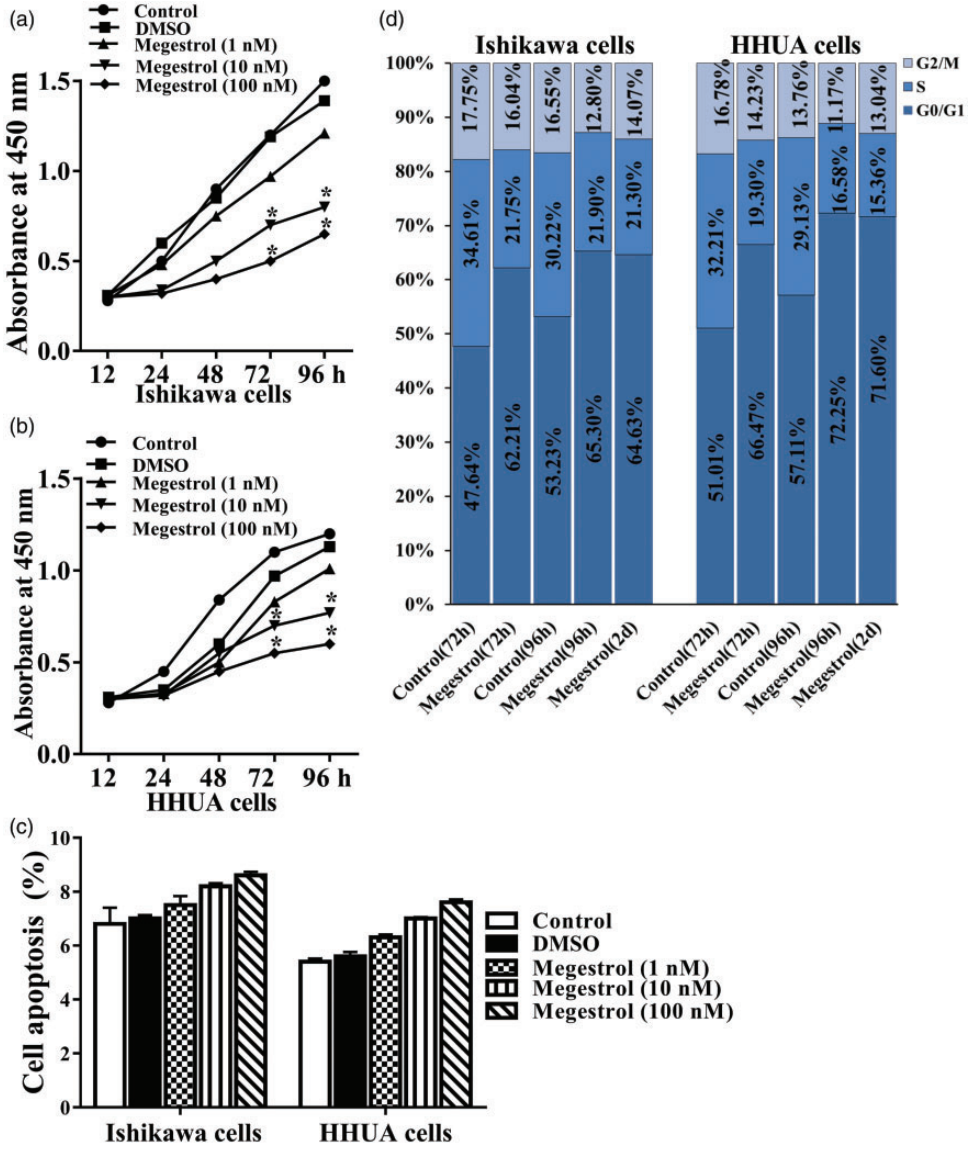

Figure 1. Megestrol acetate reduces cell survival and induces irreversible G1 arrest in human endometrial cancer cells. The human endometrial cancer Ishikawa and HHUA cells were treated without (control) or with different concentrations of megestrol (1, 10, 100 nmol/L) or the vehicle DMSO for different time, respectively. Then the proliferation assay of (a) Ishikawa and (b) HHUA cells were performed (*$P < 0.05$ vs. control). (c) Cell apoptosis was detected by flow cytometry. (d) Ishikawa and HHUA cells were treated with 10 nmol/L megestrol or the vehicle DMSO (control) for 72 or 96 h, respectively. Then the cell cycles were analyzed (*$P < 0.05$ vs. control). (A color version of this figure is available in the online journal.)

demonstrate the cellular mechanism of megestrol acetate, cell apoptosis and cell cycle were further evaluated. As shown in Figure 1(c) and Supplementary Figure S1, treatment with different concentrations of megestrol acetate for 96 h slightly increased the apoptosis of Ishikawa and HHUA cells, compared with corresponding untreated control and DMSO group. However, no significant difference was found ($P > 0.05$). The cell cycle of Ishikawa cells was arrested in G0/G1 phase after 72-h treatment with 100 nmol/L megestrol acetate, compared with the control. G0/G1 arrest was observed throughout 96-h treatment of megestrol acetate (Figure 1(d)). After 96 h-treatment, cells were washed, and re-cultured with fresh medium for another two days. The percentage of cells in G0/G1 phase remained approximately unchanged (Figure 1(d)). Similar results were found in megestrol acetate treated HHUA cells (Figure 1(d)). These results indicate that megestrol acetate has an inhibitory effect on cell growth and induces irreversible cell cycle arrest of human endometrial cancer cells.

Megestrol acetate induces senescence in endometrial cancer cells

Irreversible cell cycle arrest may result in cell senescence. We next determined the accumulation of endogenous lysosomal β-galactosidase (SAβGal), the common marker of cellular senescence, in megestrol acetate-treated endometrial cancer cells. Megestrol acetate treatment significantly increased the activity of SAβ-Gal in endometrial cancer cells (Figure 2(a) and (b)). It has been reported that increased p21 expression and activity directly induced cellular senescence. Thus, we next analyzed the expression of cyclin D1, a cell-cycle-related protein, as well as p21 and p16, hallmarks of cellular senescence, in endometrial cancer cells. Megestrol acetate downregulated the expression of cyclin D1 in Ishikawa and HHUA cells, whereas expressions of p21 and p16 were increased compared with the control (Figure 2(c) and (d)). These results suggest that megestrol acetate affects cell cycle progression and induces cellular senescence, thus suppressing human endometrial cancer.

Megestrol acetate regulates the growth and senescence of endometrial cancer cells through the PR-B isoform

To fully understand whether PR-B or PR-A is involved in the role of megestrol acetate in endometrial cancer cells, we successfully constructed PR-B and PR-A overexpressing Ishikawa and HHUA cells (Figure 3(a)). PR-B overexpression (PR-B-OE) significantly augmented the effects of megestrol acetate on senescence (B), growth (Figure 3(c) and (d)), and cell migration (Figure 3(e) and (f)) of endometrial cancer cells. In contrast, the effect of megestrol

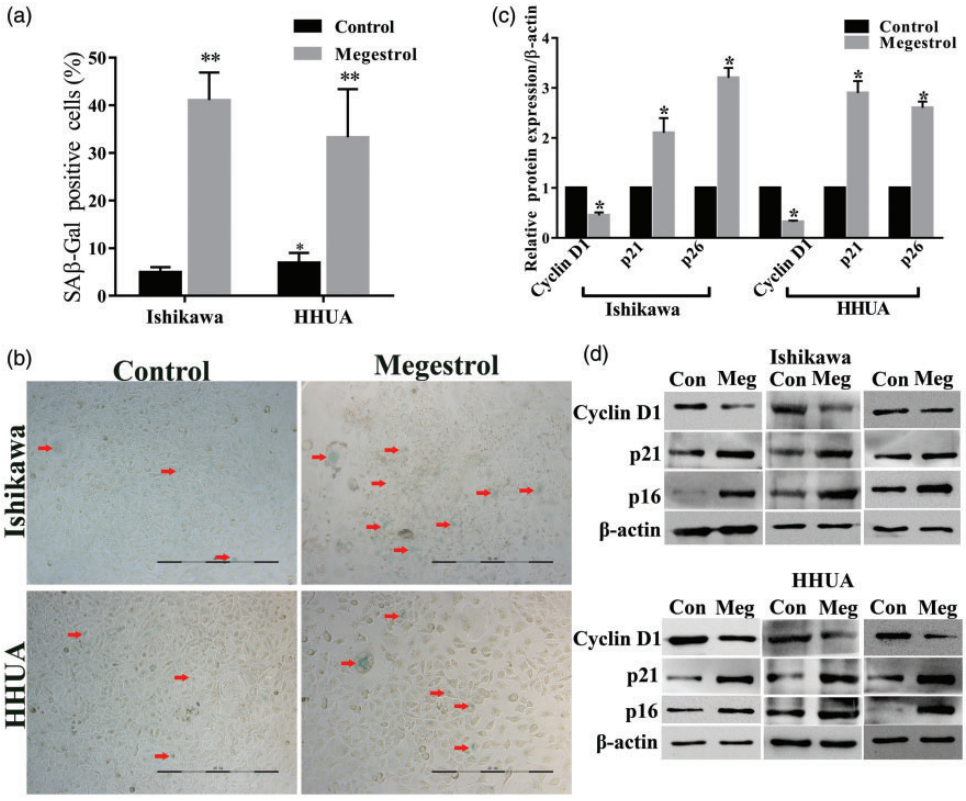

Figure 2. Megestrol acetate induces cell senescence in endometrial cancer cells. Ishikawa and HHUA cells were treated without (control) or with 10 nmol/L megestrol for 96 h. Cell senescence was detected by SAβGal staining and the protein expressions of cyclin D1, p21, and p16 were detected by Western blotting. (a) SAβGal positive cell numbers. (b) SAβGal staining. Senescent cells were SAβGal positive, and were stained with blue. Red arrows: representative senescent cells. (c) Relative expression of cyclin D1, p21, and p16. (d) The expression of cyclin D1, p21, and p16 of three repeated experiments (**$P < 0.01$ vs. control, *$P < 0.05$ vs. control). Bar = 200 μm. (A color version of this figure is available in the online journal.)

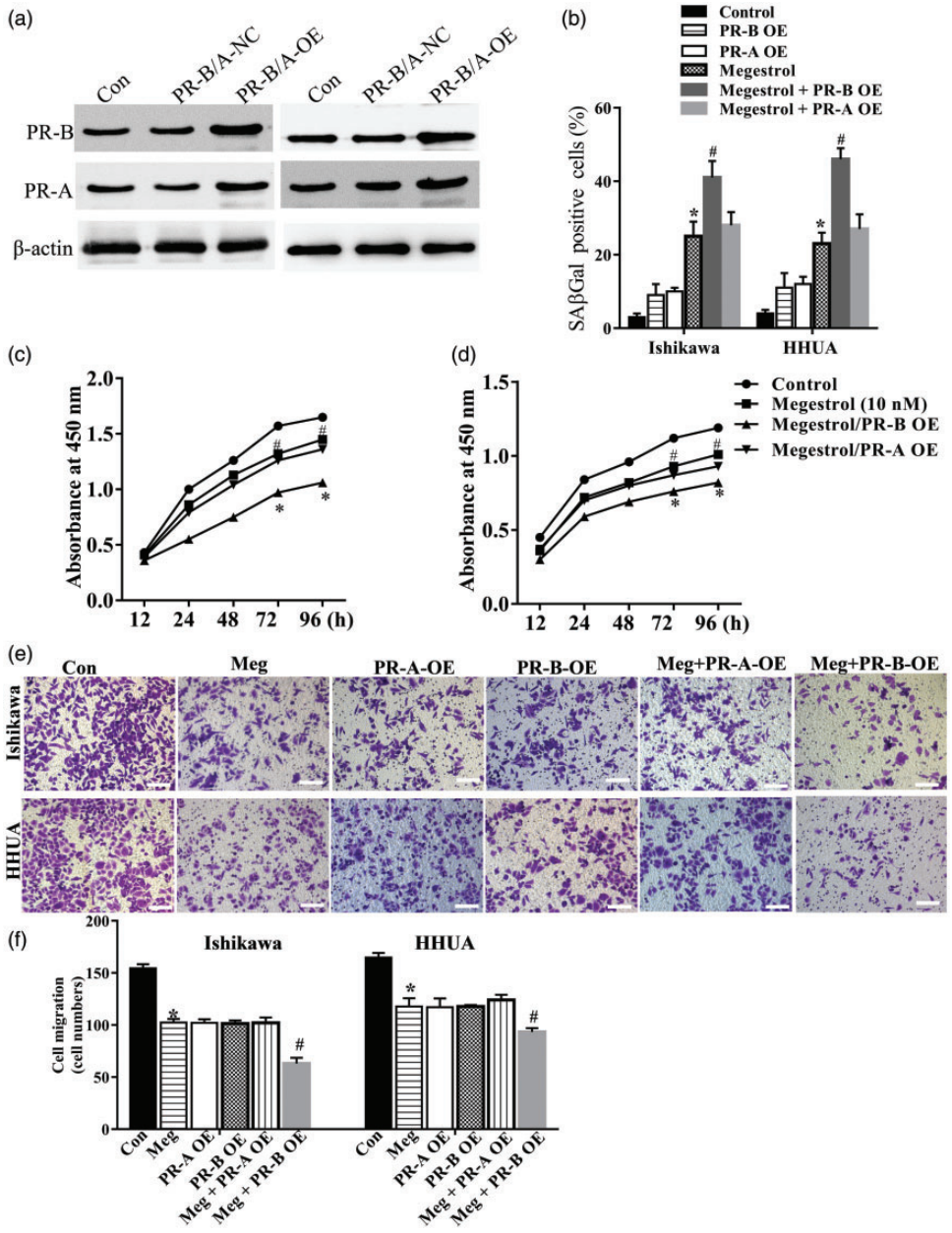

Figure 3. PR-B isoform overexpression enhances the changes of endometrial cancer cells induced by megestrol acetate. (a) The PR-A and PR-B expression in PR-B or PR-A respective plasmids transfected cells were measured by Western blotting. Cell senescence (b), cell proliferation ((c) and (d)), and migration ((e) and (f)) of PR-A and PR-B overexpressed endometrial cancer cells were then analyzed (*$P < 0.05$ vs. control; #$P < 0.05$ vs. megestrol). Bar = 50 μm. (A color version of this figure is available in the online journal.)

acetate treatment on cell growth, senescence, and cell migration in PR-A overexpression (PR-A-OE) cells was slightly enhanced or unchanged. To confirm the involvement of PR-B in megestrol acetate's effect, a PR-B specific siRNA was further utilized (Figure 4(a)). PR-B knockdown could significantly abolish megestrol acetate-induced cell activity changes, including cellular senescence (Figure 4 (b)), cell growth (Figure 4(c) and (d)), and cell migration (Figure 4(e) and (f)). These results indicated that PR-B, not PR-A, was involved in megestrol acetate's effect on endometrial cancer cells.

Megestrol acetate/PR-B axis induces senescence of endometrial cancer cells via the FOXO1 pathway

FOXO1 is a targeted gene of PR and a master regulator of cell cycle mediators (p16, p21, and p27).[26] The expression of FOXO1 protein was robustly upregulated in both Ishikawa and HHUA cells upon megestrol acetate treatment (Figure 5(a)). Using a selective small-molecular inhibitor of FOXO1, AS1842856, we found that FOXO1 inhibition alone did not exert induction or inhibition on PR-B expression (Figure 5(b)). However, the megestrol acetate-induced senescence and growth arrest were significantly abrogated

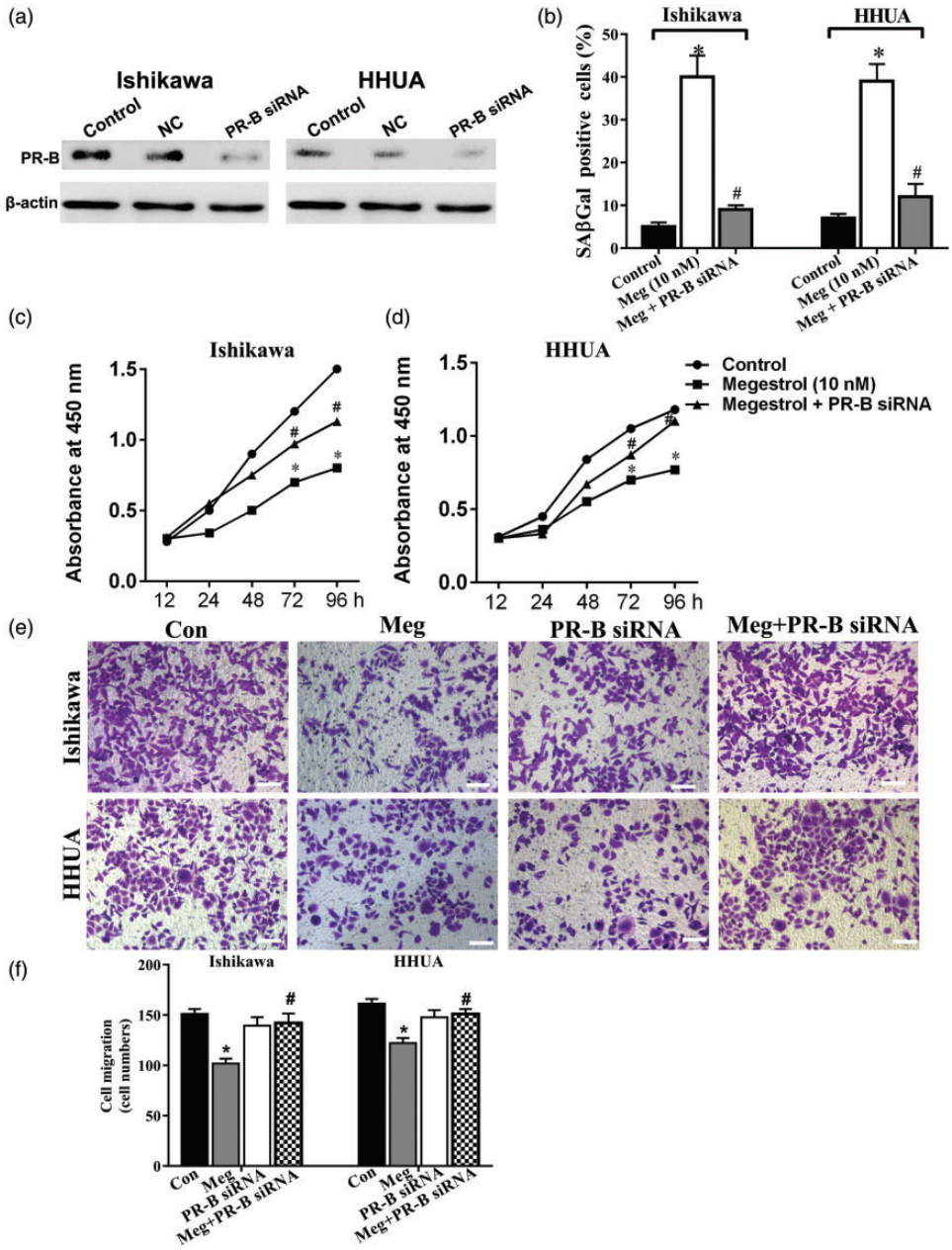

Figure 4. Megestrol acetate mediates the decreased survival of endometrial cancer cells through the PR-B isoform. (a) The PR-B expressions in PR-B specific siRNA transfected cells were measured by Western blotting. The human endometrial cancer Ishikawa and HHUA cells were treated with 10 nmol/L megestrol alone or with PR-B specific siRNA, cell senescence was detected (b), cell growth was detected by CCK8 assay ((c) and (d)), and cell migration was detected by transwell ((e) and (f)) ($^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. megestrol). Bar = 50 μm. (A color version of this figure is available in the online journal.)

upon AS1842856 treatment (Figure 5(c) to (e)). Cell migration inhibited by megestrol acetate in both cells was also alleviated upon AS1842856 treatment (Figure 6(a) and (b)). Similar changing patterns were observed in the expression of p21 and p16 in megestrol acetate-treated endometrial cancer cells (Figure 6(c)). These results indicate that megestrol acetate may induce the senescence of endometrial cancer cells by FOXO1 through PR-B isoform.

Discussion

In the present study, we demonstrate that megestrol acetate could induce irreversible G1 arrest and cellular senescence, affect the survival and growth of endometrial cancer cells, thus exhibiting anticancer property in human endometrial cancer. Moreover, megestrol acetate exerts its anticancer roles in human endometrial cancer through PR-B/FOXO1. These results provide a novel understanding for the molecular mechanism of megestrol acetate in the treatment human endometrial cancer.

Megestrol is a synthetic progesterone with high efficacy. It has short-acting contraceptive role in the general use by oral or injection manner. Notably, megestrol and other progesterones are widely used in the clinical treatment of various cancers, especially malignant gynecologic carcinoma. In the treatment of advanced breast cancer, megestrol acetate provided effective palliation. In a two-stage phase II

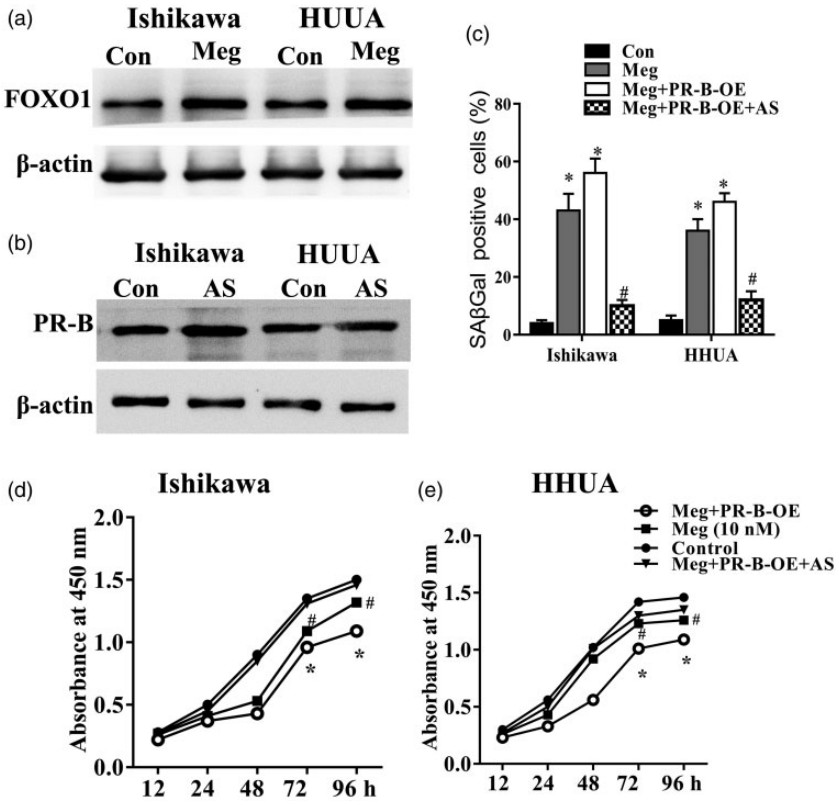

Figure 5. FOXO1 participates in megestrol/PR-B axis-induced senescence. (a) FOXO1 expression was increased in megestrol treated Ishikawa and HHUA cells. Ishikawa and HHUA cells or PR-B overexpressed Ishikawa and HHUA cells were treated with megestrol alone or pretreated with AS1842856, cell senescence was detected by SAβGal staining (b), cell growth was detected by CCK8 assay ((c) and (d)) (*$P < 0.05$ vs. control; #$P < 0.05$ vs. megestrol).

trial with postmenopausal women who had hormone-sensitive advanced breast cancer and experienced disease progression on a third-generation non-steroidal aromatase inhibitor, megestrol acetate had demonstrated activity and acceptable tolerability.[27] In a phase II trial with patients with platinum-refractory epithelial ovarian cancer, megestrol acetate showed modest but definite activity.[28] *In vitro* studies showed that progesterone treatment modulated protein expressions in human endometrial cancer cell lines. Besides their inhibitory effect on cell growth, morphologic changes such as multinucleation, multinucleolation, vacuolation, and extensive Golgi apparatus upon progesterone treatment were also reported.[29,30] The clinical safety and efficacy of megestrol application have been identified in multiple randomized, double-blind and placebo-controlled trials.[31–33] However, the exact anticancer molecular mechanism involved is not completely known.

As a frequently used drug in endometrial cancer patients, megestrol acetate, administered alone or combined with other drugs such as tamoxifen and metformin, exhibits high efficacy and low side effects.[15,34] Despite inhibiting the secretion of progesterone in pituitary gland, it is supposed that other potential mechanisms are also implicated in its anticancer roles.[31] In this study, we investigated the role of megestrol acetate in regulating survival and growth of endometrial cancer cells. Megestrol acetate treatment reduced the proliferation of Ishikawa cells and HHUA cells *in vitro*, suggesting that megestrol acetate is an anticancer agent in endometrial cancer by directly influencing tumor cell growth.

Senescence is a genetically regulated mechanism in normal development. Disruption of cell cycle progression is an important cytological factor leading to the occurrence and development of tumor.[35,36] And induction of tumor cell senescence is also a therapeutic mechanism of various antitumor drugs. Herein, we found that megestrol acetate significantly inhibited cell cycle progression, and induced irreversible G1 arrest and senescence of endometrial cancer cells. Consistently, expression of cyclin D1 was remarkably downregulated, whereas p21 and p16 were increased in megestrol acetate treated endometrial cancer cells. These findings suggest that megestrol acetate suppresses cell cycle progression, which is consistent with a previous report showing that downregulation of SUSD2, endometrial mesenchymal stem cell marker, induces endometrial cancer cells into senescence and death.[24]

PRs are the main mediators recognizing megestrol acetate and other progestagens. Decreased expressions of PR isoforms PR-A and PR-B are associated with the initiation and progression of various gynecological cancers.[20,37] In breast cancer, loss of PR-B expression is observed, and hydroxyprogesterone (OHPg) can activate PR-B to further drive the autophagy in human breast cancer cells.[38] The altered expression and functional roles of PR also have been found in endometriosis and other related diseases. Overexpressed PR-B inhibits tumor growth by suppressing

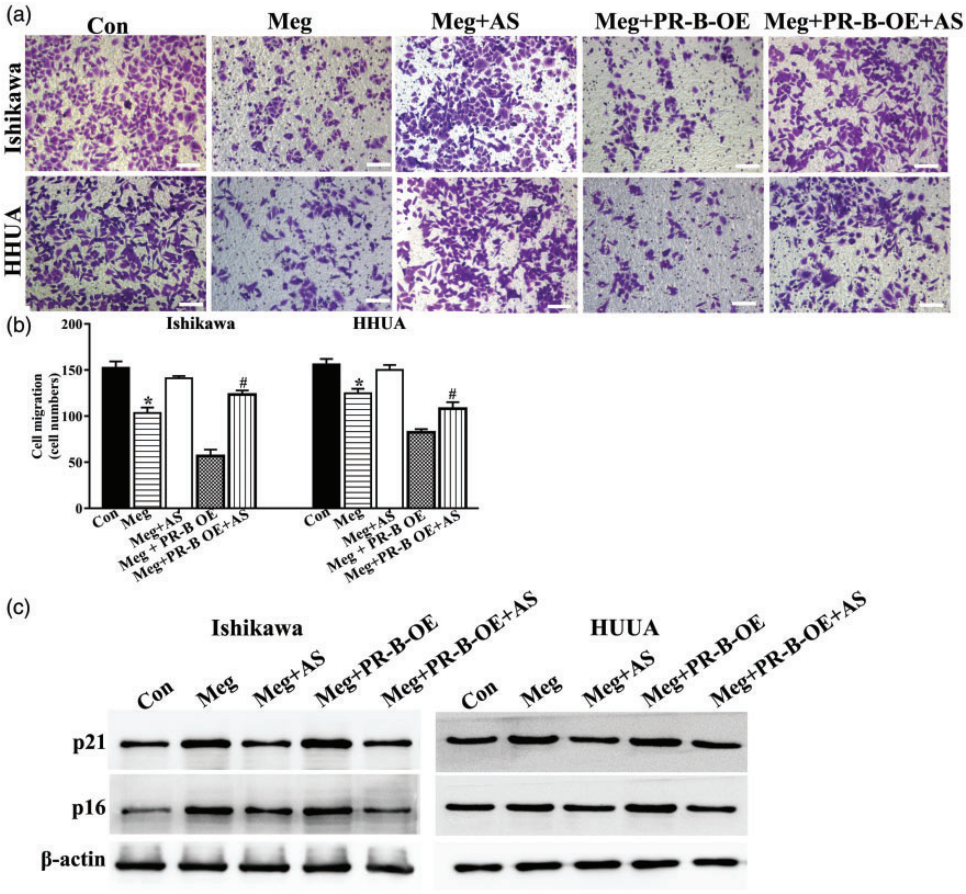

Figure 6. FOXO1 participates in megestrol/PR-B axis-induced cell cycle arrest and megestrol/PR-B axis inhibits migration of endometrial cancer cells. Ishikawa and HHUA cells or PR-B overexpressed Ishikawa and HHUA cells were treated with megestrol alone or pretreated with AS1842856, and cell migration was detected by transwell ((a) and (b)), and the expressions of p21 and p16 were detected by Western blotting ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. megestrol). Bar = 50 μm. (A color version of this figure is available in the online journal.)

the invasive activity of EC cells via affecting the expression of matrix metalloproteinases.[23] PR transcriptional activity is commonly linked to the expression of many cell cycle regulators such as cyclin-dependent kinase and p21/p27 families. In this study, we found that PR-B, not PR-A, participated in the anticancer role of megestrol acetate in endometrial cancer. FOXOs participate in cell proliferation, oxidative stress, and apoptosis. In PR-B+ ovarian cancer cells, FOXO1 and p21 are required for progestin-mediated cellular senescence.[39,40] In this study, we found that FOXO1 inhibition abrogated the megestrol acetate-induced growth arrest and senescence of endometrial cancer cells, suggesting that the FOXO1 pathway might be involved in the decreased survival of endometrial cancer cells mediated by megestrol acetate/PR-B. From a clinical perspective, FOXO1 might be a potential therapeutic target, and compounds which are capable of regulating FOXO1 activities may be necessary to enhance the efficacy of megestrol acetate in the future treatment of endometrial cancer and other cancers.

In summary, the present study reveals that megestrol acetate can induce the growth arrest and senescence of endometrial cancer cells through PR-B/FOXO1/p21 axis, thus contributing to the suppression of human endometrial cancer. These findings may provide new understanding for treating human endometrial cancer.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. SHR conceived and designed the study, and reviewed the article. WH performed the experiments and wrote the article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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(Received February 9, 2021, Accepted May 26, 2021)