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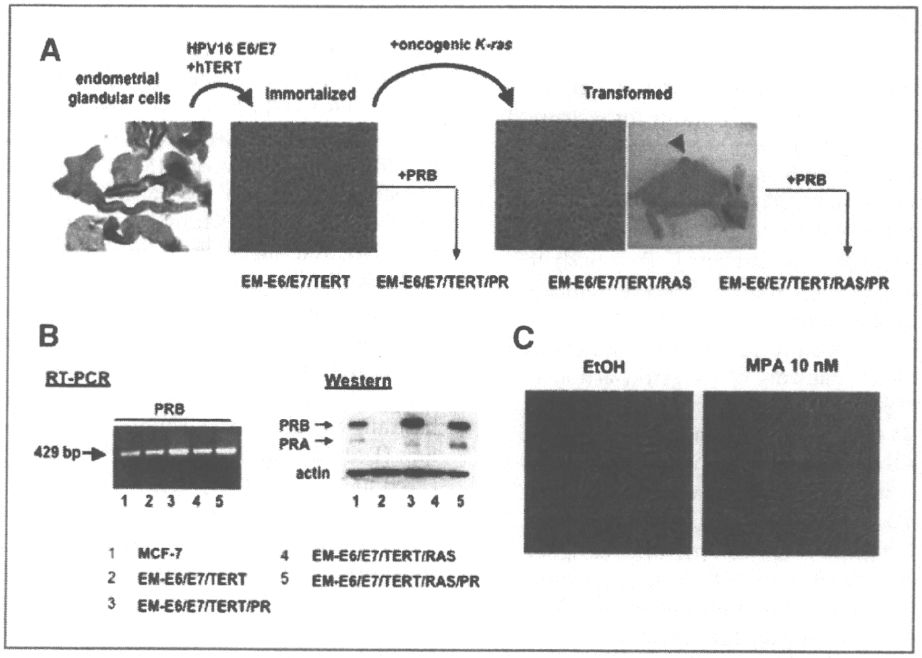

Figure 1. Endometrial epithelial cell lines used in the study. A, EM-E6/E7/TERT cells were immortalized cells generated from primary endometrial epithelial cells by the introduction of *HPV16 E6/E7* and *hTERT* genes (11). EM-E6/E7/TERT/RAS cells were generated from EM-E6/E7/TERT cells by the retroviral 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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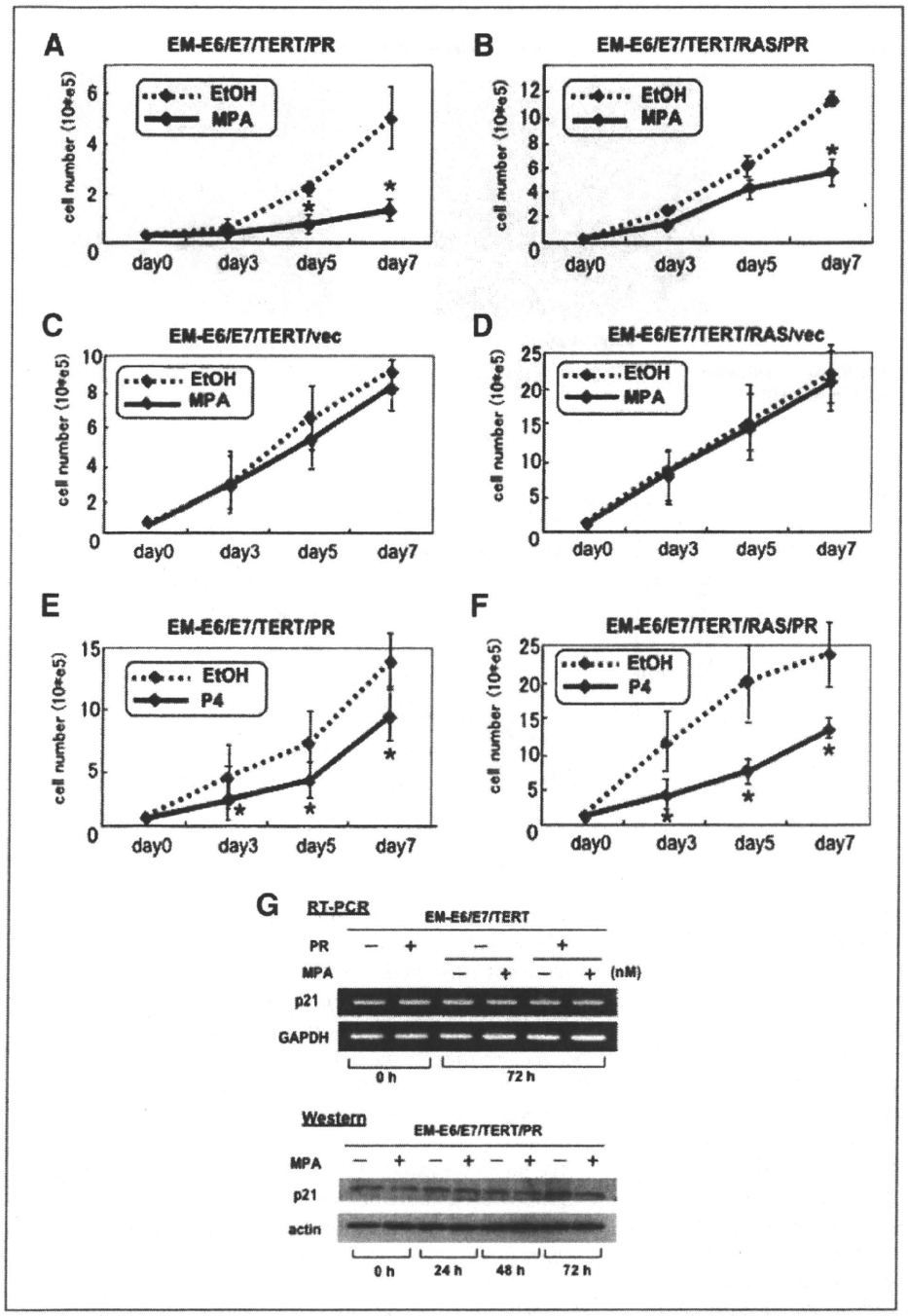

Figure 2. *In vitro* effect of progestin on the growth of endometrial epithelial cell lines. *In vitro* growth assay of endometrial epithelial cell lines treated with MPA or progesterone (P4). Cells were grown in growth media with or without 10 nM MPA (A–D) or progesterone (E and F) for different time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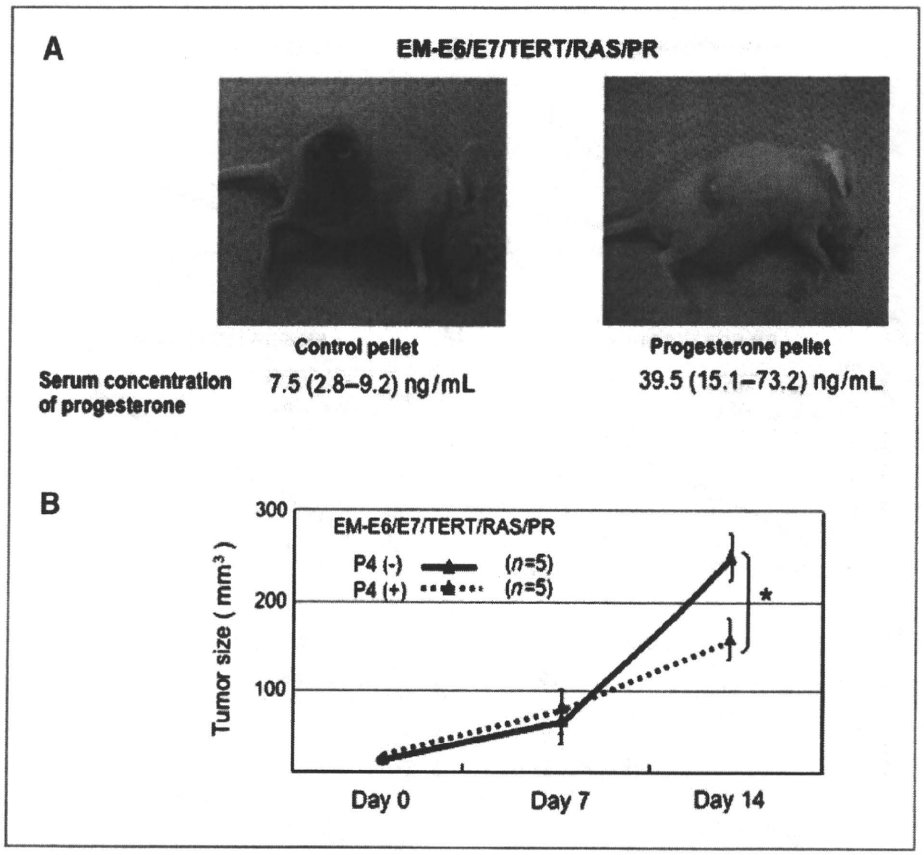

Figure 3. *In vivo* effect of MPA on the growth of endometrial epithelial cell lines. EM-E6/E7/TERT/RAS/PR cells were inoculated on the flank of mice. After tumor growth was confirmed, control placebo pellets or progesterone pellets were administrated subcutaneously, and then tumor growth was monitored. 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

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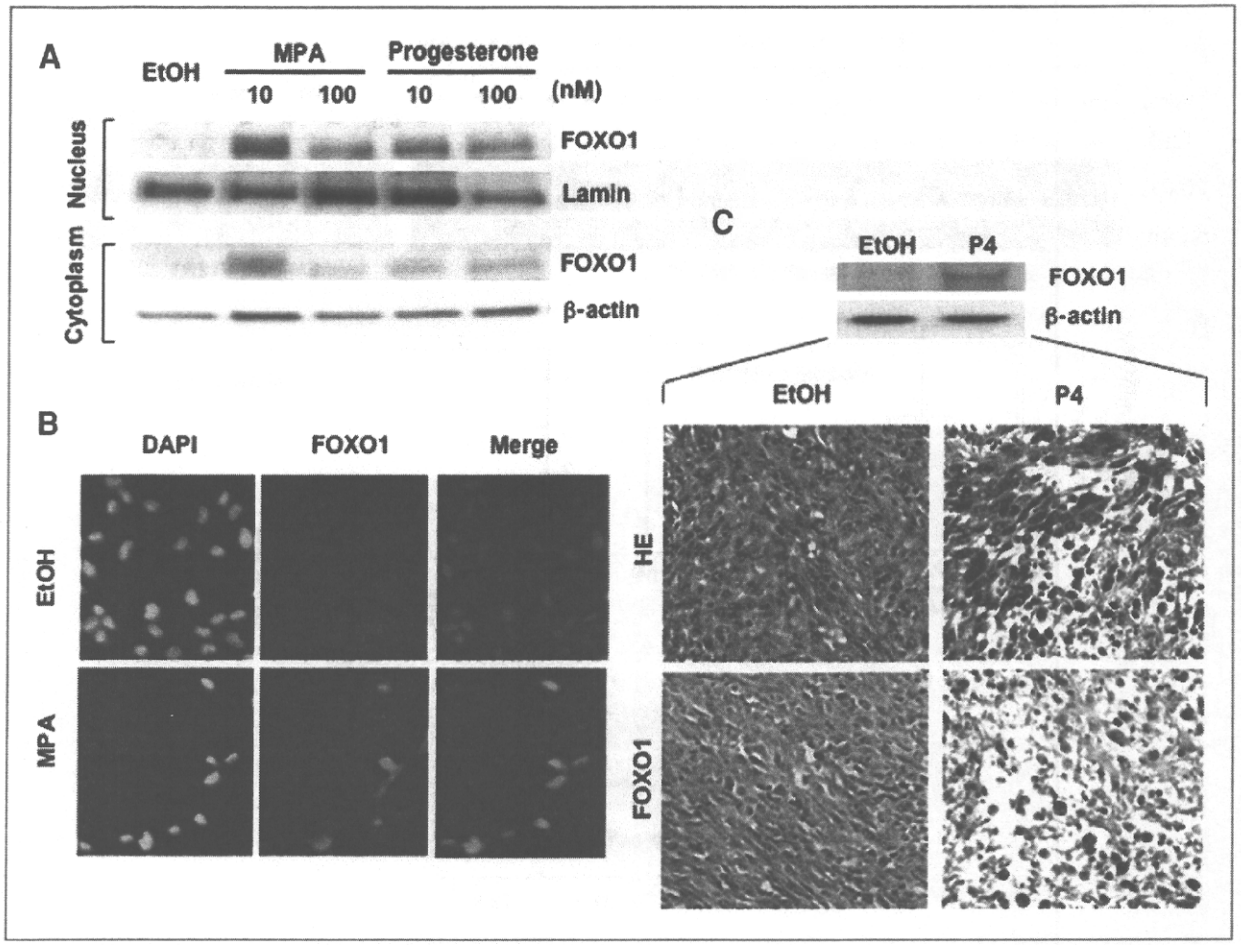

Figure 4. Induction of FOXO1 by progestin. A, EM-E6/E7/TERT/PR cells preincubated in growth media for 24 h were incubated in the serum-depleted phenol red–free DMEM in the absence or presence of MPA or progesterone for 48 h at the indicated concentrations. Nuclear or cytoplasmic extracts were collected from cells and the western blot analysis was performed using FOXO1 antibody. Lamin and β–actin were used as controls for nuclear or cytoplasmic protein loading, respectively. B, immunocytochemistry of FOXO1. EM-E6/E7/TERT/PR cells preincubated in growth media for 24 h were incubated on LAB TEK chamber slides for 24 h in the absence or presence of MPA for 24 h. After fixation, the cells were incubated with primary antibody to FOXO1, followed by fluorescent anti-IgG secondary antibody. The cells were also incubated with DAPI for nuclear staining and observed under a fluorescence microscope. Note that FOXO1 expression is induced preferentially in the nuclei (consistent with DAP staining) by MPA treatment. D, mouse tumors formed with EM-E6/E7/TERT/RAS/PR cells were treated with or without progesterone (P4) via subcutaneous injection of hormone pellets and were collected 4 weeks after the treatment. Then, whole-cell extracts were prepared, followed by western blot analysis for FOXO1. Immunohistochemistry of FOXO1 was also performed with matched samples of the EM-E6/E7/TERT/RAS/PR tumors treated with or without P4. HE staining of the tumor samples and induced FOXO1 expression mainly in the nuclei of the tumor cells are shown.

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

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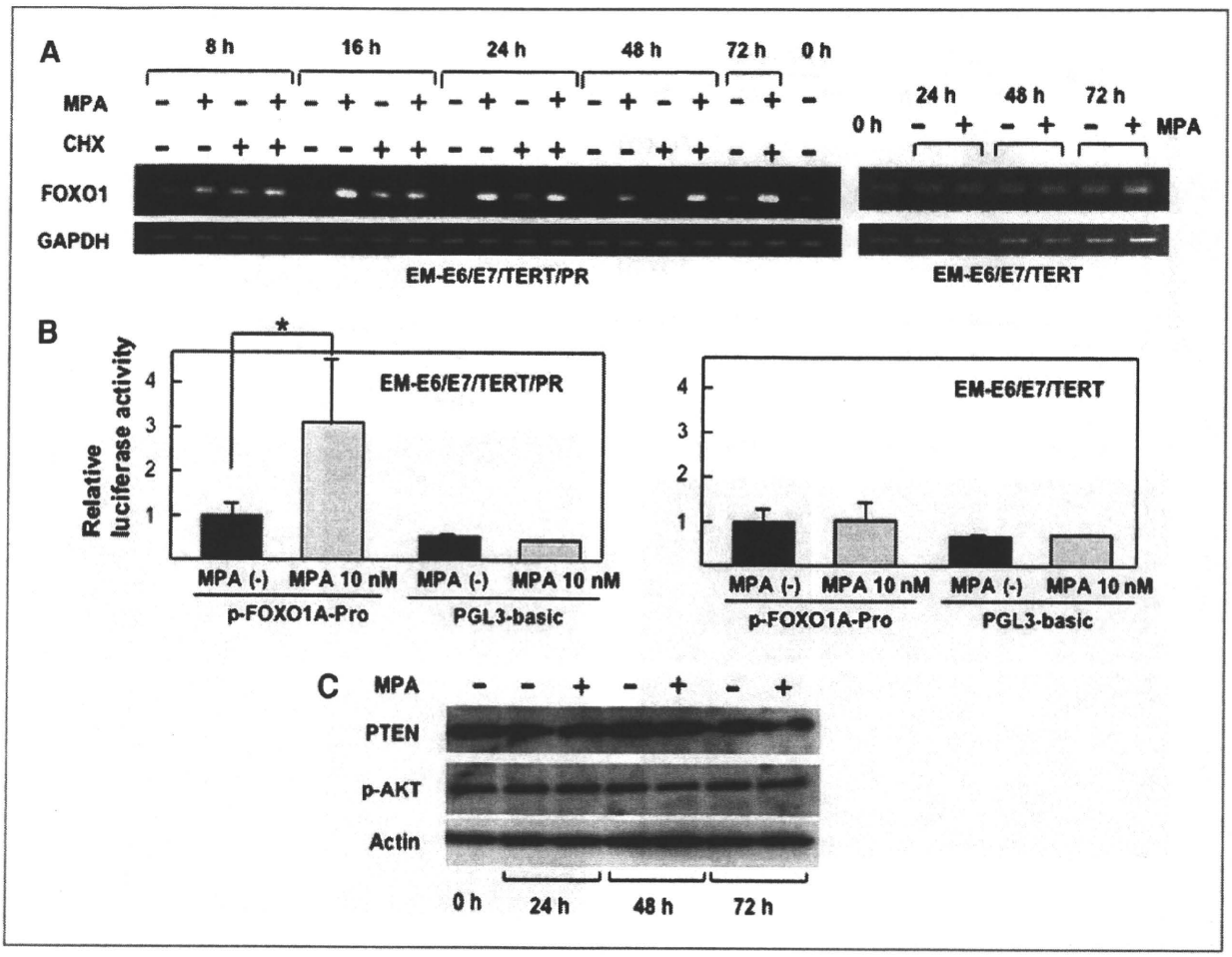

Figure 5. Mechanisms of FOXO induction by progestin. A, RT-PCR analysis of FOXO1 in EM-E6/E7/TERT/PR or EM-E6/E7/TERT cells. The cells were cultured with or without MPA in the presence or absence of cycloheximide (CHX) for different time periods and RNAs were collected and subjected to the RT-PCR for FOXO1. B, luciferase reporter assay using FOXO promoter. EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells were transfected with luciferase reporter plasmid containing 2.0 kb of FOXO promoter or with blank reporter plasmid (pGL3-basic), followed by treatment with or without MPA. Seventy-two hours after treatment, cells were recovered and luciferase assays were performed. Relative luciferase activity is shown in each reporter plasmid. Data are presented as mean ± SD of the three independent experiments. *$P < 0.05$. C, EM-E6/E7/TERT/PR cells were treated with or without MPA at 10 nM and incubated at different time periods, followed by the western blot analysis for PTEN or p-AKT.

with or without MPA at 10 nM, followed by monitoring cell growth. Western blot analysis confirmed that knockdown 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/E7/TERT/PR/DN-AKT, was established with an introduced dominant

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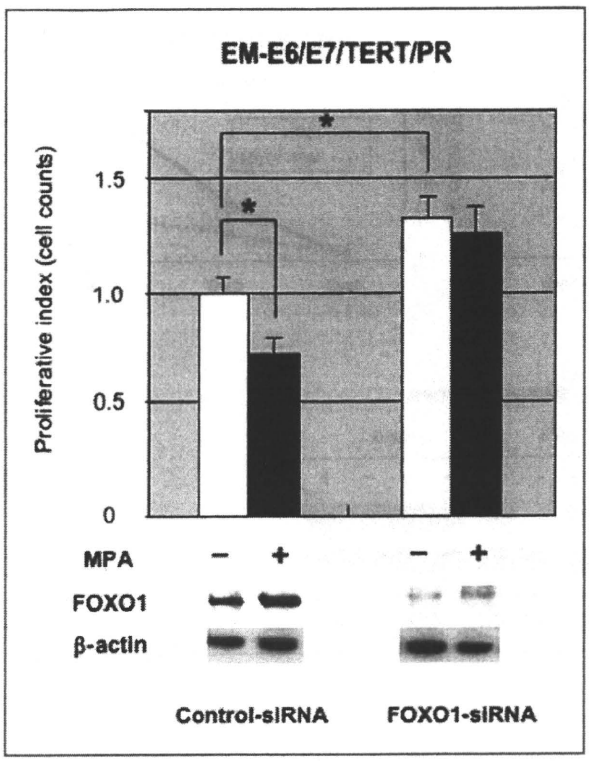

Figure 6. Role of FOXO1 in the action of progestin. EM-E6/E7/TERT/PR cells were transfected with non-specific scramble $siRNA_{control}$ or FOXO1-specific siRNA. Forty-eight hours after transfection, the cells were incubated with or without MPA at 10 nM for 72 h. Then, western blot analysis was performed to confirm the levels of FOXO1 expression. Simultaneously, cell number was counted in paired samples and shown as the relative value (proliferation index) in each sample to evaluate the effects of MPA. Data are presented as mean ± SD of the three independent experiments. *$P < 0.05$.

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-l1), 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 *FOXO* 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 *FOXO* 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

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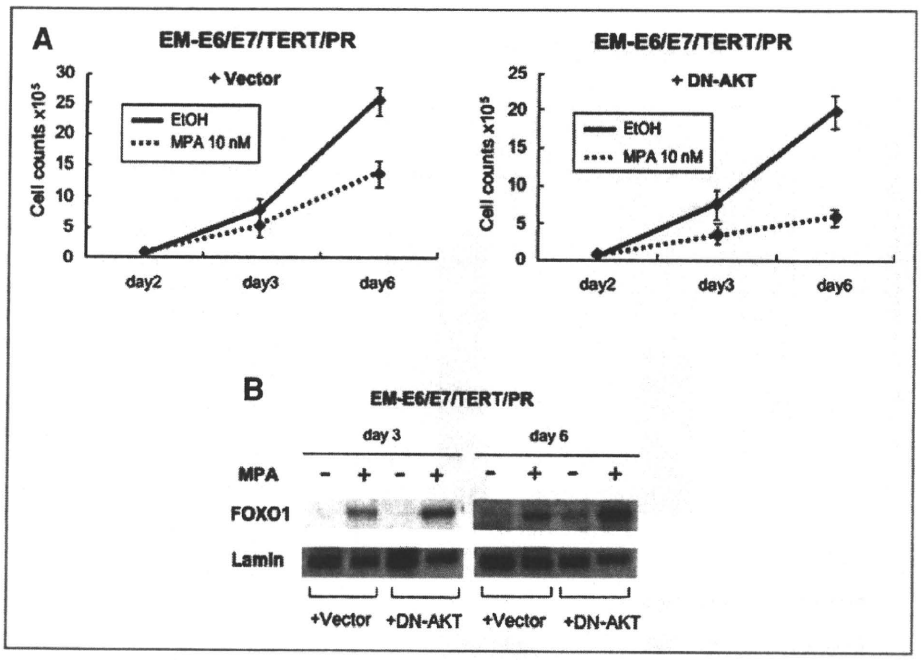

Figure 7. Akt activity limits the MPA effect. A, EM-E6/E7/TERT/PR/DN-AKT or EM-E6/E7/TERT/PR/vector cells were established from EM-E6/E7/TERT/PR cells by retroviral transfection of dominant negative *Akt* gene or blank vector, respectively. These cells were treated with or without MPA for different periods, and the cell number was counted to evaluate the proliferative activity. Data are presented as mean ± SD of the three independent experiments. B, western blot analysis was performed using nuclear extracts of EM-E6/E7/TERT/PR/DN-AKT or EM-E6/E7/TERT/PR/vector cells in the absence or presence of MPA on day 3 or day 6 to compare the levels of nuclear FOXO1 induction. Data are presented as mean ± SD of the three independent experiments.

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-1® 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 hemangiomas, 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

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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.

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Clinical Cancer Research

Activation of NF-κB Is a Novel Target of *KRAS*-Induced Endometrial Carcinogenesis

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Clinical Cancer Research

Human Cancer Biology

Activation of NF-κB Is a Novel Target of *KRAS*-Induced Endometrial Carcinogenesis

Yasunari Mizumoto[1], Satoru Kyo[1], Tohru Kiyono[2], Masahiro Takakura[1], Mitsuhiro Nakamura[1], Yoshiko Maida[1], Noriko Mori[1], Yukiko Bono[1], Hiroaki Sakurai[3], and Masaki Inoue[1]

Abstract

Purpose: Although the *KRAS* mutation is one of critical genetic alterations in endometrial carcinogenesis, the downstream targets are not known.

Experimental Design: In this study, we investigated the molecular targets of *KRAS* signals, using tumorigenic cells with oncogenic *KRAS* mutation established from telomerase reverse transcriptase (*TERT*)-immortalized endometrial epithelial cells.

Results: We first confirmed that the RAF-ERK pathway, but not the PI3K-Akt pathway, was activated in *KRAS* tumorigenic cells. However, the introduction of constitutively active MAP/ERK kinase into immortalized cells to mimic RAF-ERK activation failed to obtain tumorigenic phenotypes, indicating the existence of other carcinogenic pathways triggered by *KRAS*. Recent evidence suggestive of linkage with *KRAS* signals prompted us to examine the involvement of NF-κB in endometrial carcinogenesis. We found that the DNA-binding activity of NF-κB was markedly elevated in *KRAS* tumorigenic cells compared with *TERT*-immortalized cells. Furthermore, the ability of NF-κB to activate the target gene promoters significantly increased in *KRAS* tumorigenic cells. Introduction of a mutant IκB that is resistant to degradation and thereby enhances the inhibitory effect on NF-κB largely abrogated the transformed phenotypes of *KRAS* tumorigenic cells. Thus, oncogenic *KRAS* signals contributed to the tumorigenic phenotypes of endometrial cells by activating the transcription function of NF-κB.

Conclusions: These findings clearly show that NF-κB activation is a novel target of oncogenic *KRAS* in endometrial carcinogenesis, implying the potential utility of NF-κB inhibitors for endometrial cancer chemoprevention, especially with *KRAS* mutation. *Clin Cancer Res; 17(6); 1341–50. ©2011 AACR.*

Introduction

The genetic alterations frequently observed in endometrial cancer involve microsatellite instability and mutations in *PTEN*, *PIK3CA*, *β-catenin*, and *KRAS*, whereas a relatively small percentage of endometrial cancers have *p53* mutations (1, 2). Because some of these gene mutations, including *KRAS* mutation, were detected in precursor lesions, they are thought to be early events in endometrial carcinogenesis (1–4). Ras signals activate various effector pathways in a species- or tissue-specific manner (5). However, the Ras downstream signals essential for endometrial carcinogenesis remain unclear.

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The study of human tumor specimens has provided much of our current understanding of the molecular basis of carcinogenesis. However, most human cancers harbor complex karyotypes and multiple genetic mutations, so the specific types and mechanisms of genetic alterations contributing to carcinogenesis remain unclear. One potential way to overcome these issues is to develop a carcinogenesis model, using defined genetic elements. We have previously created an *in vitro* model of endometrial carcinogenesis in which purified endometrial epithelial cells were immortalized by stably introducing HPV16 *E6* and *E7* and the catalytic subunit of telomerase (*hTERT*; resulting in EM-E6/E7/TERT cells; ref. 6), followed by the additive introduction of oncogenic *KRAS* alleles to obtain tumorigenic cells with anchorage-independent growth and tumorigenicity on nude mice (EM-E6/E7/TERT/RAS cells; ref. 7). One of the most important characteristics of the EM-E6/E7/TERT/RAS cells is their genetic purity with intact chromosomes. Therefore, these immortalized and tumorigenic endometrial epithelial cell lines created with defined genetic rearrangements are advantageous and available for analyzing the oncogenic pathways of endometrial carcinogenesis.

NF-κB has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory

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Translational Relevance

The signal transduction or oncogenic pathways in endometrial carcinogenesis remain unclear, although some genetic factors, including *PTEN* and *KRAS* mutations and microsatellite instability, have been identified to play etiologic roles in the development of this tumor type. Most researchers believed that KRAS-ERK1/2 pathway plays central roles in it, but few studies have directly proved it. In this study, we for the first time found that the conventional KRAS-ERK1/2 pathway is insufficient for endometrial carcinogenesis and that NF-κB is a critical target of KRAS-induced endometrial carcinogenesis. This information implies the novel molecular mechanisms of endometrial carcinogenesis and the future therapeutic direction for cancer prevention by suppressing this novel pathway, such as with NF-κB inhibitors.

responses. Accumulating evidence supports a key role of the constitutive activation of NF-κB in controlling the initiation and progression of human cancer (8). NF-κB has also been documented both to be activated downstream of oncogenic Ras signals in some types of human cancers and to participate in the transformation of rodent cells (9–11). However, the role of NF-κB in endometrial carcinogenesis remains unclear. In this study, we show for the first time that NF-κB activation plays a central role in *KRAS*-mediated endometrial carcinogenesis.

Materials and Methods

Electrophoretic mobility shift assay

The nuclear extracts were prepared as previously described (12). A consensus oligonucleotide containing the NF-κB binding site (Promega) was end labeled with the kit (MEGALABEL; Takara Bio Inc.). For the assay, 50 μg of nuclear protein extract was incubated for 30 minutes at room temperature in a final volume of 25 μL containing 10,000 cpm of labeled oligonucleotides, 1 μg of poly (dI-dC), 0.5 mmol/L of phenylmethylsulfonylfluoride, 1 mmol/L of dithiothreitol, 10% glycerol, 25 mmol/L of HEPES (pH 7.9), and 50 mmol/L of KCl. DNA–protein complexes were then separated from free probes by electrophoresis on a 5% polyacrylamide gel. For competition assays, 100-fold molar excess of unlabeled consensus oligonucleotides for AP2, SP1, or NF-κB were used as competitors. For supershift assays, the nuclear extracts were incubated with specific antibodies against NF-κB for 30 minutes before addition of the labeled oligonucleotides. Antibodies against p65 (sc-109X) and p50 (sc-114X) were purchased from Santa Cruz Biotechnology, Inc.

Luciferase reporter assay

Cells were cultured in 24-well culture plates and transfected with 0.4 μg of luciferase reporter plasmid driven by NF-κB–responsive elements (Panomics, Inc.), using Lipofectamine Plus (Invitrogen Corp.), according to the manufacturer's protocol. After 48 hours of incubation, the cells were harvested in passive lysis buffer (Promega) and luciferase assays were carried out. To examine the role of IKK (IκB kinase complex) pathways in promoter activation, 5 μmol/L of IKK inhibitor X (Calbiochem) was added to the medium after the reporter transfection. All experiments were carried out at least 3 times, and the results represent average relative luciferase activity.

Establishment of stable transfectants

The plasmid encoding a constitutively active mutant of *MEK1* (HA-MEK1DD; ref. 13) was kindly provided by Dr. S. Meloche (Université de Montréal, Quebec, Canada). HA-MEK1DD and the mutant IκBα cDNA-encoding superrepressor (IκBα-SR) harboring S32A and S36A mutations (Clontech; catalogue no. 6319233) were cloned and recombined into retroviral vectors to generate pCMSCVpuro-HA-MEK1DD and pCMSCVbsd-IκBα (Ser32/36Ala) as described previously (14). The production and infection of recombinant retroviruses have been described previously (6). These retroviruses and backbone vectors were infected into EM-E6/E7/TERT/RAS cells. The infected cells were selected in the presence of 1 mg/mL of puromycin and 8 mg/mL of blasticidin S.

Immunoblot and immunoprecipitation

Whole-cell extracts were prepared as previously described (12), with specific antibodies against phospho-p44/42MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), phospho-NF-κB p65 (Ser536), phospho-NF-κB p65 (Ser276), IκBα (Cell Signaling Technology), IκBε (Abcam), IκBβ (Delta Biolabs), NF-κB p65 phospho-Ser529 (Millipore), NF-κB p52, NF-κB p50, and actin (Santa Cruz Biotechnology). The LAS3000 CCD-Imaging System (Fujifilm Co. Ltd.) was used for the detection and quantification of proteins visualized by ECL Plus Western blotting detection reagents (GE Healthcare UK Ltd.).

Immunoprecipitation was done using Dynabeads Protein G kit (Invitrogen) with antibodies against p65 (sc-8008; Santa Cruz Biotechnology) or normal mouse IgG (sc-2025; Santa Cruz Biotechnology), according to the manufacturer's protocol. Immunoprecipitated lysates were subjected to the Western blot analysis with antibodies against p65 (sc109; Santa Cruz Biotechnology) or IκB (Cell Signaling Technology).

Cell culture and *in vitro* growth assay

Establishment of immortalized (EM-E6/E7/TERT) and tumorigenic (EM-E6/E7/TERT/RAS) endometrial epithelial cells has been described elsewhere (6, 7). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin–streptomycin in an atmosphere of 5% CO_2 at 37°C. Growth activity of EM-E6/E7/TERT/RAS cells with overexpressed mutant IκBα (IκBαM) or with control vectors was evaluated in normal serum (10% FBS) or low serum (0.5% FBS) conditions by

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counting cell number on days 3, 4, 5, and 6 after the seeding of 5×10^4 cells in 6-cm dishes.

Anchorage independence of growth

A total of 1×10^4 cells was seeded in 60-mm dishes containing a top layer of 0.33% noble agar in DMEM supplemented with 10% FBS and a bottom layer of 0.5% base agar in DMEM supplemented with 10% FBS as described elsewhere (7). The number of colonies larger than 0.05 mm in size after 4 weeks of incubation was counted under a microscope.

Nude mouse xenograft experiments

Cells were resuspended in growth media (10^7 cells/mL) and injected (0.1 mL) subcutaneously at the base of the trunk of female BALB/c *nu/nu* mice (age range, 7–9 weeks; Japan SCL). Tumor size, if any, was monitored weekly for 8 weeks.

Matrigel invasion assay

The invasive ability of cells was assayed *in vitro* using a BioCoat Matrigel Invasion Chamber (Becton Dickinson Biosciences), as described elsewhere (15). Cells were suspended in the upper wells of Matrigel chambers at 2.5×10^4 cells/chamber in DMEM containing 0.1% bovine serum albumin. Chambers were set into 24-well plates with DMEM containing 10% FBS. After 22 hours of incubation, cells on the upper surface of the membrane were removed by wiping with cotton swabs and cells that had migrated through the membrane containing Matrigel to the lower surface were fixed with methanol and stained with Mayer's hematoxylin. The cells on the lower surface of the membrane were counted microscopically as the invasion index. Chemotaxis assays were conducted in the same manner as for chemoinvasion, except that the filters were not coated with Matrigel, and the number of cells on the lower surface of the membrane was counted as the migration index. The invasive ability of cells was described as the relative value of invasion index versus migration index.

Statistical analysis

The data from the anchorage-independent growth assay and Matrigel invasion assay were presented as the mean ±SD of triplicate assays per group. Differences between groups were evaluated using Student's *t* test. The value of $P < 0.05$ was considered to be statistically significant.

Results

RAF-ERK and PI3K-Akt pathways do not play major roles in *KRAS*-induced endometrial carcinogenesis

Numerous effector pathways have been shown downstream of oncogenic *KRAS* signals, including the RAF-ERK and PI3K-Akt pathways. Activation of ERK upregulates the transcription of genes associated with cell proliferation, whereas the activation of Akt leads to the induction of antiapoptotic genes; thus, both ERK and Akt play crucial roles in cancer initiation. We first examined whether either or both pathways were activated by oncogenic *KRAS* signals during endometrial carcinogenesis. As shown in Figure 1, the expression of phosphorylated ERK apparently increased in tumorigenic EM-E6/E7/TERT/RAS cells compared with immortalized control EM-E6/E7/TERT cells whereas phosphorylated Akt expression was not detected in both cell types, suggesting that the RAF-ERK pathway, but not the PI3K-Akt pathway, was activated by oncogenic *KRAS* signals in endometrial cancer cells.

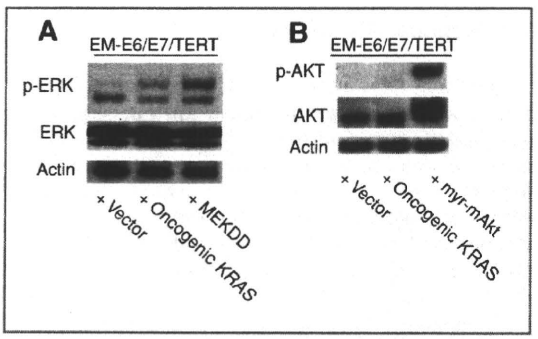

Figure 1. The RAF-ERK pathway, but not the PI3K-Akt pathway, is activated in endometrial epithelial cells transformed by oncogenic *KRAS*. Immortalized endometrial epithelial (EM-E6/E7/TERT) cells were stably transfected with oncogenic *KRAS*, constitutively activated *MEK* (MEKDD), or constitutively activated *Akt* (myr-mAkt) alleles and the expressions of phosphorylated ERK (A) and phosphorylated Akt (B) were examined by Western blot analysis. Cells with constitutively activated *Akt* alleles were used for the positive control of the activated PI3K-Akt pathway.

We next examined the biological roles of RAF-ERK activation in *KRAS*-induced endometrial carcinogenesis. To clarify the role of RAF-ERK signaling, a constitutively active form of the *MEK1* (MEK1 S218D/S222D) allele (16) was retrovirally introduced into EM-E6/E7/TERT cells; these cells were confirmed to express stable and strong levels of p-ERK comparable with those of EM-E6/E7/TERT/RAS cells (Fig. 1A). Then, the phenotypic changes were observed. However, these cells completely lacked anchorage-independent growth or tumorigenicity in mice (Table 1). Thus, ERK activation is not a critical factor to induce transformed phenotypes on oncogenic *KRAS* signals in endometrial epithelial cells.

Table 1. Change in transformed phenotypes of endometrial epithelial cell lines by introducing defined genetic elements

EM-E6/E7/TERT cells	Anchorage-independent growth	Tumorigenicity (BALBc *nu/nu*)
+ Vector	No	No
+ Oncogenic *KRAS*	Yes (100%)	Yes (100%)
+ Active MEK	No	No

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Oncogenic *KRAS* enhances the transcriptional function of NF-κB in endometrial cancer cells

On the basis of these results, we sought other candidate factors involved in *KRAS*-induced endometrial carcinogenesis. One such factor is NF-κB, based on emerging evidence that NF-κB is one of the putative effectors of Ras-mediated cellular transformation in rodent cells (9–11). Therefore, we investigated whether the introduction of oncogenic *KRAS* regulates NF-κB activity in EM-E6/E7/TERT cells. First, we examined the change in the DNA-binding activity of NF-κB by electrophoretic mobility shift assay (EMSA), using consensus oligonucleotides for NF-κB and nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells. As shown in Figure 2A, binding complexes were clearly observed in extracts of EM-E6/E7/TERT/vec cells. These bands were apparently intensified in extracts of EM-E6/E7/TERT/RAS cells. However, these bands were completely inhibited in competition assays by the addition of excess amounts of NF-κB consensus oligonucleotides but not by unrelated SP1 or AP2 oligonucleotides. Furthermore, they were supershifted by the addition of antibodies against NF-κB p50 or p65 subunits (Fig. 2B). We confirmed that endogenous expression levels of NF-κB were equivalent in EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. These findings indicate that oncogenic *KRAS* facilitates the DNA binding of NF-κB to its target sequences in endometrial cancer cells.

Next, we examined the change in the ability of NF-κB to transactivate the target promoters by oncogenic *KRAS*. Both EM-E6/E7/TERT/KRAS and EM-E6/E7/TERT/vec cells were transfected with the luciferase reporter plasmid containing the NF-κB–responsive elements (pNFκB-luc), and the relative luciferase activities of cell lysates were measured 48 hours after transfection. As shown in Figure 2C, the luciferase activity significantly increased (up to 5-fold) in EM-E6/E7/TERT/RAS cells compared with EM-E6/E7/TERT/vec cells, showing that oncogenic *KRAS* enhances the ability of NF-κB to transactivate the target gene promoter in endometrial cancer cells. Interestingly, this upregulation of NF-κB transcriptional activity was not cancelled by the addition of the MAP/ERK kinase (MEK)-inhibitor U0126, indicating that the RAF-ERK pathway is not involved in this activation. Taken together, we concluded that oncogenic *KRAS* functionally activates NF-κB in endometrial epithelial cells in a RAF-ERK pathway–independent manner.

Inhibition of NF-κB activity abrogates the transformed phenotypes of endometrial cancer cells

Regulation of NF-κB activity is controlled mainly by the inhibitory function of the IκB family, including IκBα. Phosphoryation of IκBα at 2 serine residues (Ser32 and

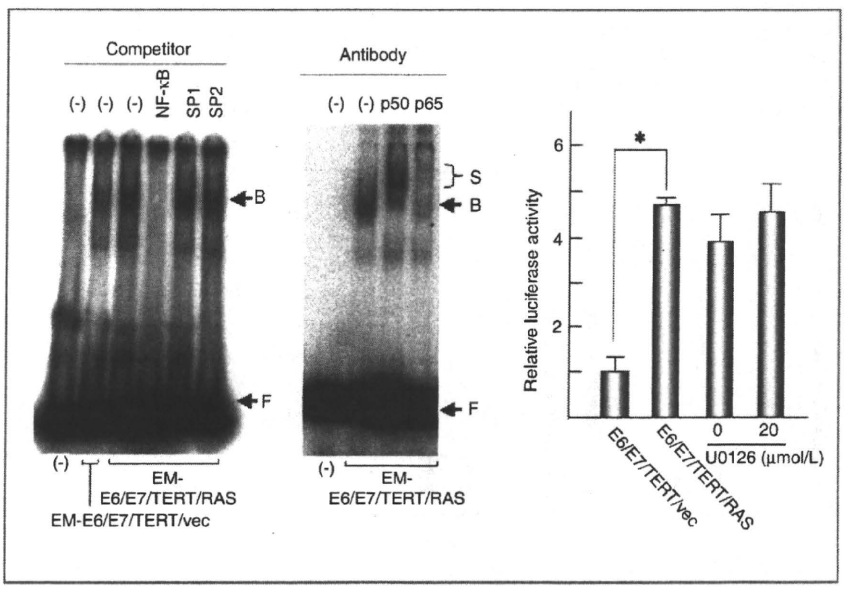

Figure 2. Oncogenic *KRAS* activates NF-κB in endometrial epithelial cells. A and B, EMSA to examine the DNA-binding activity of NF-κB. Nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were incubated with γ^{32}-P–labeled consensus oligonucleotides containing the NF-κB–responsive elements, followed by electrophoresis. For competition assays, 100-fold molar excess of unlabeled consensus oligonucleotides for NF-κB, AP2, and SP1 were used as competitors (A). For the supershift analysis, specific antibodies against NF-κB subunit p50 or p65 were added in the reactions. B, binding complexes; F, labeled free probes; S, supershifted complexes. C, luciferase reporter assays to examine the ability of NF-κB to activate the target promoters. EM-E6/E7/TERT/RAS cells and the vector control EM-E6/E7/TERT/vec cells were transfected with luciferase reporter plasmids containing the NF-κB–responsive elements. Plates were harvested 48 hours after transfection and luciferase assays were conducted. For inhibition of the RAF-ERK pathway, 20 μmol/L of U0126 or dimethyl sulfoxide was added to the medium 6 hours after transfection. The results are presented as relative luciferase activity in which the activity from EM-E6/E7/TERT/vec cells was normalized to 1.0. Values are represented as the means of 3 independent experiments. Bars, SD. *, $P < 0.05$.

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Ser36) leads to ubiquitination of IκB and subsequent proteasome-mediated degradation in the canonical NF-κB induction pathway (17). A dominant-negative mutant of IκBα, named IκBαM, has been engineered to be protected from phosphorylation and degradation. The introduction of this mutant form tightly represses the nuclear translocation and DNA binding of NF-κB (18). To elucidate the role of NF-κB in *KRAS*-induced endometrial carcinogenesis, we established cell lines with disabled NF-κB function by introducing IκBαM into EM-E6/E7/TERT/RAS cells. We first confirmed the inhibitory effect of this mutant, using the luciferase reporter assay. As shown in Figure 3A, the introduction of IκBαM significantly repressed the ability of NF-κB to activate the target promoters. We also confirmed that ERK activity was not affected by the introduction of IκBαM by Western blot analyses (Fig. 3B). Transformed phenotypes of this transfectant were evaluated by cell growth *in vitro* and the soft agar colony formation assay, tumor formation assay in nude mice, and Matrigel invasion assay. Under normal serum conditions, there was no significant increase in exponential growth rate by the introduction of IκBαM (data not shown). However, in low-serum conditions with 0.5% FBS, cells with overexpressed IκBαM exhibited decreased growth rate (Fig. 4A). We also observed that anchorage-independent growth in soft agar was almost completely abolished in these mutant cells (Fig. 4B). Furthermore, these cells completely lost their tumorigenic potential in mice (Fig. 4C). Similarly, their invasive ability significantly decreased, as evaluated by the Matrigel invasion assay (Fig. 4D). These findings indicate the crucial roles of NF-κB in *KRAS*-mediated endometrial carcinogenesis.

NF-κB activation by oncogenic *KRAS* is IKK dependent but independent of p65 phosphorylation or IκBα degradation/dissociation

We next sought to identify the molecular mechanisms of NF-κB activation by oncogenic *KRAS*. We first tested whether IKK signaling involves this activation. EM-E6/E7/TERT/RAS cells were treated with or without the IKK inhibitor X, the molecule known to inactivate IKKβ and IKKα. As shown in Figure 5A, the addition of IKK inhibitors largely inhibited the activity of NF-κB–responsive promoter in *KRAS*-introduced cells but not vector cells, indicating that this activation was IKK dependent.

Recent studies have focused on IκB subtype regulation (19) or p65 nuclear modification which can affect DNA binding and interactions with coactivators and corepressors (20, 21), Thus, we compared the basal expression levels of IκB subtypes or p65 modification between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. As shown in Figure 5B, the expression levels of IκBα, β, ε, and p105 and those of phospho-p65 at Ser276, 529, and 536 were basically equivalent in both cells except p100. These findings suggest that IκB subtype regulation or p65 nuclear modification does not significantly contribute to *KRAS*-induced NF-κB activation during endometrial carcinogenesis.

One potential mechanism of NF-κB activation includes the degradation of IκB by its ubiquitination. Therefore, we further evaluated the change in degradation rate of IκBα by Western blot analyses, using the protein synthesis inhibitor emetine. As expected, the treatment of cells with emetine resulted in the decreased expression of IκBα in both EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells but not in

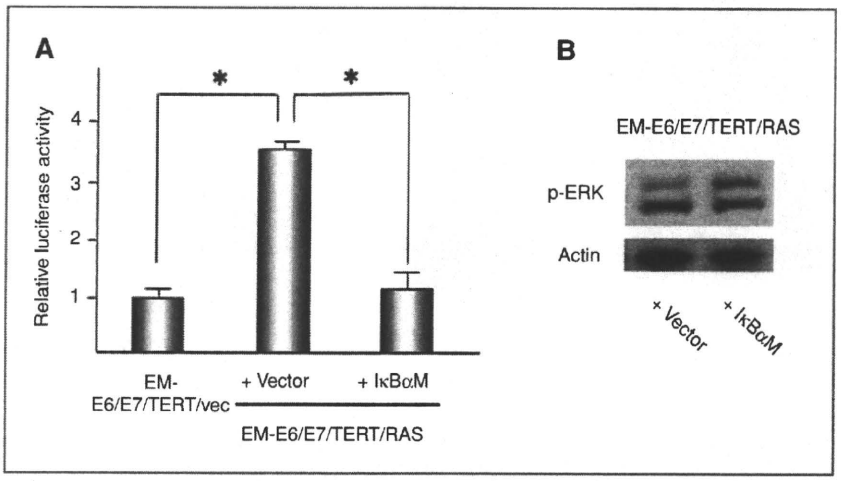

Figure 3. Introduction of IκBαM effectively inhibits NF-κB activity in transformed endometrial epithelial cells. EM-E6/E7/TERT/RAS cells were stably overexpressed with IκBαM lacking phosphorylation sites essential for its degradation, thereby becoming stable against degradation signals. Eventually, these transfectants were expected to have disabled NF-κB by IκB activity. To confirm the *in vivo* function of NF-κB in these cells, luciferase assays were done, in which EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were transfected with reporter plasmids containing the NF-κB–responsive elements and the luciferase assays were conducted. A, relative luciferase activities are shown as the mean values of 3 independent experiments, in which those of E6/E7/TERT/vec cells were normalized to 1.0. Bars, SD. *, $P < 0.05$. B, states of the RAF-ERK pathway were compared between EM-E6/E7/TERT/RAS cells transfected with IκBαM and the vector control cells, examining the phosphorylated ERK expression by Western blot analysis.

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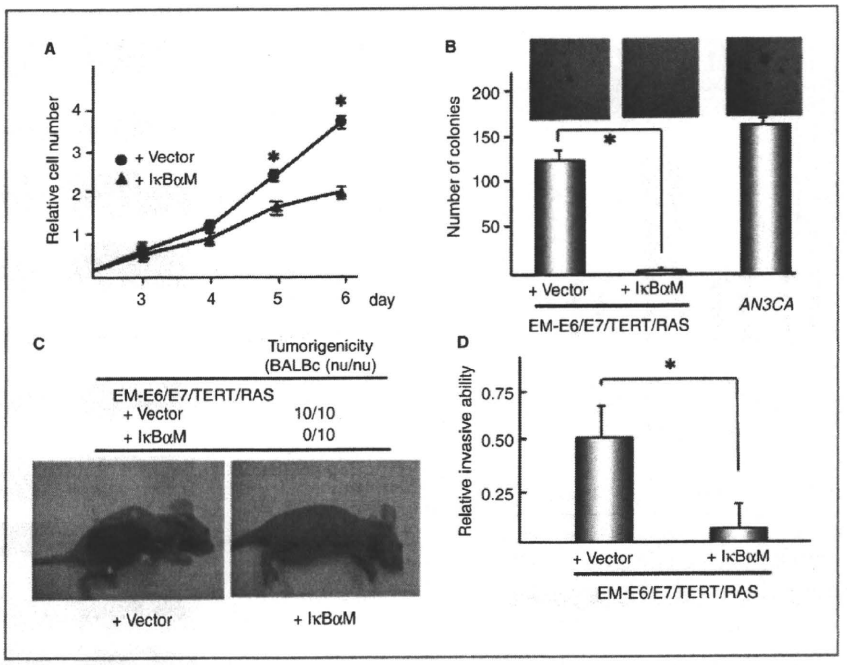

Figure 4. Transforming activity of oncogenic *KRAS* is disturbed by inhibiting NF-κB activity in endometrial epithelial cells. Transformed phenotypes of EM-E6/E7/TERT/RAS cells overexpressed with IκBαM were evaluated by various experiments. A, cell growth assay in a low-serum condition. The growth curve is shown in which EM-E6/E7/TERT/RAS cells transfected with or without IκBαM were cultured at a low-serum concentration with 0.5% FBS. Bars, SD. *, statistically significant decrease ($P < 0.05$) in the number of EM-E6/E7/TERT/RAS cells overexpressed with IκBαM compared with the number of vector control cells on days 5 and 6. B, soft agar colony formation assay. A total of 10,000 cells were seeded on soft agar in 6-cm dishes and colonies with a diameter of more than 0.05 mm 4 weeks after seeding were counted. Bars, SD. *, $P < 0.01$. C, nude mice xenograft experiment. A total of 10^7 cells of EM-E6/E7/TERT/RAS with IκBαM or control vector were inoculated subcutaneously into the right trunk of immunodeficient mice. Tumor formation was monitored for 8 weeks after inoculation. Breast cancer AN3CA cells were used as a positive control for colony formation. D, Matrigel invasion assay. EM-E6/E7/TERT/RAS cells with IκBαM or control vector were suspended in the upper wells of Matrigel chambers at 250,000 cells/chamber. After incubation, cells on the upper surface of the membrane were removed, and the cells that had migrated through the membrane to the lower surface were counted microscopically. The numbers are shown as relative invasive ability. Bars, SD. *, $P < 0.05$.

cells with overexpressed IκBαM lacking phosphorylation sites responsible for degradation (Fig. 5C). However, the degradation ratio was equivalent in EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. These results show that the activation of NF-κB by oncogenic *KRAS* is not due to accelerated degradation of IκBα.

The remaining possibility of activation mechanism might be an enhanced dissociation of p65 with IκBα (22). We tested this possibility by immunoprecipitation with p65 antibody, followed by the Western blot analysis with IκBα antibody, using extracts from EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. As shown in Figure 5D, the ratio of IκBα associated with p65 was similar between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells, denying the involvement of enhanced dissociation of p65 with IκBα.

Discussion

Using an *in vitro* carcinogenesis model with human endometrial epithelial cells, we first investigated the status of 2 major signaling pathways, the RAF-MEK-ERK and PI3K-Akt pathways, downstream of Ras. As expected, phosphorylated ERK expression significantly increased in EM-E6/E7/TERT/RAS cells (Fig. 1A). In contrast, phosphorylated Akt expression was not detected in both EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells (Fig. 1B). However, the introduction of a constitutively active form of MEK, mimicking the activated RAF-MEK-ERK pathway, failed to show transformed phenotypes. Thus, activation of ERK alone was not sufficient to transform EM-E6/E7/TERT cells. There are several reports showing that constitutive activation of MEK successfully transformed rodent epithelial cells (23–26). In contrast, Boehm and colleagues showed that the introduction of a constitutively active form of MEK failed to transform immortalized human embryonic kidney epithelial cells (27). These results together with our results suggest that activation of the MEK-ERK pathway alone may not be sufficient to transform human epithelial cells and that the activation of other oncogenic pathways is required. Thus, we sought novel effectors involved in KRAS-mediated endometrial carcinogenesis.

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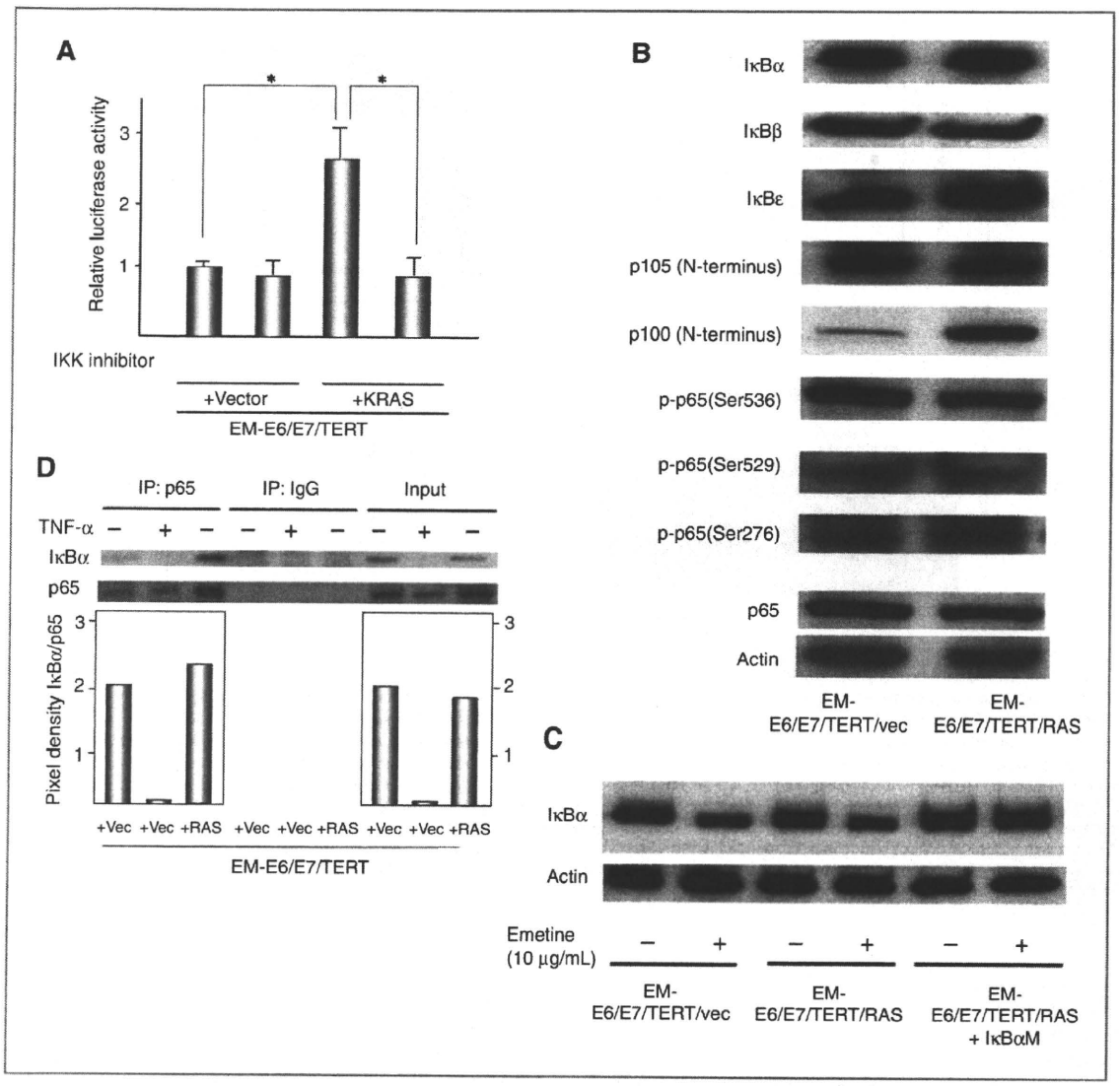

Figure 5. *KRAS*-induced activation of NF-κB in endometrial carcinogenesis is IKK dependent but not on known canonical pathways. A, IKK dependence of NF-κB activation. EM-E6/E7/TERT/vec cells or EM-E6/E7/TERT/RAS cells were transfected with reporter plasmids containing the NF-κB–responsive elements and were incubated with or without 5 μmol/L of IKK inhibitor X. Luciferase assays were carried out after 48 hours of incubation. Relative luciferase activities are shown as the mean values of 3 independent experiments, in which those of E6/E7/TERT/vec cells were normalized to 1.0. Bars, SD. *, $P < 0.05$. B, expression levels of IκB family proteins and phosphorylated p65 in *KRAS*-introduced cells. Whole-cell extracts of EM-E6/E7/TERT/vec cells or EM-E6/E7/TERT/RAS cells were subjected to the Western blot analysis, and the levels of expression in each factor are compared. C, change in IκBα turnover rate was compared between cells with or without oncogenic *KRAS*. EM-E6/E7/TERT/RAS cells or EM-E6/E7/TERT/vec cells were treated with or without 10 μg/mL of emetine and the whole-cell extracts were subjected to the Western blot analysis for IκBαM. As a control, EM-E6/E7/TERT cells with overexpressed IκBαM were used, in which IκBα level is stable even in the presence of emetine due to the lack of the specific phosphorylation site essential for degradation. D, change in dissociation rate of p65 with IκBα was compared between cells with or without oncogenic *KRAS*. Immunoprecipitation (IP) was carried out with antibodies against p65 or control IgG, using whole-cell lysates from EM-E6/E7/TERT/vec or EM-E6/E7/TERT/RAS cells. TNF-α (20 ng/mL for 5 minutes) stimulation was carried out in vector (Vec) cells to facilitate the degradation of IκBα, used as a positive control of degradation status. Western blot analysis was subsequently carried out on immunoprecipitants with antibodies to p65 or IκBα. The pixel densities of IκBα and p65 Western blots were quantified using NIH Scion software. Graph represents the relative pixel density of IκBα normalized to p65 levels in each sample.

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NF-κB transcriptional factor is a putative effector of Ras-mediated transformation (8–10). We showed that oncogenic *KRAS* enhanced the NF-κB binding to its responsive elements and facilitated the transactivation of the target promoters. The introduction of IκBαM successfully inhibited transactivation of NF-κB without affecting ERK activity, and we found that such inhibition completely abrogated the anchorage-independent growth and tumor-forming ability of EM-E6/E7/TERT/RAS cells, suggesting a major contribution of NF-κB activity to *KRAS*-induced carcinogenesis of endometrial epithelial cells.

What is the molecular mechanism of NF-κB activation in *KRAS*-induced endometrial carcinogenesis? The transactivation of promoters by NF-κB is directly controlled by its nuclear translocation and its modification in the nucleus. In most cell types, NF-κB dimers are sequestered in the cytoplasm and inactivated by IκB proteins, which bind to the NF-κB and mask the nuclear localization signal (28). The phosphorylation of a specific serine residue, Ser32/36, in IκBα by the upstream regulators such as IKK results in polyubiquitination and subsequent degradation by 26 S proteasomes, causing release of the NF-κB dimer and promoting its translocation to the nucleus, activating various κB-responsive gene expressions (29, 30). Thus, we first tested the involvement of IKK in the activation. The treatment of EM-E6/E7/TERT/RAS cells with the IKK inhibitor X significantly suppressed NF-κB transcriptional activity, confirming that *KRAS*-induced NF-κB activation was IKK dependent. We further examined the basal expression levels of IκB family proteins in the presence or absence of oncogenic *KRAS* and found that the expression levels of IκB proteins, including IκBα, β, ε, and p105, were not affected by oncogenic *KRAS*. Expression of p100 protein, which is a member of the IκB protein family and a precursor of NF-κB subunit p52 (31), increased in EM-E6/E7/TERT/RAS cells. This is probably because the p100 promoter contains a κB site (32). We do not consider that this phenomenon is involved in NF-κB activation, because the increased expression of p100 may inhibit NF-κB activity but never activates NF-κB. In addition, the expression of p52 was unchanged (data not shown). Therefore, we speculate that the p100/p52 subunit is not likely to participate in NF-κB activation by oncogenic *KRAS*. Furthermore, we focused on the modification of the NF-κB subunit itself. Nuclear NF-κB modification, especially p65/RelA subunit modification, has been investigated and found to affect DNA binding and interactions with coactivators and corepressors and the termination of the NF-κB response (33). These modifications include phosphorylation of Ser536 and Ser529 in the C-terminal transactivation domains and Ser276 in the Rel homology domain (17, 34–36). However, the expression of phosphorylated p65 was not elevated in the *KRAS*-introduced cells. We further confirmed the possibility of increased IκB degradation that might result in NF-κB activation. The IκB turnover assay with protein synthesis inhibitor revealed that the turnover was not accelerated by oncogenic *KRAS*, again denying the possibility as an activation mechanism. Recently proven additional mechanisms of NF-κB activation is a dissociation of IκB from p65 in the absence of IκB degradation (22, 37). We examined the interaction of IκBα and p65 by immunoprecipitation. The expression levels of IκBα attached to p65 was, however, equivalent between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells, showing that such dissociation is not involved in *KRAS*-mediated NF-κB activation. Thus, we concluded that *KRAS*-induced activation of NF-κB during endometrial carcinogenesis is IKK dependent but not on known canonical pathways. Identification of such unknown mechanisms is needed using our model for understanding not only of the activation mechanisms of NF-κB but also of the carcinogenesis of endometrium.

So far, only one study has addressed *KRAS* mutation and NF-κB activation in endometrial cancer (38). This report examined surgically resected cancer tissues and reported the high frequency of nuclear location of NF-κB families. However, no correlation was found between the nuclear immunostaining of NF-κB and *KRAS* mutation. These findings do not conflict with our results, because their analyses were carried out using specimens of progressive cancers and not samples at the stage of cancer initiation or development, in which network of etiologic factors might be modified because of acquired genetic alterations during the late stage of cancer development.

This study may provide a clinical implication for NF-κB as a novel molecular target for cancer chemoprevention of the endometrium. Accumulating evidence has clarified chemopreventive effects of anti-inflammatory agents such as aspirin or other nonsteroidal anti-inflammatory drugs on various cancer types partially via inhibition of NF-κB (39, 40). As for endometrial cancer, Moysich and colleagues reported the risk reduction by regular use of aspirin among obese women (41). Interestingly, multiple signaling pathways, including PTEN-PI3K-Akt pathway, are known to activate NF-κB in endometrial cancer cells (42). Loss of function mutation in *PTEN* and activating mutation in *PIK3CA* are putative activator of NF-κB through Akt expression in endometrial cancer and in the precursor lesions (43). Therefore, it is possible that NF-κB plays a role in endometrial carcinogenesis via various pathways other than *KRAS*-driven pathways, giving light to the potential role of NF-κB inhibitors in preventing endometrial carcinogenesis.

In summary, we for the first time show that the activation of NF-κB is a novel target of oncogenic *KRAS* in endometrial carcinogenesis. Blockade of NF-κB activity led to effective inhibition of transformed phenotypes of endometrial cells. These findings may add the novel information on the molecular pathway of endometrial carcinogenesis, implying the potential utility of NF-κB inhibitors for endometrial cancer chemoprevention, especi'ally with *KRAS* mutation.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.