

Activation of NF- κ B Is a Novel Target of KRAS-Induced Endometrial Carcinogenesis

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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- κ B in endometrial carcinogenesis. We found that the DNA-binding activity of NF- κ B was markedly elevated in *KRAS* tumorigenic cells compared with *TERT*-immortalized cells. Furthermore, the ability of NF- κ B to activate the target gene promoters significantly increased in *KRAS* tumorigenic cells. Introduction of a mutant I κ B that is resistant to degradation and thereby enhances the inhibitory effect on NF- κ B 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- κ B.

Conclusions: These findings clearly show that NF- κ B activation is a novel target of oncogenic *KRAS* in endometrial carcinogenesis, implying the potential utility of NF- κ B 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.

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- κ B has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory

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doi: 10.1158/1078-0432.CCR-10-2291

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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- κ B 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- κ B inhibitors.

responses. Accumulating evidence supports a key role of the constitutive activation of NF- κ B in controlling the initiation and progression of human cancer (8). NF- κ B 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- κ B in endometrial carcinogenesis remains unclear. In this study, we show for the first time that NF- κ B 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- κ B binding site (Promega) was end labeled with the kit (MEGALABEL; Takara Bio Inc.). For the assay, 50 μ g of nuclear protein extract was incubated for 30 minutes at room temperature in a final volume of 25 μ L containing 10,000 cpm of labeled oligonucleotides, 1 μ g of poly (dI-dC), 0.5 mmol/L of phenylmethylsulfonyl fluoride, 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- κ B were used as competitors. For supershift assays, the nuclear extracts were incubated with specific antibodies against NF- κ B 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 μ g of luciferase reporter plasmid driven by

NF- κ B-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 (I κ B kinase complex) pathways in promoter activation, 5 μ mol/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 I κ B α cDNA-encoding superrepressor (I κ B α -SR) harboring S32A and S36A mutations (Clontech; catalogue no. 6319233) were cloned and recombined into retroviral vectors to generate pCMSCVpuro-HA-MEK1DD and pCMSCVbsd-I κ B α (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- κ B p65 (Ser536), phospho-NF- κ B p65 (Ser276), I κ B α (Cell Signaling Technology), I κ B ϵ (Abcam), I κ B β (Delta Biolabs), NF- κ B p65 phospho-Ser529 (Millipore), NF- κ B p52, NF- κ B 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 I κ B (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% CO₂ at 37°C. Growth activity of EM-E6/E7/TERT/RAS cells with overexpressed mutant I κ B α (I κ B α M) or with control vectors was evaluated in normal serum (10% FBS) or low serum (0.5% FBS) conditions by

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 \pm 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

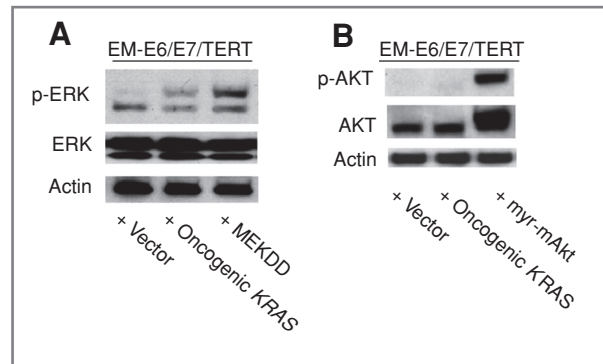


Figure 1. The RAF-ERK pathway, but not the PI3K-Akt pathway, is activated in endometrial epithelial cells transformed by oncogenic *KRAS*. Immortalized endometrial epithelial (EM-E6/E7/TERT) cells were stably transfected with oncogenic *KRAS*, constitutively activated *MEK* (MEKDD), or constitutively 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.

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.

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 <i>nu/nu</i>)
+ Vector	No	No
+ Oncogenic <i>KRAS</i>	Yes (100%)	Yes (100%)
+ Active MEK	No	No

Oncogenic *KRAS* enhances the transcriptional function of NF- κ B 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- κ B, based on emerging evidence that NF- κ B 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- κ B activity in EM-E6/E7/TERT cells. First, we examined the change in the DNA-binding activity of NF- κ B by electrophoretic mobility shift assay (EMSA), using consensus oligonucleotides for NF- κ B 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- κ B consensus oligonucleotides but not by unrelated SP1 or AP2 oligonucleotides. Furthermore, they were supershifted by the addition of antibodies against NF- κ B p50 or p65 subunits (Fig. 2B). We confirmed that endogenous expression levels of NF- κ B 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- κ B to its target sequences in endometrial cancer cells.

Next, we examined the change in the ability of NF- κ B 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- κ B-responsive elements (pNF κ B-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- κ B to transactivate the target gene promoter in endometrial cancer cells. Interestingly, this upregulation of NF- κ B 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- κ B in endometrial epithelial cells in a RAF-ERK pathway-independent manner.

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

Regulation of NF- κ B activity is controlled mainly by the inhibitory function of the I κ B family, including I κ B α . Phosphorylation of I κ B α at 2 serine residues (Ser32 and

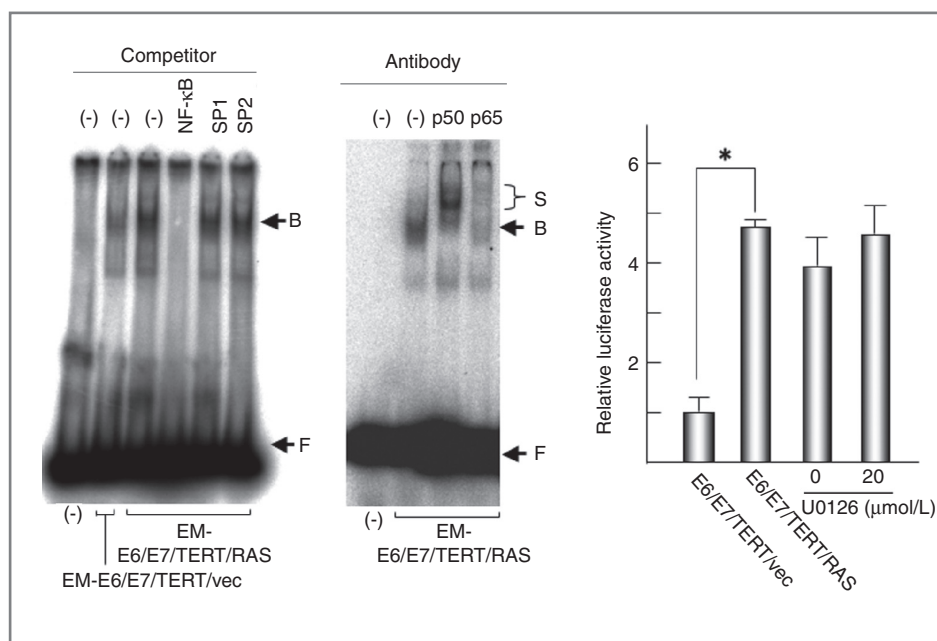


Figure 2. Oncogenic *KRAS* activates NF- κ B in endometrial epithelial cells. A and B, EMSA to examine the DNA-binding activity of NF- κ B. Nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were incubated with γ -³²P-labeled consensus oligonucleotides containing the NF- κ B-responsive elements, followed by electrophoresis. For competition assays, 100-fold molar excess of unlabeled consensus oligonucleotides for NF- κ B, AP2, and SP1 were used as competitors (A). For the supershift analysis, specific antibodies against NF- κ B 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- κ B 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- κ B-responsive elements. Plates were harvested 48 hours after transfection and luciferase assays were conducted. For inhibition of the RAF-ERK pathway, 20 μ mol/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. *, $P < 0.05$.

Ser36) leads to ubiquitination of I κ B and subsequent proteasome-mediated degradation in the canonical NF- κ B induction pathway (17). A dominant-negative mutant of I κ B α , named I κ B α M, 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- κ B (18). To elucidate the role of NF- κ B in *KRAS*-induced endometrial carcinogenesis, we established cell lines with disabled NF- κ B function by introducing I κ B α M 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 I κ B α M significantly repressed the ability of NF- κ B to activate the target promoters. We also confirmed that ERK activity was not affected by the introduction of I κ B α M 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 I κ B α M (data not shown). However, in low-serum conditions with 0.5% FBS, cells with overexpressed I κ B α M 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- κ B in *KRAS*-mediated endometrial carcinogenesis.

NF- κ B activation by oncogenic *KRAS* is IKK dependent but independent of p65 phosphorylation or I κ B α degradation/dissociation

We next sought to identify the molecular mechanisms of NF- κ B 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 IKK β and IKK α . As shown in Figure 5A, the addition of IKK inhibitors largely inhibited the activity of NF- κ B-responsive promoter in *KRAS*-introduced cells but not vector cells, indicating that this activation was IKK dependent.

Recent studies have focused on I κ B 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 I κ B 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 I κ B α , 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 I κ B subtype regulation or p65 nuclear modification does not significantly contribute to *KRAS*-induced NF- κ B activation during endometrial carcinogenesis.

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

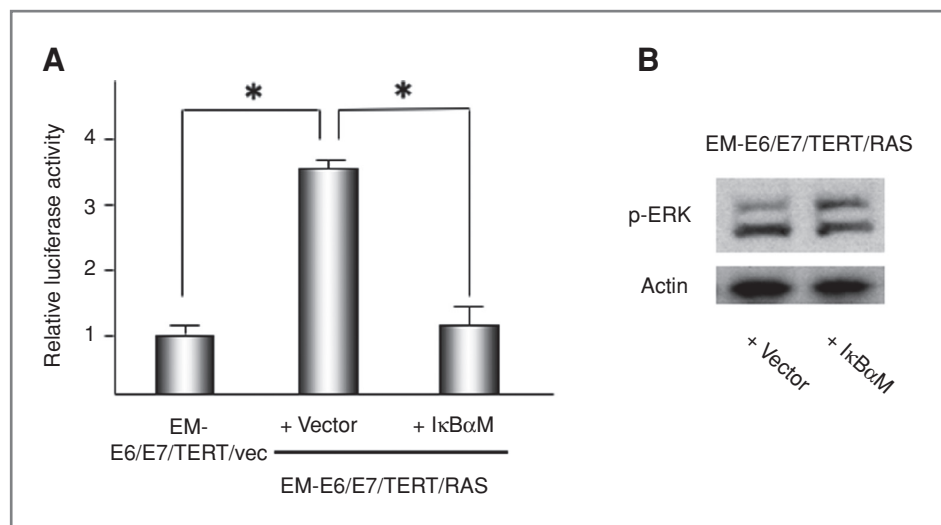


Figure 3. Introduction of I κ B α M effectively inhibits NF- κ B activity in transformed endometrial epithelial cells. EM-E6/E7/TERT/RAS cells were stably overexpressed with I κ B α M lacking phosphorylation sites essential for its degradation, thereby becoming stable against degradation signals. Eventually, these transfectants were expected to have disabled NF- κ B by I κ B activity. To confirm the *in vivo* function of NF- κ B 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- κ B-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 I κ B α M and the vector control cells, examining the phosphorylated ERK expression by Western blot analysis.

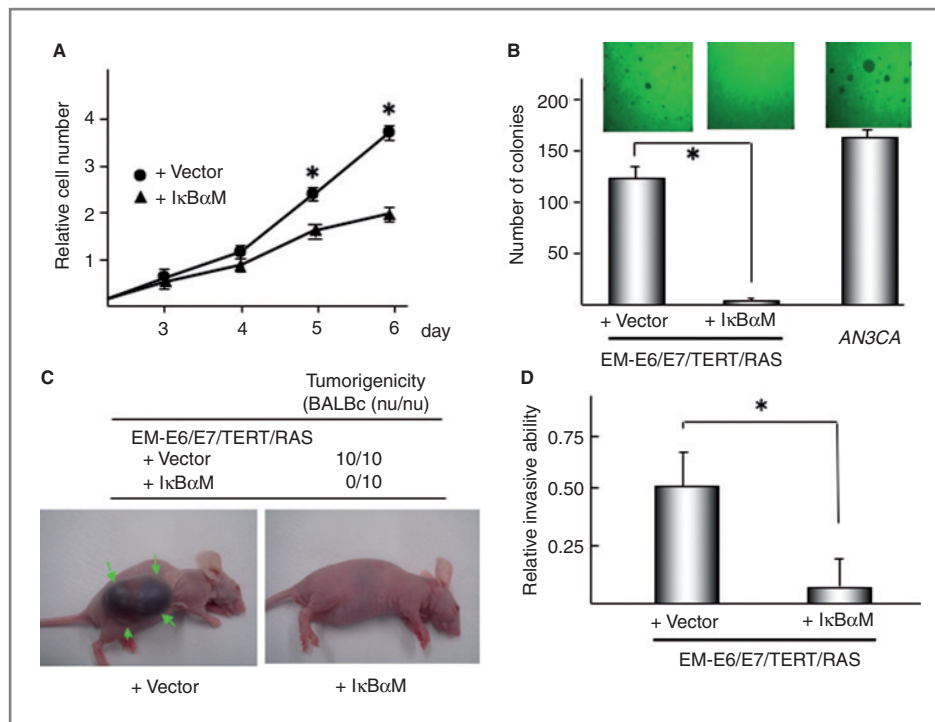


Figure 4. Transforming activity of oncogenic *KRAS* is disturbed by inhibiting NF- κ B activity in endometrial epithelial cells. Transformed phenotypes of EM-E6/E7/TERT/RAS cells overexpressed with I κ B α M were evaluated by various experiments. **A**, cell growth assay in a low-serum condition. The growth curve is shown in which EM-E6/E7/TERT/RAS cells transfected with or without I κ B α M 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 I κ B α M 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 I κ B α M 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 I κ B α M 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 I κ B α M 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- κ B by oncogenic *KRAS* is not due to accelerated degradation of I κ B α .

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

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.

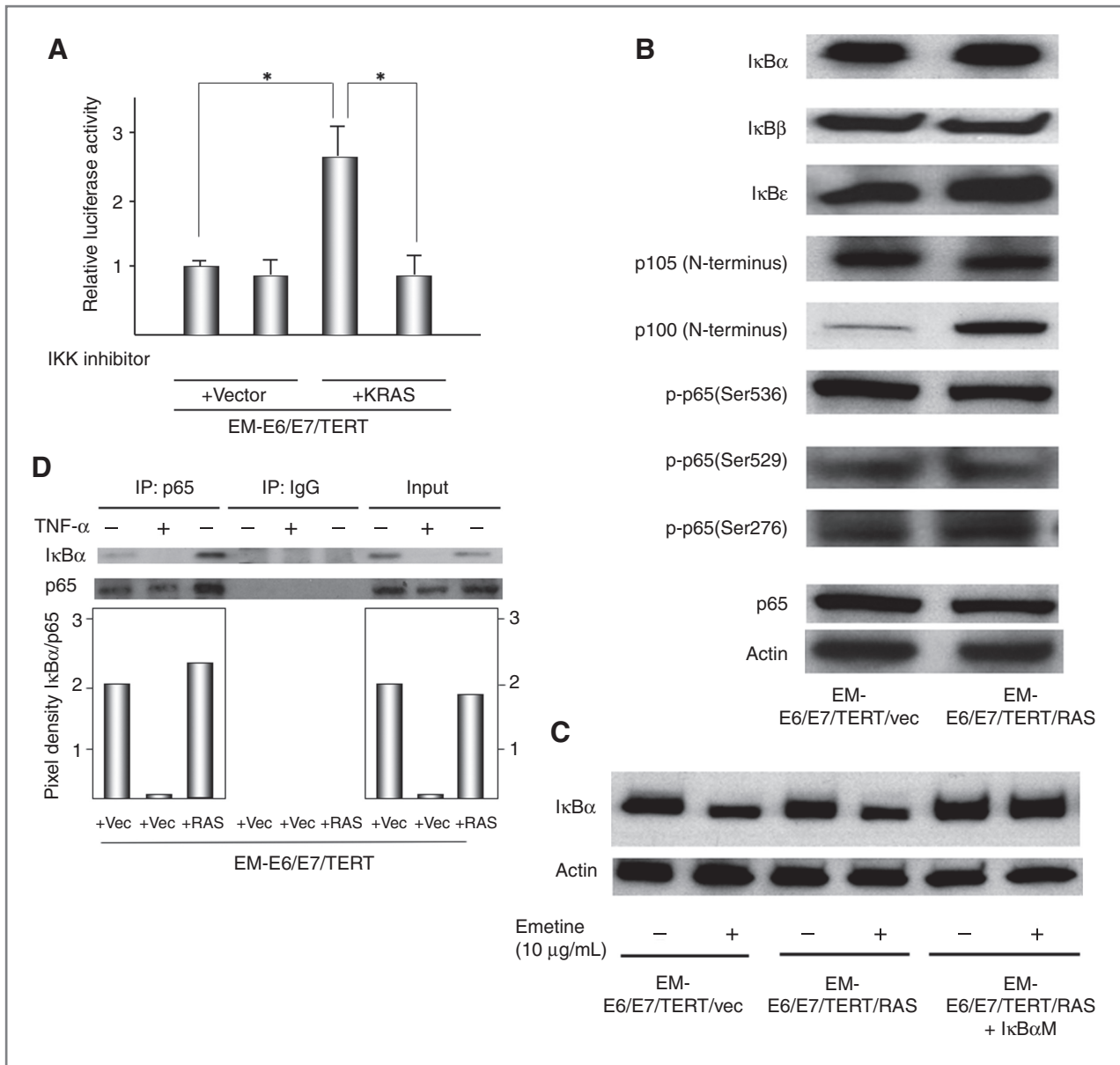


Figure 5. KRAS-induced activation of NF- κ B in endometrial carcinogenesis is IKK dependent but not on known canonical pathways. **A**, IKK dependence of NF- κ B activation. EM-E6/E7/TERT/vec cells or EM-E6/E7/TERT/RAS cells were transfected with reporter plasmids containing the NF- κ B-responsive elements and were incubated with or without 5 μ mol/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 I κ B 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 I κ B α 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 μ g/mL of emetine and the whole-cell extracts were subjected to the Western blot analysis for I κ B α M. As a control, EM-E6/E7/TERT cells with overexpressed I κ B α M were used, in which I κ B α 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 I κ B α 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 I κ B α , used as a positive control of degradation status. Western blot analysis was subsequently carried out on immunoprecipitants with antibodies to p65 or I κ B α . The pixel densities of I κ B α and p65 Western blots were quantified using NIH Scion software. Graph represents the relative pixel density of I κ B α normalized to p65 levels in each sample.

NF- κ B transcriptional factor is a putative effector of Ras-mediated transformation (8–10). We showed that oncogenic *KRAS* enhanced the NF- κ B binding to its responsive elements and facilitated the transactivation of the target promoters. The introduction of I κ B α M successfully inhibited transactivation of NF- κ B 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- κ B activity to *KRAS*-induced carcinogenesis of endometrial epithelial cells.

What is the molecular mechanism of NF- κ B activation in *KRAS*-induced endometrial carcinogenesis? The transactivation of promoters by NF- κ B is directly controlled by its nuclear translocation and its modification in the nucleus. In most cell types, NF- κ B dimers are sequestered in the cytoplasm and inactivated by I κ B proteins, which bind to the NF- κ B and mask the nuclear localization signal (28). The phosphorylation of a specific serine residue, Ser32/36, in I κ B α by the upstream regulators such as IKK results in polyubiquitination and subsequent degradation by 26 S proteasomes, causing release of the NF- κ B dimer and promoting its translocation to the nucleus, activating various κ B-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- κ B transcriptional activity, confirming that *KRAS*-induced NF- κ B activation was IKK dependent. We further examined the basal expression levels of I κ B family proteins in the presence or absence of oncogenic *KRAS* and found that the expression levels of I κ B proteins, including I κ B α , β , ϵ , and p105, were not affected by oncogenic *KRAS*. Expression of p100 protein, which is a member of the I κ B protein family and a precursor of NF- κ B subunit p52 (31), increased in EM-E6/E7/TERT/RAS cells. This is probably because the p100 promoter contains a κ B site (32). We do not consider that this phenomenon is involved in NF- κ B activation, because the increased expression of p100 may inhibit NF- κ B activity but never activates NF- κ B. 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- κ B activation by oncogenic *KRAS*. Furthermore, we focused on the modification of the NF- κ B subunit itself. Nuclear NF- κ B 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- κ B 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 I κ B degradation that might result in NF- κ B activation. The I κ B 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- κ B activation is a dissociation of I κ B from p65 in the absence of I κ B degradation (22, 37). We examined the interaction of I κ B α and p65 by immunoprecipitation. The expression levels of I κ B α 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- κ B activation. Thus, we concluded that *KRAS*-induced activation of NF- κ B 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- κ B but also of the carcinogenesis of endometrium.

So far, only one study has addressed *KRAS* mutation and NF- κ B activation in endometrial cancer (38). This report examined surgically resected cancer tissues and reported the high frequency of nuclear location of NF- κ B families. However, no correlation was found between the nuclear immunostaining of NF- κ B 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- κ B 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- κ B (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- κ B in endometrial cancer cells (42). Loss of function mutation in *PTEN* and activating mutation in *PIK3CA* are putative activator of NF- κ B through Akt expression in endometrial cancer and in the precursor lesions (43). Therefore, it is possible that NF- κ B plays a role in endometrial carcinogenesis via various pathways other than *KRAS*-driven pathways, giving light to the potential role of NF- κ B inhibitors in preventing endometrial carcinogenesis.

In summary, we for the first time show that the activation of NF- κ B is a novel target of oncogenic *KRAS* in endometrial carcinogenesis. Blockade of NF- κ B 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- κ B inhibitors for endometrial cancer chemoprevention, especially with *KRAS* mutation.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Grant Support

This study was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the Megumi Medical Foundation of Kanazawa University.

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Received August 26, 2010; revised December 17, 2010; accepted December 29, 2010; published online March 16, 2011.

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Clin Cancer Res 2011;17:1341-1350.

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