Forkhead Transcription Factor FOXO1 is a Direct Target of Progestin to Inhibit Endometrial Epithelial Cell Growth

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Abstract

Purpose and experimental design: Despite the therapeutic utility of progestin in invasive and preinvasive endometrial neoplasias, the molecular mechanisms through which it exerts inhibitory effects on endometrial epithelial growth are largely unknown. The aim of the study was to clarify the molecular mechanisms of progestin action to endometrial epithelial cells using originally established in vitro and in vivo treatment models for immortalized and transformed endometrial epithelial cell lines that express progesterone receptor.

Results: In this model, progestin effectively inhibited the cell growth, inducing G0/G1 arrest rather than apoptosis without p21/WAF-1 induction. Using DNA microarray analysis, we identified 24 genes whose expression increased more than 10-fold on progestin treatment. Of these genes, we paid special attention to forkhead box transcription factor FOXO1, known as a key gene for endometrial decidualization. Progestin markedly induced FOXO1 gene expression mainly in the nuclei in vitro and in vivo. This induction was not due to the canonical activation of FOXO1 via protein dephosphorylation but due to FOXO1 promoter activation and mRNA induction. siRNA inhibition of FOXO1 significantly attenuated the effects of progestin to inhibit endometrial epithelial cell growth. Disrupting Akt activity by the introduction of the dominant negative form of Akt increased nuclear FOXO1 accumulation and enhanced the effect of progestin.

Conclusion: These findings suggest that FOXO1 is a direct target of progestin, implicating novel molecular mechanisms of progestin to eradicate endometrial neoplasia. Clin Cancer Res; 17(3); 1–13. ©2010 AACR.

Introduction

Endometrial cancer accounts for approximately 50,000 annual deaths worldwide and is the most common invasive neoplasia of the female genital tract in North America, where the incidence is highest (22.0 per 100,000 people per year) (1). The prognosis of this type of cancer is relatively favorable, mainly because the disease is usually diagnosed while it is limited to the corpus of the uterus, in which case the lesions are surgically treated by hysterectomy and bilateral salpingo-oophorectomy with or without retroperitoneal lymphadenectomy. However, patients with disseminated disease or those who have recurrence at distant sites after initial surgery have few options for systemic therapy. Because low-grade endometrial cancer is susceptible to hormonal influences in a significant proportion of progesterone receptor (PR)-positive cases, progestin has been used as a treatment with recurrent or disseminated disease with such characteristics. Most studies have employed oral progestogens, including medroxyprogesterone acetate (MPA), which have response rates in the range of 11% to 56% (2).

Unlike invasive endometrial cancer, the prognosis of well-differentiated endometrial cancer without myometrial invasion is excellent, with a 5-year survival rate of more than 90% (3). Therefore, younger patients with these noninvasive diseases can opt for progestin therapy instead of surgery if they wish to preserve their potential for fertility. Superior response rates ranging from 70% to 90% have been reported for progestin therapy in patients with such early stages of endometrial cancer or preinvasive hyperplasia (4, 5).

Despite the therapeutic utility of progestin to invasive and preinvasive endometrial neoplasia, the molecular mechanisms of progestin’s inhibitor effects on endometrial glands are largely unknown. Initial studies have demonstrated that
Translational Relevance

Endometrial cancer patients with recurrence at distant sites after initial surgery have few options for systemic therapy. Progestin [medroxyprogesterone acetate (MPA)] has been frequently used for such patients. Furthermore, younger patients with early stage endometrial cancer or endometrial hyperplasia can opt for progestin therapy instead of surgery if they wish to preserve their potential for fertility. However, efficacy of MPA varied among patients and no predictive parameter has been known, mainly due to lack of knowledge on the molecular mechanisms of progestin action to inhibit endometrial cell growth.

Our in vitro and in vivo treatment model has revealed that progestin directly induces FOXO via transcriptional activation to inhibit the growth of cancer and precancerous endometrial epithelial cells, indicating that FOXO is a novel target of MPA therapy, and Akt signaling, an upstream inhibitor of FOXO, is a potential predictor of MPA efficacy. This information will help define patient selection for progestin therapy.

Progestin activates p21/WAF-1 expression, triggering cell cycle inhibition; therefore, p21/WAF-1 is a key effector of progestin action (6, 7). However, most studies have used breast cancer cell lines, whose response to progestin must be different from that of endometrial cells; for example, unlike breast cancer cell lines, whose response to progestin must be characterized by the additional introduction of oncogenic mutant K-ras alleles into EM-E6/E7/TERT cells (12). Although parental EM-E6/E7/TERT cells constitutively express weak levels of PR, EM-E6/E7/TERT or EM-E6/E7/TERT/RAS cells were further transfected with retroviral progestin receptor B (PRB) expression vector (MSCVbsd-PRB) so that these transfectants, named EM-E6/E7/TERT/PR or EM-E6/E7/TERT/RAS/PR cells, strongly and stably expressed PRB (Fig. 1A). Akt signaling is one of the major inhibitory pathways for FOXO1 activity. To clarify the role of FOXO1 as a target of progestins, we introduced dominant negative alleles of Akt (DN-Akt) by retroviral transfer (12) into EM-E6/E7/TERT/PR cells to establish EM-E6/E7/TERT/PR/DN-AKT cells. These endometrial epithelial cell lines were basically maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO₂ at 37°C. Tumorigenic endometrial epithelial cells were maintained in DMEM with 10% FBS. MCF-7 (Michigan Cancer Foundation-7) cells were purchased from the American Type Culture Collection and used as a positive control for progesterone receptor A (PRA) and PRB expressions. Ishikawa cells were kindly provided by Dr. Masato Nishida (National Kasumigaura Hospital, Tsuchiura, Japan).

RT-PCR assay

The expression of p21/WAF-1, PRB, and FOXO1 mRNAs was analyzed by reverse transcriptase-PCR (RT-PCR) amplification. The primer pairs used were: 5'-CTTCTTGTGGCCCGTGACGAC-3' (forward), 5’–CCGTITITCAGACCCCTGAGAG-3' (reverse) for p21/WAF-1, 5’– ACAGAATTCAGACTGAGCTGAAGGCAAAGGGT - 3' (forward), 5’–ACAGAATTCAAAAACGGAAGACAGCTGCTAGA - 3' (reverse) for PRB (744–1173, 429 bp) (13), 5’–TGAGACTGTCCAGGAGATC-3'(forward), 5’-TTGGTCAGCCGGCCTTC-3' (reverse) for FOXO1.

For the detection of FOXO1 mRNA, EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells were cultured in growth medium for 24 h and then incubated with or without MPA (10 nM) for different time periods. To examine whether FOXO1 mRNA expression depended on de novo protein synthesis, cycloheximide was simultaneously added with MPA at a final concentration of 10 μg/mL. Total RNA was then isolated from the cells using Isogen (Nippon Gene) according to the manufacturer’s protocol, and cDNA was synthesized from 1 μg of RNA using the RNA PCR kit version 2 (TakaRa) with random primers. Typically, 2 μl aliquots of the reverse-transcribed cDNA were amplified by 28 cycles of PCR in 50 μL of 1× buffer [10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, and 50 mM KCl] containing 1 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Gene Taq (Nippon Gene), and 0.2 μM primers. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s for p21/WAF-1 or FOXO1, or at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min for PRB or PRAB. The PCR products were resolved by electrophoresis.
on 7% polyacrylamide gels and stained with SYBR green I (FMC BioProducts). The efficiency of cDNA synthesis from each sample was estimated by PCR using glyceraldehyde 3 phosphate dehydrogenase (GAPDH)-specific primers as described previously (14).

**Cell growth assay**

Cells preincubated in growth media for 24 h were incubated in 6-well plates in growth media or in serum-depleted media with phenol red-free DMEM in the absence or presence of MPA (MPA; 10 nM) or progesterone (10 nM), and cell growth was evaluated by counting the number of cells or measuring the incorporation of 5-Bromo-2-0-deoxyuridine (BrdU) on the indicated days. The BrdU incorporation was detected using the DELFIA cell proliferation kit (PerkinElmer) according to the manufacturer's protocol as previously reported (15). EtOH was added so that its concentration was normalized to 0.1% in control and MPA- or progesterone-treated samples.

**Cell cycle analysis**

EM-E6/E7/TERT/PR cells were cultured in growth media in 6-well plates to 70%-80% confluence; then the media were replaced with serum-depleted phenol red-free DMEM and incubated for 24 h in the absence or presence of MPA at 10 nM. The cells were harvested and fixed overnight with 3 mL of ice-cold 80% ethanol. The fixed cells were then centrifuged, suspended in lysis buffer (100 mmol/L sodium citrate and 0.1% Triton X-100), and incubated for 15 min at room temperature before incubating with RNase A (10 mg/mL; Sigma Chemical) for 10 min at room temperature. DNA was stained with propidium iodide (50 μg/mL) for at least 1 h at 4°C. The DNA content was determined by flow cytometry (Beckman Coulter) and EXPO 32 software.

**Western blot analysis**

For examining PRA and PRB expressions, nuclear extracts were prepared from EM-E6/E7/TERT cells (15). The antibody against PR used in the western blot analysis recognized both PRA (81 kDa) and PRB (116 kDa), distinguishing each isoform by band size. MCF-7 cells were used as a positive control for PRA and PRB. C, change in cell shape of EM-E6/E7/TERT/PR cells following treatment with MPA for 72 h. Cells treated with MPA exhibited thinner and longer morphology.
Membranes were blocked by immersion in TBST [150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 0.1% Tween] containing 5% non-fat dried milk. They were then incubated with a specific antibody against PR [Progesterone Receptor Ab-8 (clone hPRa2+ hPRa3; Lab Vision), p21/WAF (SC-469; Santa Cruz Biotechnology), PTEN (ABM-2052 (clone 6H12.1); Cascade BioScience), p-AKT (#4058; Cell Signaling Technology), or FOXO1 (#2880, Cell Signaling Technology). Next, the membranes were reacted with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories). The membranes were also probed with anti-actin antibody (Sigma) to normalize the differences among the samples. The LAS3000 CCD-Imaging System (Fujiﬁlm Co. Ltd.) was used for detection and quantiﬁcation of proteins visualized by Lumi-LightPlus Western Blotting Substrate (Roche).

Nude mice xenograft experiments

EM-E6/E7/TERT/RAS or EM-E6/E7/TERT/RAS/PR cells were resuspended in a Hanks’ balanced salt solution (Sigma) and subcutaneously injected (5 × 10⁴ cells/mouse) at the base of the left flank of female 7- to 9-week-old ovariectomized BALB/c nu/nu mice (SLC). When tumors were seen after 3 weeks, hormone pellets consisting of placebo or progesterone (200 mg/pellet, 60-day release) (Innovative Research of America) were placed subcutaneously into the backs of the mice. Blood samples were collected from the tail vein, 10–14 days after pellet implantation, for measuring serum concentration of progesterone by ELISA (SRL, Inc.). Tumors were then monitored weekly for growth and were collected 4 weeks after pellet implantation, for measuring serum concentration of progesterone (Biogenex). Then, endogenous peroxidase was blocked by immersing the sections in 0.3% H₂O₂ methanol for 30 min. The reaction was visualized with the EnVision Detection Kit (DAKO Cytometion) using diaminobenzidine tetrahydrochloride as the enzyme substrate. All sections were counterstained with GM hematoxylin stain solution (Muto Pure Chemicals Co., Ltd.). For negative controls, the non-reactive rabbit immunoglobulin fractions (X0903; Daco) or mouse IgG2a (X0943; Daco) was used, instead of the primary FOXO1 or cytokeratin antibodies, respectively.

DNA microarray analysis

For MPA-responsive gene expression proﬁling, EM-E6/E7/TERT/PR cells were cultured in growth medium for 24 h and then incubated with or without 10 nM MPA for 24 h.

The Agilent Whole Human Genome Oligo Microarrays (G4112A) containing 44,000 60-mer oligonucleotide probes representing 41,000 unique genes and transcripts were used for the experiments. Two types of total RNA samples were prepared: total RNA samples extracted from cells after 24 h with or without MPA treatment. Sample labeling and microarray processing was performed as detailed in the “One-Color Microarray-Based Gene Expression Analysis” (version 1.0, part number G4410-90040) protocol. Briefly, the Agilent One-Color Spike-Mix (part number 5188-5282) was diluted to 5,000-fold and 5 L of the diluted spike-in mix was added to 500 ng of each of the total RNA samples prior to labeling reactions. The spike-in mix consists of a mixture of 10 in vitro synthesized, polyadenylated transcripts derived from the adenosine E1A gene. The labeling reactions were performed using the Agilent Low RNA Input Linear Ampliﬁcation Kit (part number 5183-3523) in the presence of cyanine 3-CTP (Perkin Elmer part number NEL 580). For microarray hybridization, 500 ng of cyanine-3-labeled cRNA was fragmented and hybridized on the Agilent Whole Human Genome microarrays at 65°C for 17 h using the Agilent Gene Expression Hybridization Kit (part number 5188-5242). The hybridized microarrays were dissembled at room temperature in Gene Expression Wash Buffer 1 (part number 5188-5325), and then washed in the same buffer at room temperature for 1 min. This was followed by a 1-min wash in Gene Expression Wash Buffer 2 (part number 5188-5326) at an elevated temperature. The processed microarrays were scanned with the Agilent DNA microarray scanner (part number G2565BA) and extracted with Agilent Feature Extraction software (version 8.5, part number 25675AA). The resulting text ﬁles were loaded into the Agilent GeneSpring GX software (version 7.3) for further analysis.

The microarray data set was normalized in GeneSpring GX using the following scheme: First, data transformation:
Intensity measurements less than 0.01 were set to 0.01. Second, per-chip normalization: Normalize to 70 percentile. Each intensity measurement on a microarray was divided by the 70-percentage intensity of all measurements on that microarray. Per-chip normalization removes any systemic error in signal intensities between chips. Third, per-gene normalization: Normalize to specific samples. For each gene, intensity values in all samples were normalized to the intensity value for that gene in the control samples. Per-gene normalization was carried out to investigate the relative gene expression of each sample after MPA treatment compared with specific control samples. We compared microarray data between samples with and without MPA treatment. Microarray data are supplied on our Supplemental Materials site.

**Luciferase reporter assay**

The 2.0 kb 5’-upstream region of the FOXO1 gene (-1993 to -18: numbering based on the first ATG of FOXO1 gene) was PCR-amplified from genomic DNA using primer set, 5’-CTAATTTATTTTCCCTTCCCCT-3’ (forward) and 5’-AGGGGCGGGGGGTACCC-3’ (reverse), and inserted into the luciferase reporter plasmid pGL3-basic (Promega), named p-FOXO1-pro. EM-E6/E7/TERT/PR cells were preincubated in growth media in 24-well plates for 24 h were incubated in serum-depleted phenol red-free DMEM and transfected with 0.4 µg of reporter plasmid using Lipofectamine PLUS (Invitrogen Corp.) according to the manufacturer’s protocol. Cells were simultaneously treated with 10 nM MPA for 48 h before being harvested and cell extracts prepared. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega), in which Renilla luciferase plasmids were cotransfected as the Dual-Luciferase Reporter Assay System (Promega), in which Renilla luciferase plasmids were cotransfected as controls to standardize transcription efficiency. All experiments were performed at least three times for each plasmid, controls to standardize transcription efficiency. All experiments were performed at least three times for each plasmid, and the relative luciferase activity reported here is the mean of the three results.

**siRNA inhibition assay**

EM-E6/E7/TERT/PR cells were seeded overnight in the growth media in 6-well plates and transfected the next day with 25 nmol/L of non-specific scramble siRNAcontrol (Ambion) or FOXO1A-specific siRNA (Ambion) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s recommendations. Forty-eight hours after transfection, cells were incubated in serum-depleted phenol red-free DMEM in the absence or presence of 10 nM MPA for 72 h before the cell number was counted in each sample to evaluate the effects of MPA on cell growth.

**Statistical analysis**

The data were basically presented as the mean ± SD of triplicated assays. Differences between groups were evaluated using Student’s t-test. A P value of less than 0.05 was considered to indicate statistical significance.

**Results**

**Progestin inhibits the growth of endometrial epithelial cell lines without p21/WAF-1 induction**

We have previously established immortalized endometrial epithelial cells (EM-E6/E7/TERT cells) (Fig. 1A) (11) that sustain the functional characteristics of primary cells, including steroid responsiveness; estrogen treatment promoted cell growth in vitro whereas progesterin treatment inhibited it, although these responsiveness weakened with increased population doublings. Tumorigenic endometrial epithelial cells (EM-E6/E7/TERT/RAS) were established by the additive introduction of oncogenic mutant K-RAS alleles into EM-E6/E7/TERT cells, which have the potential to form colonies on soft agar and tumors on nude mice (12). Basically, these immortal and transformed cells express weak levels of estrogen receptor α (ERα) as well as PR. To enhance the effect of progesterin, stable cell lines expressing higher levels of PR were established by the additive introduction of PRB cDNA into immortal and transformed cells, named EM-E6/E7/TERT/PR and EM-E6/E7/TERT/RAS/PR cells, respectively. The expression of PR was then confirmed in these cells. RT-PCR assays confirmed that all these immortal cells expressed PRB. Western blot analysis revealed that EM-E6/E7/TERT/PR or EM-E6/E7/TERT/RAS/PR cells exhibited high levels of PRB expression with weak or faint levels of PRA expression (Fig. 1B), as shown in our recent study (15).

We first examined the effect of progesterin on these cells in vitro. EM-E6/E7/TERT/PR or EM-E6/E7/TERT/RAS/PR cells were cultured in growth media and treated with 10 nM MPA for different time periods and the cell growth was monitored. The cells exhibited a longer and thinner morphology by the treatment with MPA (Fig. 1C). Significant growth retardation was observed after 4–5 days of treatment (Fig. 2A and B). In contrast, cells without PR overexpression (EM-E6/E7/TERT/vec or EM-E6/E7/TERT/RAS/vec) lacked the inhibitory effect of MPA (Fig. 2C and D), indicating that the growth inhibition was PR-dependent. We also confirmed MPA responsiveness in serum-depleted conditions as well (Supplementary Fig. 1). Significant growth inhibition was similarly observed in cells with PR overexpression but not those without it, although the extent of inhibition was lesser than cells incubated in growth media, probably because decreased proliferative activity by serum depletion masked the inhibitory effect of MPA. Furthermore, we tested the effect of another progestin, progesterone, on these cells. Progesterone inhibited the growth of EM-E6/E7/TERT/PR or EM-E6/E7/TERT/RAS/PR cells in a similar fashion (Fig. 2E and F) but not EM-E6/E7/TERT/vec or EM-E6/E7/TERT/RAS/vec cells (data not shown). Flow cytometric analysis revealed that MPA increased G0/G1 fractions by 5% to 6% and sub-G0/G1 fractions by 1% to 2% (Supplementary Fig. 2). To examine whether or not p21/WAF-1 was involved in MPA-induced growth retardation, we measured the change in p21/WAF-1 expression on treatment with MPA. RT-PCR assays or western blot analysis revealed that there was no
significant induction of p21/WAF-1 mRNA or protein expression (Fig. 2G). Thus, p21/WAF-1 does not appear to play critical role in MPA-induced growth retardation.

We then examined the in vivo effects of progestin on the growth of tumorigenic endometrial cells. EM-E6/E7/TERT/RAS/PR cells were implanted to the flank of nude mice, and then progesterone pellets were inoculated after 3 weeks. Progesterone rather than MPA was selected because it is easy to measure its serum concentration using our available kit. As expected, progesterone concentration was significantly higher in treated groups than untreated groups [39.5 (15.1–73.2) vs. 7.5 (2.8–9.2) ng/mL, respectively]. As shown in Figure 3, tumors significantly decreased in size in treated groups. Taken together, these findings clearly show that progesterin exerted an inhibitory effect on the cell growth of endometrial epithelial cell lineages both in vitro and in vivo in a p21/WAF-1-independent manner.

Figure 2. In vitro effect of progestin on the growth of endometrial epithelial cell lines. In vitro growth assay of endometrial epithelial cell lines treated with MPA or progesterone (P4). Cells were grown in growth media with or without 10 nM MPA (A–D) or progesterone (E and F) for different time periods; cell growth was determined by counting the cell number for each period. Note that MPA as well as progesterone significantly inhibited the growth of cells with PRB overexpression (A, B, E, and F) but not those without it (C and D). Each point represents the means ± SD of triplicate determinations in three independent experiments. *P < 0.05. G, analyses of the p21 expression upon MPA stimulation. EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells were treated with or without MPA at 10 nM at different time periods, and RT-PCR and western blot analyses were performed.
Progestin induces FOXO1 expression via transcriptional activation in endometrial epithelial cell lines

To identify the molecular mechanisms of progestin’s growth inhibitory effect on endometrial epithelial cell lines, we compared gene expression profiles of EM-E6/E7/TERT/PR cells treated and untreated with MPA. Of 44,000 transcripts included in the DNA microarray, we first defined the genes induced more than 10-fold by MPA stimulation and identified 24 of them (Supplementary Table 1). Of these genes, we noticed the FOXO1 gene, because previous studies have found that progestin regulates the expression of FOXO1 in endometrial stromal cells through subcellular translocation linked to its phosphorylation status, triggering endometrial decidualization and menstruation (17).

We therefore examined the change in expression and subcellular localization of FOXO1 in endometrial epithelial cell lines following treatment with progestin. EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells were treated with MPA and western blot analyses performed using FOXO1-specific antibody. FOXO1 expression was significantly induced by treatment with MPA as well as progestosterone at 10 or 100 nM in EM-E6/E7/TERT/PR cells (Fig. 4A) but not EM-E6/E7/TERT cells (data not shown). Induction was mainly observed in the nuclei, with only weak or faint levels of FOXO1 expression in the cytoplasm. Immunocytochemical analysis using EM-E6/E7/TERT/PR cells clearly showed that FOXO1 was mainly induced in the nuclei by MPA (Fig. 4B). Thus, progestin facilitates FOXO1 expression in a PR-dependent manner in vitro. This was not due to a change in the subcellular localization of FOXO1 (from cytoplasm to nucleus) because no significant FOXO1 expression was observed in the cytoplasm of untreated cells (Fig. 4A).

We then confirmed the induction of FOXO1 expression in vivo by the treatment with progesterone. Mouse tumors of EM-E6/E7/TERT/RAS/PR cells treated or untreated with progesterone pellets were collected and subjected to western blot analysis. As shown in Figure 4C, FOXO1 protein expression was upregulated by progesterone treatment in tumor tissues. Immunohistochemistry also showed that tumors exhibited marked induction of FOXO1 mainly in the nuclei when treated with progesterone (Fig. 4C).

We investigated the molecular mechanisms of FOXO1 induction upon MPA treatment. RT-PCR analysis was performed for FOXO1 mRNA expression using EM-E6/E7/TERT/PR cells treated or untreated with MPA. FOXO1 mRNA was significantly upregulated 8–72 h after the treatment (Fig. 5A). This activation was not blocked by treatment with cycloheximide, indicating that MPA directly activates FOXO1 mRNA expression without de novo protein
synthesis. We confirmed that this activation was not obvious in cells lacking PR-overexpression (EM-E6/E7/TERT cells) (Fig. 5A), suggesting that FOXO1 activation by MPA is PR-dependent. We further performed the luciferase reporter assays using FOXO1 gene promoter spanning 2.0 kb sequences upstream of the first ATG. As shown in Figure 5B, MPA treatment of EM-E6/E7/TERT/PR cells activated the FOXO1 promoter approximately by 3-fold, while no activation was observed in EM-E6/E7/TERT cells. These findings indicate that MPA directly induces FOXO1 expression via the transcriptional activation of FOXO1. To analyze the status of signaling pathway, which regulates subcellular localization of FOXO1, we examined the expression of PTEN and p-AKT as critical components of phosphatidylinositol 3-kinase (PI3K)/AKT pathway. As shown in Figure 5C, activation of PTEN expression or reduction of p-AKT expression, both of which facilitate nuclear retention of FOXO1, was not observed by the treatment with MPA.

**FOXO1 mediates progestin to inhibit epithelial cell growth**

To investigate the role of FOXO1 in the effect of progestin, a knockdown experiment for FOXO1 was performed via siRNA inhibition. EM-E6/E7/TERT/PR cells were transfected with siRNA against FOXO1 and treated...
with or without MPA at 10 nM, followed by monitoring cell growth. Western blot analysis confirmed that knock-down was successful, exhibiting apparently decreased FOXO1 expression. These cells showed only minimally inducible FOXO1 expression on treatment with MPA (Fig. 6). In the absence of MPA, cells with knocked-down FOXO1 had increased growth rate compared with those with control siRNA, indicating that endogenous FOXO1 plays some role in cell proliferation. Treatment with MPA significantly inhibited the growth of cells transfected with control siRNA, while the inhibition was largely abrogated in those with knocked-down FOXO1. Thus, the effect of MPA was attenuated via knockdown of FOXO1, supporting the role of FOXO1 in progestin action. We sought to confirm whether similar effect was observed in other endometrial cancer cell lines as well. FOXO1 knockdown was performed in Ishikawa cells in the same way and MPA effect was examined (Supplementary Fig. 3). Ishikawa cells exhibited growth inhibition as well as FOXO1 induction by the treatment with MPA, but with lesser extent, probably due to very low levels of PR expression. FOXO1 knockdown effectively cancelled growth inhibition by MPA.

**Akt signaling limits progestin action on endometrial epithelial cell growth**

FOXO family members are direct downstream targets of the PI3K/Akt signal transduction pathway. Activation of PI3K/Akt signals phosphorylates FOXO proteins, resulting in cytoplasmic retention and inhibiting their transcriptional activity. Therefore, we speculated that Akt signaling might affect the action of progestin. To investigate this possibility, a special cell line, named EM-E7/TERT/PR/DN-AKT, was established with an introduced dominant...
negative allele of Akt gene from EM-E7/E7/TERT/PR cells. Introduction of the dominant negative allele of Akt gene has been confirmed to inhibit Akt function in endometrial epithelial cell lines (12). Both cells exhibited similar growth rate in the absence of MPA (Fig. 7A). We then compared the effects of MPA on these cells. Treatment with MPA at 10 nM led to 35% growth inhibition of EM-E7/E7/TERT/PR/vector cells on day 3 and 46% on day 6 (Fig. 7A). The same treatment in EM-E7/E7/TERT/PR/DN-AKT cells resulted in 56% growth inhibition on day 3 and 66% on day 6. Thus, introducing DN-AKT caused enhanced growth inhibition by MPA.

We next examined the extent of FOXO1 induction by MPA in the nuclei of both cells (Fig. 7B). The western blot analysis revealed the enhanced FOXO1 induction in EM-E7/E7/TERT/PR/DN-AKT compared with EM-E7/E7/TERT/PR/vector cells on days 3 and 6. Especially, most prominent induction of FOXO1 was observed in EM-E7/E7/TERT/PR/DN-AKT on day 6, when the maximal growth inhibition was confirmed in Figure 7A. These findings proved that Akt signaling is a critical factor that limits the progestin action to endometrial epithelial cells.

Discussion

FOXO1 is a member of the FOXO subfamily of the Forkhead/winged helix family of transcription factors that is involved in cell cycle regulation and apoptosis (17): the subfamily contains the mammalian members FOXO1 (Fkhr), FOXO3a (Fkhr-11), and FOXO4 (Afx) (18, 19). The role of FOXO1 in endometrial biology has been known in relation to the process of decidualization (17, 20, 21). FOXO1 induces the expression of decidualization-specific genes of endometrial stromal cells, such as insulin-like growth factor binding protein 1 (IGFBP1), decorin (DCN), and prolactin (PRL); this is enhanced by the action of cyclic AMP (cAMP). Here, we focused on the roles of FOXO1 in progestin action on endometrial epithelial cells and clearly showed it to be a novel target of progestin to inhibit the growth of both non-tumorigenic and tumorigenic endometrial epithelial cells.

The canonical pathway of FOXO1 regulation has been thought to be on the PI3K pathway. Growth-factor–dependent activation of the PI3K pathway blocks the function of all FOXO members by Akt-dependent phosphorylation of their three conserved residues, which leads to inhibition of DNA binding, nuclear exclusion, and subsequent sequestration in the cytoplasm (22–24). Recently, a unique role of progestin in the survival of endometrial stromal cells has been reported by Labied and colleagues (17). According to their results, progestin treatment of stromal cells enhanced the expression of phosphorylated FOXO1, which, because it is strictly localized to the cytoplasm, is considered to be an inactive form. Withdrawal of progestin induced rapid nuclear translocation of FOXO1, which activated expression of BIM, a known FOXO target gene encoding for a proapoptotic Bcl-2 homology 3 domain-only protein (25). This unique role of FOXO1 in response to progestin withdrawal was demonstrated in differentiating stromal cells of the endometrium. On the other side, we found that progestin induced FOXO1 expression mainly in the nuclei upon progestin stimulation in endometrial epithelial cells. RT-PCR assay clearly showed that FOXO1 mRNA is upregulated approximately 4–8 h after treatment with MPA, even in the presence of cycloheximide. Luciferase reporter assays demonstrated that MPA upregulated the transcriptional activity of FOXO1 promoter. Therefore, our results support the direct transcriptional activation of FOXO1 gene by progestin. Computer-assisted homology search found potential PR-binding sites that have a homology with the glucocorticoid receptor-responsive element on the FOXO1 promoter (data not shown). We are currently confirming the specific interaction of PR with such sites on the FOXO1 promoter. What is the molecular mechanism of the nuclear FOXO1 accumulation upon progestin stimulation? The most probable scenario might be that MPA inhibits PI3K/AKT signaling pathway, leading to the nuclear FOXO1 translocation. However, we confirmed that PI3K/AKT pathway was not inhibited by MPA in endometrial epithelial cells (Fig. 5D). Alternatively, it is known that FOXO1 binds to PR in the nuclei (26) and this physical
interaction may account for the nuclear accumulation. Further mechanistic study will be needed to clearly dissect molecular mechanisms of nuclear accumulation of FOXO1 by progestin in endometrial epithelial cells.

A role for FOXO1 in inhibiting cell growth has recently been reported using endometrial cancer cell lines in vitro (27). Overexpression of a gain-of-function mutant of the FOXO family inhibited the growth of Ishikawa cells that constitutively express low levels of FOXO1, while siRNA inhibition of the FOXO gene in HEC-1B cells that express high levels of FOXO1 enhanced their growth. Furthermore, Ward and colleagues also demonstrated that progestins increased FOXO1 protein levels in endometrial cancer cell lines, specifically through PRB (28), supporting our data. A growth inhibitory effect of FOXO family members has been proposed in other cell types, in particular, in vascular cells (29, 30). More recently, a role of the FOXO family as a tumor suppressor has been proposed (31). To circumvent embryonic lethality, Paik and colleagues used an inducible Cre-lox system to knock out the FOXO family: the widespread somatic deletion of these genes caused thymic lymphomas and hemangiomatas, which were associated with increased cell proliferation and survival in these lineages (31). What are the downstream targets of FOXO for inhibiting cell growth? Recently, sprouty (Spry2), a negative regulator of receptor tyrosine kinases, was validated as a direct FOXO target to inhibit cell cycle progression and induce apoptosis (31). Several other forkhead-responsive genes have been reported, including insulin-like growth factor-binding protein-1 (IGBP-1), glucose-6-phosphatase, Fasl, Trail, and Bim (20, 25, 32–35). However, in DNA microarray and RT-PCR analyses, we failed to observe upregulation of these candidate genes upon MPA stimulation (data not shown). Therefore, at present, it remains unclear how cell cycle arrest at G0/G1 is conferred by FOXO1 in endometrial epithelial cells.

In clinical practice of cancer treatment, we have no reliable parameter to predict the efficacy of MPA therapy. We found that the Akt signal, an upstream inhibitory factor of FOXO family, limits the effect of progestin. As we cannot predict the activation of FOXO by MPA before the treatment, the status of Akt activation could be an alternative predictor of MPA therapy. Our preliminary data show that patients responsive to MPA therapy have decreased p-AKT expression confirmed by immunohistochemistry in pretreated samples. It is well known that RAS signalings lead to AKT activation in various cancers via cross-talk signalings (36). It is therefore possible that activated RAS signalings (such as via RAS mutation) disturb MPA responsiveness and is an additional predictor of MPA therapy. In the present study, EM-E6/E7/TERT/RAS/PR cells with oncogenic KRAS mutation well responded to MPA (Figs. 2 and 3). These cells exhibit only weak levels of p-AKT expression, lacking AKT activation even with KRAS mutation (data not shown). Furthermore, in clinical samples, KRAS mutation is not always associated with AKT activation in endometrial cancer (37). Therefore, the status of RAS does not appear to be a strong predictor of MPA response, but this point requires further investigation.

In summary, our in vitro and in vivo treatment model has, for the first time, revealed that progestin targets FOXO via transcriptional activation to inhibit the growth of both non-transformed and transformed endometrial...
epithelial cells without p21/WAF-1 induction. Further investigations of the FOXO1 target genes as well as of AKT signaling as a predictor of MPA efficacy are required to fully understand the molecular mechanisms of progestin effects and help define patient selection for progestin therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

We greatly thank Ms. Tamami Ryu for her technical assistance. This study was supported by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (JSPS) and the Megumi Medical Foundation of Kanazawa University.

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Received May 14, 2010; revised August 3, 2010; accepted August 5, 2010; published OnlineFirst December 3, 2010.


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Clin Cancer Res  Published OnlineFirst December 3, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-1287

Supplementary Material  Access the most recent supplemental material at:
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