Table W1. Primers and Antibodies Used in This Study.

Gene Name	Forward PCR Primer	Reverse PCR Primer	Roche Universal, No.
GCLC	ggatgatgctaatgagtctgacc	tctactctccatccaatgtctgag	25
GSR	aacaacatcccaactgtggtc	ccatatttatgaatggcttcatctt	83
GAPDH	ccaaccgcgagaagatga	ccagaggcgtacagggatag	64

Antibodies Used in This Study

Antigen	Vendor		Clone No.	Dilution for Immunoblot
NRF2	Santa Cruz	Polyclonal		×500
β-Actin	Sigma	Monoclonal	AC-15	×1000
Lamin B1	Santa Cruz	Polyclonal		×500

Table W2. Pathologic Diagnosis of the Analyzed Sarcoma Cases.

Case No.	Histology
028-L	Leiomyosarcoma
029-L	Leiomyosarcoma
032-L	Leiomyosarcoma
034-L	Leiomyosarcoma
035-L	Leiomyosarcoma
037-L	Leiomyosarcoma
038-L	Leiomyosarcoma
039-L	Leiomyosarcoma
040-L	Leiomyosarcoma
093-mL	Liposarcoma, myxoid
094-mL	Liposarcoma, myxoid
095-mL	Liposarcoma, myxoid
096-mL	Liposarcoma, myxoid
097-mL	Liposarcoma, myxoid
098-mL	Liposarcoma, myxoid
099-mL	Liposarcoma, myxoid
100-mL	Liposarcoma, myxoid
101-mL	Liposarcoma, myxoid
102-mL	Liposarcoma, myxoid
103-mL	Liposarcoma, myxoid
105-mL	Liposarcoma, myxoid
106-mL	Liposarcoma, myxoid
108-mL	Liposarcoma, myxoid
020-M	MPNST
022-M	MPNST
023-M	MPNST
024-M	MPNST
025-M	MPNST
026-M	MPNST
056-mM	Myxoid MFH
057-mM	Myxoid MFH
060-mM	Myxoid MFH
062-mM	Myxoid MFH
063-mM	Myxoid MFH
065-mM	Myxoid MFH
066-mM	Myxoid MFH
067-mM	Myxoid MFH
068-mM	Myxoid MFH
069-mM	Myxoid MFH
070-mM	Myxoid MFH
073-pM	Pleomorphic MFH
074-pM	Pleomorphic MFH
075-pM	Pleomorphic MFH
077-pM	Pleomorphic MFH
078-pM	Pleomorphic MFH
079-pM	Pleomorphic MFH

Table W2. (*continued*)

Case No.	Histology
080-pM	Pleomorphic MFH
081-pM	Pleomorphic MFH
082-pM	Pleomorphic MFH
083-pM	Pleomorphic MFH
084-pM	Pleomorphic MFH
085-pM	Pleomorphic MFH
086-pM	Pleomorphic MFH
087-pM	Pleomorphic MFH
088-pM	Pleomorphic MFH
RMS-16	Rhabdomyosarcoma
RMS-17	Rhabdomyosarcoma
RMS-18	Rhabdomyosarcoma
043-S	Synovial sarcoma
044-S	Synovial sarcoma
045-S	Synovial sarcoma
046-S	Synovial sarcoma
049-S	Synovial sarcoma
051-S	Synovial sarcoma
052-S	Synovial sarcoma
054-S	Synovial sarcoma
055-S	Synovial sarcoma
SS1	Synovial sarcoma
SS11	Synovial sarcoma
SS12	Synovial sarcoma
SS13	Synovial sarcoma
SS14	Synovial sarcoma
SS2	Synovial sarcoma
SS3	Synovial sarcoma
SS4	Synovial sarcoma
SS5	Synovial sarcoma
SS6	Synovial sarcoma
SS7	Synovial sarcoma
SS8	Synovial sarcoma
SS9	Synovial sarcoma
OS-41	Osteosarcoma
OS-44	Osteosarcoma
OS-45	Osteosarcoma
OS-46	Osteosarcoma
OS-47	Osteosarcoma
OS-49	Osteosarcoma
ES-01	Epithelioid sarcoma
ES-02	Epithelioid sarcoma
ES-04	Epithelioid sarcoma
ES-05	Epithelioid sarcoma
ES-06	Epithelioid sarcoma
ES-10	Epithelioid sarcoma

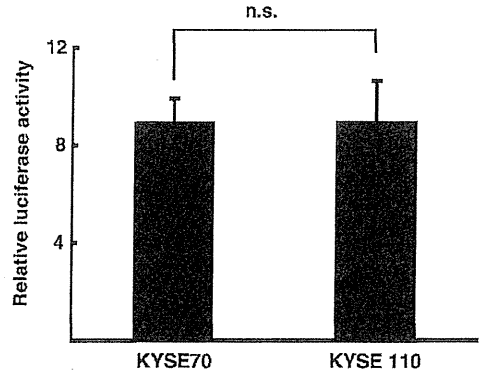

Figure W1. NRF2-dependent transcriptional activity in homozygous (KYSE70) and heterozygous (KYSE110) cells. n.s. indicates not significant.

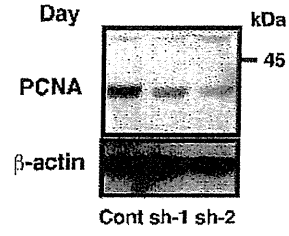

Figure W2. The expression of proliferating cell nuclear antigen (PCNA) in control (Cont) and *NRF2* (sh-1 and sh-2) shRNA expression clones. Protein extracted from each clone were immunoblotted with anti-PCNA mouse antibody (clone PC10; Cell Signaling Technologies, Danvers, MA). β-Actin was used as a loading control. Molecular marker is indicated on the right (kDa).

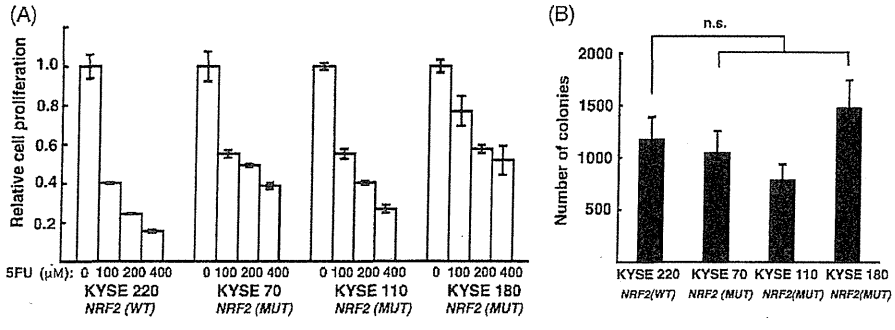

Figure W3. 5-FU sensitivity and basal colony formation capacity of *NRF2* wild-type (KYSE220) and mutated (KYSE70, KYSE110, KYSE180) ESC cell lines. (A) ESC cells were treated with various concentrations of 5-FU, and relative cell proliferation was shown. (B) Number of colony formation of each ESC cell was shown.

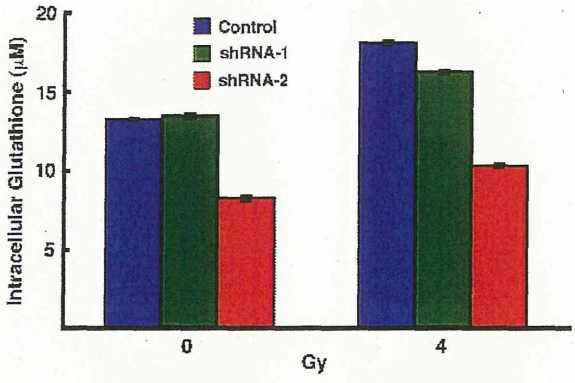

Figure W4. KYSE70 clones were irradiated (4 Gy), and the amount of intracellular reduced glutathione was measured after 24 hours.

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Clinical Cancer Research

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); 525–37. ©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 salphingo-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

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 endometrial cells, progestin occasionally has growth stimulatory effect on breast cancer cells *in vitro* and *in vivo*. Furthermore, either overexpression of ckd4 and/or cyclin D or dysfunction has been reported to frequently occur in a significant proportion of hormone-sensitive endometrial cancer, opposing p21/WAF-1 function (8–10), indicating that the p21/WAF-1 pathway is disrupted in endometrial carcinogenesis, and factors other than p21 may play crucial roles in exerting progestin action.

We have aimed to establish an *in vitro* and *in vivo* treatment model of progestin using immortalized and transformed endometrial epithelial cells expressing PR, and investigated the molecular mechanisms of progestin to inhibit the growth of endometrial glands. The microarray analyses identified the genes upregulated by progestin treatment, among which we noticed that the forkhead box O1 (*FOXO1*) gene plays a pivotal role in the progestin-mediated growth inhibition of the endometrial gland.

Experimental Procedures

Endometrial epithelial cell lineage and cell culture

EM-E6/E7/TERT cells are immortalized endometrial epithelial cells established by retroviral transduction of human papilomavirus (HPV) *E6/E7* genes together with human telomerase reverse transcriptase *(hTERT)* cDNA into primary endometrial epithelial cells purified from surgical specimens (11). EM-E6/E7/TERT/RAS cells are transformed cells with tumorigenicity in mice established 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 progesterone 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 modied Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 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′-CCTCTTCGG-CCCAGTGGAC-3′ (forward),
5′–CCGTTTTCGACCCTGAGAG-3′ (reverse) for *p21/WAF-1*,
5′ – ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT - 3′ (forward),
5′ – ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA -3′ (reverse) for *PRB* (744–1173, 429 bp) (13),
5′–TGGACATGCTCAGCAGACATC-3′ (forward),
5′-TTGGGTCAGGCGGTTCA-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 $MgCl_2$, 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

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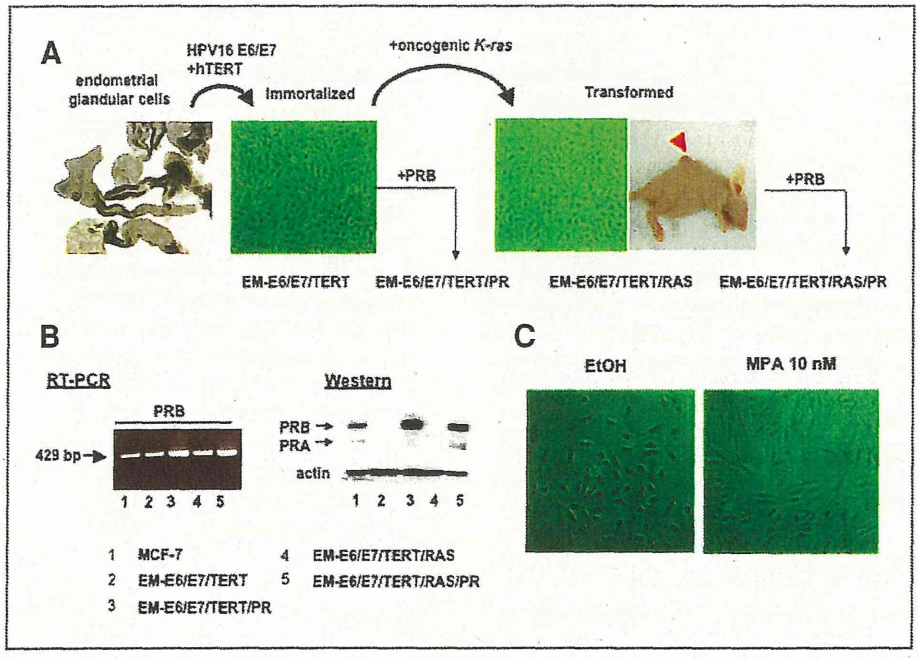

Figure 1. Endometrial epithelial cell lines used in the study. A, EM-E6/E7/TERT cells were immortalized cells generated from primary endometrial epithelial cells by the introduction of *HPV16 E6/E7* and *hTERT* genes (11). EM-E6/E7/TERT/RAS cells were generated from EM-E6/E7/TERT cells by the retroviral introduction of oncogenic mutant *K-ras* alleles, which produced transformed phenotypes, including anchorage-independent growth and tumorigenicity on nude mice (12). EM-E6/E7/TERT or EM-E6/E7/TERT/RAS cells were retrovirally transfected with *PRB* expression vector to achieve higher levels of PR expression. B, RT-PCR and western blot analyses were performed to confirm the expression of PR subtypes. PRA is an isoform of PR, lacking the N-terminal 164 amino acids of the larger isoform, PRB. The RT-PCR primers were designed to generate PRB products (429 bp) (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.

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 precultured 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′-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, EM-E6/E7/TERT/PR, EM-E6/E7/TERT/RAS, or EM-E6/E7/TERT/RAS/PR cells as previously described (16). For examining p21/WAF-1, PTEN, p-AKT, or FOXO1 expression, EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells preincubated in growth media for 24 h were incubated in serum-depleted phenol red-free DMEM in the absence or presence of MPA or progesterone (10 or 100 nM) for various time periods; whole-cell extracts or nuclear or cytoplasmic extracts were prepared as previously described (16). Fifteen micrograms of the nuclear extracts or 50–100 μg of whole-cell or cytoplasmic extracts were electrophoresed on a SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes.

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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 6H2.1); 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-Light^{Plus} 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 (5×10^5 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. Half of the collected samples were stored for histological analysis after formalin fixation, while the other half were stored at −70°C until protein extraction.

Immunocytochemistry and immunohistochemistry

EM-E6/E7/TERT/PR cells were cultured on LAB TEK chamber slides (Nalge Nunc International) for 24 h and treated with or without MPA (10 nM) for 24 h. Then, the cells were fixed with 10% formaldehyde neutral buffer solution (37152-51; Nacalai Tesque, Inc.), immersed in methanol for 10 min at −20°C, blocked with PBS containing 10% goat serum and 0.3% Triton X-100 (166-11805; Wako Pure Chemical Industries, Ltd.) for 1 h at room temperature, and stained with monoclonal antibody to FOXO1 (#9462; Cell Signaling Technology) at 1:100 dilution for 12 h at 4°C. Next, they were incubated with fluorescent anti-rabbit IgG conjugates [Alexa Fluor 568 goat anti-rabbit IgG (H+L) highly cross-adsorbed, A11036; Invitrogen] for 1 h at room temperature in the dark. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (D1306; Invitrogen) for 3 min. Representative images were captured with a fluorescent microscope (Olympus BX-50; Olympus).

Immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded specimens from mouse tumors formed with EM-E6/E7/TERT/RAS/PR cells. Sections were stained with a monoclonal antibody to FOXO1 (#9462; Cell Signaling Technology) at a 1:100 dilution for 12 h at 4°C. After the specimens were deparaffinized in xylene and graded alcohols, epitope retrieval was performed, in which the sections were heated in a microwave oven at 700 W for 10 min in 1× Antigen Retrieval Solution (Biogenex). Then, endogenous peroxidase was blocked by immersing the sections in 0.3% H_2O_2 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 adenovirus 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 2567AA). 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:

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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-percentile 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′-CTAATTTTTCCTTTTTTCCCCTC-3′ (forward) and 5′-AGGGGCGGGGGTCACC-3′ (reverse), and inserted into the luciferase reporter plasmid pGL3-basic (Promega), named p-FOXO1-pro. EM-E6/E7/TERT/PR cells preincubated in growth media in 24-well dishes for 24 h were incubated in serum-depleted phenol red-free DMEM and transfected with 0.4 μg of reporter plasmid using Lipofectamin 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 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 $siRNA_{control}$ (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 progestin 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 progestin, 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 progestin 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

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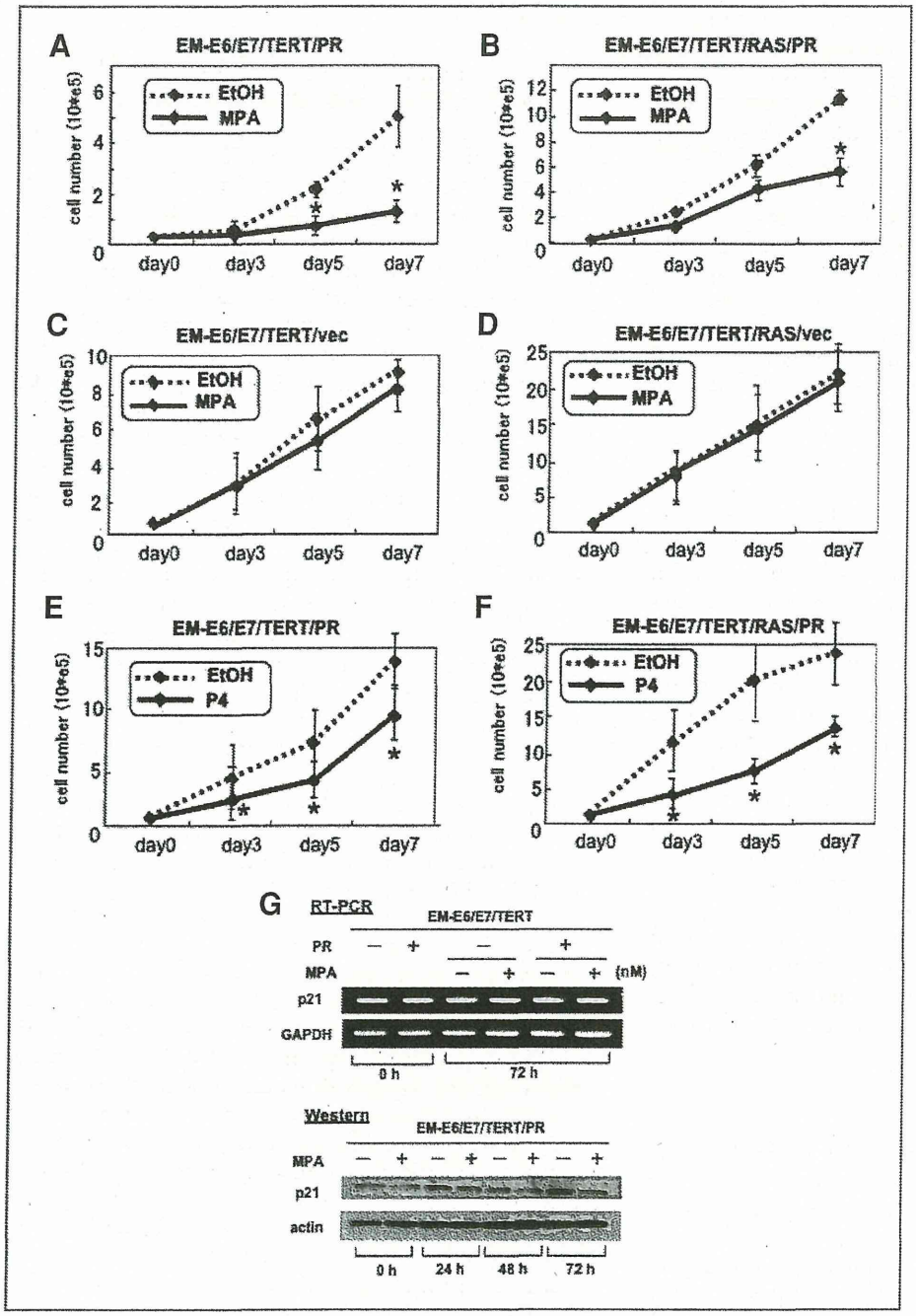

Figure 2. *In vitro* effect of progestin on the growth of endometrial epithelial cell lines. *In vitro* growth assay of endometrial epithelial cell lines treated with MPA or progesterone (P4). Cells were grown in growth media with or without 10 nM MPA (A–D) or progesterone (E and F) for different time 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.

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 progestin 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.

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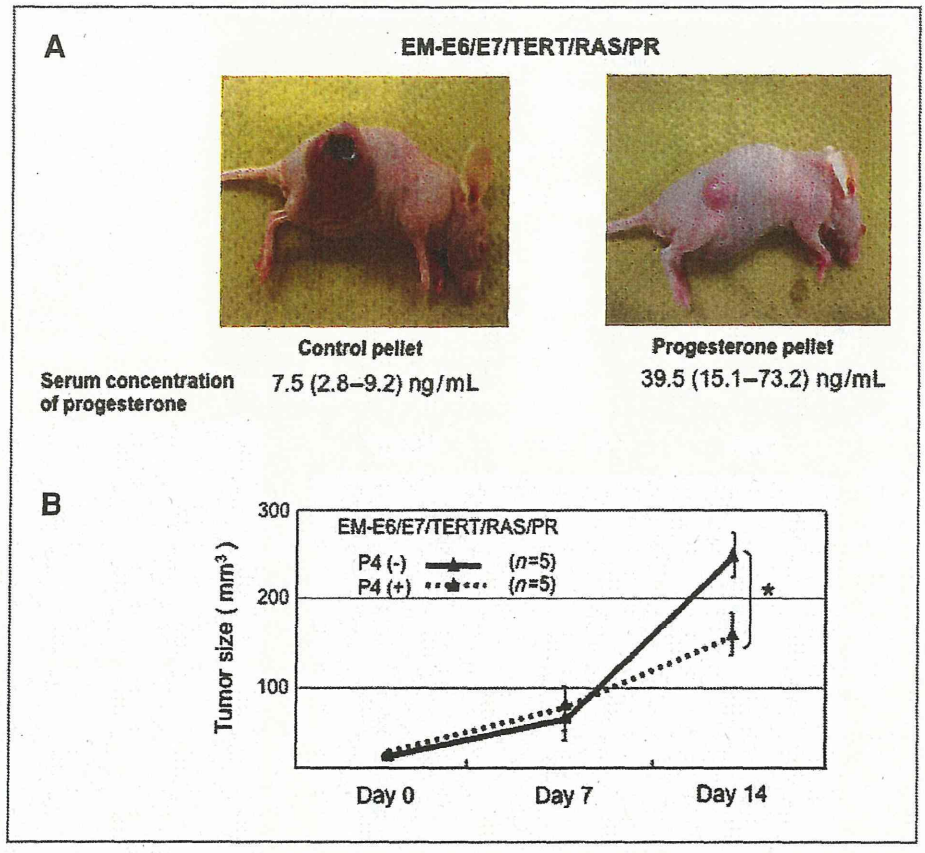

Figure 3. *In vivo* effect of MPA on the growth of endometrial epithelial cell lines. EM-E6/E7/TERT/RAS/PR cells were inoculated on the flank of mice. After tumor growth was confirmed, control placebo pellets or progesterone pellets were administrated subcutaneously, and then tumor growth was monitored. Mean serum concentration of progesterone was shown for each group. Data are presented as mean ± SD; *$P < 0.05$.

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 progesterone 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