Mig-6 Suppresses Endometrial Cancer Associated with Pten Deficiency and ERK Activation

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Abstract

PTEN mutations are the most common genetic alterations in endometrial cancer. Loss of PTEN and subsequent AKT activation stimulate estrogen receptor α–dependent pathways that play an important role in endometrial tumorigenesis. The major pathologic phenomenon of endometrial cancer is the loss of ovarian steroid hormone control over uterine epithelial cell proliferation and apoptosis. However, the precise mechanism of PTEN/PI3K/AKT signaling in endometrial cancer remains poorly understood. The progesterone signaling mediator Mig-6 suppresses estrogen signaling and it has been implicated previously as a tumor suppressor in endometrial cancer. In this study, we show that Mig-6 also acts as a tumor suppressor in endometrial cancers associated with PTEN deficiency. Transgenic mice, where Mig-6 was overexpressed in progesterone receptor–expressing cells, exhibited a relative reduction in uterine tumorigenesis caused by Pten deficiency. ERK1/2 was phosphorylated in uterine tumors and administration of an ERK1/2 inhibitor suppressed cancer progression in Pten+/- Pten/+/ mice. In clinical specimens of endometrial cancer, Mig-6 expression correlated inversely with ERK1/2 phosphorylation during progression. Taken together, our findings suggest that Mig-6 regulates ERK1/2 phosphorylation and that it is crucial for progression of Pten-mutant endometrial cancers, providing a mechanistic rationale for the evaluation of ERK1/2 inhibitors as a therapeutic treatment in human endometrial cancer. Cancer Res; 74(24); 7371–82. ©2014 AACR.

Introduction

Endometrial cancer is the most common gynecologic cancer (1). In the United States, approximately 47,130 cases will be diagnosed and 8,190 women die from the disease in 2013 (2).

Endometrioid carcinoma comprises 70% to 80% of cases and originates in epithelial cells of the endometrium (1). An increased incidence of endometrial cancer has been found in association with prolonged, unopposed estrogen exposure (3), and aberrant activation of the PTEN/Pi3K/AKT and ErbB/ERK signaling pathways (1). PTEN is one of the most frequently mutated tumor-suppressor genes in human cancers (4). PTEN deficiency. Transgenic mice, where Mig-6 was overexpressed in progesterone receptor–expressing cells, exhibited a relative reduction in uterine tumorigenesis caused by Pten deficiency. ERK1/2 was phosphorylated in uterine tumors and administration of an ERK1/2 inhibitor suppressed cancer progression in Pten+/- Pten/+/ mice. In clinical specimens of endometrial cancer, Mig-6 expression correlated inversely with ERK1/2 phosphorylation during progression. Taken together, our findings suggest that Mig-6 regulates ERK1/2 phosphorylation and that it is crucial for progression of Pten-mutant endometrial cancers, providing a mechanistic rationale for the evaluation of ERK1/2 inhibitors as a therapeutic treatment in human endometrial cancer. Cancer Res; 74(24); 7371–82. ©2014 AACR.
to progestin treatment (12). However, objective responses were detected in 30% to as many as 56% of patients with metastatic or recurrent endometrial carcinoma (13). Therefore, the effectiveness of progestin for women with endometrial cancer is less clear.

The mitogen-inducible gene 6 (Mig-6, ErfR1, RALT, or gene 33) is an immediate early response gene that can be induced by various mitogens and commonly occurring chronic stress stimuli (14). Ablation of Mig-6 in mice leads to the development of animals with epithelial hyperplasia, adenoma, and adenocarcinomas in organs (15, 16). Decreased expression of Mig-6 is observed in human breast carcinomas that correlate with reduced overall survival of patients with breast cancer (17). Previously, we demonstrated that Mig-6 is an important mediator of progesterone signaling to suppress estrogen signaling in the uterus (16). Ablation of Mig-6 in the murine uterus leads to the development of endometrial hyperplasia and estrogen-induced endometrial cancer (16, 18). Mig-6 plays a tumor-suppressor function in the context of Pten ablation (19). However, the mechanism by which Mig-6 alters endometrial cancer pathophysiology remains unknown.

In this study, we used conditional overexpression of Mig-6 in the PR<sup>+/−</sup>/Pten<sup>f/f</sup> (Pten<sup>d/d</sup>) mice to demonstrate that Mig-6 is an important tumor suppressor in Pten-deficient cancer. PR<sup>+/−</sup>/Mig-6<sup>+/+</sup>/Pten<sup>f/f</sup> (Mig-6<sup>+/−</sup>/Pten<sup>d/d</sup>) mice suppressed to develop endometrial cancer through inhibition of ERK1/2 phosphorylation. The correlation studies showed an inverse correlation between Mig-6 and phospho-ERK1/2 in human endometrial cancer. Thus, these results demonstrate that Mig-6 mediates progesterone action to suppress development and progression of endometrial cancer by inhibiting ERK1/2 signaling.

**Materials and Methods**

**Construction of the targeting vectors and generation of Mig-6<sup>LSL</sup> mice**

The targeting vector contains Mig-6 cDNA, two ROSA26 genomic sequences for gene targeting (5′- and 3′-arms), a cytomegalovirus early enhancer/chicken β-actin (CAG) promoter, and a loxP-STOP-loxP (LSL) cassette. The linearized targeting construct was transfected into R1 ES cells by electroporation (Supplementary Fig. S1A). Puromycin-resistant clones were screened by Southern blot analysis using 5′ external probes with EcoRV-digested genomic DNA (Supplementary Fig. S1B). Correctly targeted clones were microinjected into blastocysts derived from C57BL/6 mice. Chimeras were bred to C57BL/6 mice and F1 agouti offspring mice were analyzed by PCR genotyping to validate the germ line transmission of the Mig-6<sup>LSL</sup> allele. For genotyping analysis, PCR analysis was used to genotype DNA extracts from tail biopsies (Supplementary Fig. S1C and S1D). To detect the Mig-6<sup>LSL</sup> allele (Supplementary Fig. S1), primers P1, P2, and P3 were designed to amplify the fragment from wild-type (300 bp) and Mig-6<sup>LSL</sup> allele (450 bp).

**Animals and tissue collection**

Mice were cared for and used in the designated animal care facility according to the Michigan State University institutional guidelines. Mice of various genotypes were sacrificed at 2 and 3 months of age (n = 5/genotype). For the U0126 treatment study. Pten<sup>f/f</sup> and Pten<sup>d/d</sup> mice at 2 months of age were injected with either vehicle (PBS) or U0126 (50 μmol/kg) for 3 months (n = 5/genotype/treatment). Inhibitor injections were repeated every week. After the fourth injection, mice were killed at 5 months of age. Uterine tissues were flash-frozen at the time of dissection and stored at −80°C for RNA or fixed with 4% (v/v) paraformaldehyde for histology analysis.

**Cell cultures**

HeLa, Ishikawa, and 293T were obtained from the ATCC. Cell lines were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin (100 μU/mL). All cells were cultured at 37°C under 5% CO<sub>2</sub>. All cells were used within 6 months from the time when they were obtained, expanded, and resuscitated from the ATCC.

**Quantitative real-time PCR**

RNA was extracted from the uterine tissues using the RNeasy Total RNA Isolation Kit (Qiagen). RT-PCR was performed using RT-PCR Universal Master Mix reagent (Applied Biosystems) according to the manufacturer’s instructions. All RT-PCR TaqMan analysis was done by using five independent RNA sets. mRNA quantities were normalized against 18S RNA using ABI rRNA control reagents.

**Immunohistochemistry**

Uterine sections from paraffin-embedded tissue were cut at 5 μm and mounted on silane-coated slides, deparaffinized, and rehydrated in a graded alcohol series. Sections were preincubated with 10% normal serum in PBS (pH 7.5) and then incubated with primary antibody diluted in 10% normal goat serum in PBS (pH 7.5) overnight at 4°C. On the following day, sections were washed in PBS and incubated with a secondary antibody (Vector Laboratories) for 1 hour at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB Kit (Vector Laboratories).

**Western blot analysis**

Samples containing 15-μg proteins were applied to SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore). Membranes were blocked overnight with 0.5% casein (w/v) in PBS with 0.1% Tween 20 (v/v; Sigma-Aldrich) and probed with antibodies. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody and treatment with enhanced chemiluminescence reagents. To control for loading, the membrane was stripped and probed with anti-actin (Santa Cruz Biotechnology) and developed again.

**Reverse phase protein array**

Reverse phase protein array (RPPA) was performed as previously described (20). Briefly, tissue lysates were printed on nitrocellulose-coated slides and probed with validated antibodies. Signals were captured by tyramide dye deposition.
(CSA System; DAKO). Data were collected and analyzed using quantification software.

**In vitro kinase assay**

GST-fusion proteins were incubated with 30 μg of FLAG-MIG-6–transfected cell lysates in the presence of kinase reaction buffer (10 μL 5× kinase buffer, 10 μL magnesium/ATP cocktail solution 90 μL, 75 mmol/L MgCl2/500 mmol/L ATP plus 10 μL (100 μCi) of γ-32P-ATP (3000 Ci/mmol) in a total volume of 50 μL for 30 minutes at 30° C. Reactions were terminated by washing twice with 1× kinase buffer. Samples were resuspended in 15 μL 5× SDS sample loading buffer and boiled for 5 minutes. After electrophoresis, SDS polyacrylamide gels were stained with Coomassie blue and dried, and the phosphorylated products were visualized by autoradiography.

**Immunofluorescence analysis**

Ishikawa cells were plated at 20-mm glass coverslip and transfected with FLAG-tagged MIG-6 or/and V5-tagged ERK2 plasmids. After incubation for 24 hours, cells were fixed for 30 minutes in cold 4% (w/v) paraformaldehyde, and permeabilized for 5 minutes in 1/40 Triton X-100. Primary antibodies to FLAG (Sigma-Aldrich) and V5 (Bethyl Laboratories) were added and overnight at 4°C. After three washes in PBS, coverslips were incubated for 1 hour at 37°C with Alexa Fluor 555 donkey anti-rabbit and Alexa Fluor 488 donkey anti-mouse (Invitrogen). Cells were then mounted with mounting media containing 4, 6-diamino-2-phenylindole (Invitrogen) for fluorescence analysis on glass slides and visualized using a Zeiss 510 Meta Confocal Microscope (Carl Zeiss).

**Duolink in situ proximity ligation assay analysis**

Duolink in situ proximity ligation assay (PLA) analysis was performed according to the manufacturer’s instructions (Olink Biosciences). Briefly, paraformaldehyde-fixed cells were washed with PBS, incubated for 10 minutes at 0.1% (v/v) Triton X-100, washed, and blocked with blocking solution. Primary antibodies, against ERK1/2 and Phospho-ERK1/2 antibodies, were applied, and the cells were incubated with PLUS and MINUS secondary PLA probes against rabbit and mouse IgGs. The incubation was followed by hybridization and ligation, and then amplification was performed. After mounting with Duolink mounting medium, samples were examined using a Zeiss 510 Meta (NLO) Confocal Microscope (Carl Zeiss).

**Statistical analysis**

Statistical analysis was performed with one of following: one-way ANOVA analysis followed by the Tukey post hoc test, and the log-rank test using the Instat package from GraphPad.

**Results**

**Overexpression of Mig-6 suppresses tumorigenesis in Pten-deficient endometrial cancer**

To determine the tumor-suppressor function of Mig-6 in the development of endometrial cancer, we generated conditional overexpression of Mig-6 mice by using embryonic stem cell targeting on the ROSA26 locus, inserting a CAGGS promoter, LSL cassette, and Mig-6 CDNA (Mig-6LSL). ROSA26 is a locus used for constitutive, ubiquitous gene expression in mice (21). The mouse Rosa26 locus is particularly useful for genetic modification as it can be targeted with high efficiency and is expressed in most cell types tested (22). The CAG promoter is a strong synthetic promoter frequently used to drive high levels of gene expression in mammalian expression vectors (23). Before activation, Mig-6 is silent due to a floxed STOP cassette inserted between the promoter and the transgene. Upon co-mediated excision of the STOP cassette, Mig-6 is constitutively expressed by the ubiquitous CAG promoter. Therefore, this transgenic mouse model can be used to express Mig-6 in any tissue in a spatial and/or temporal manner if respective cre mouse lines are available (24). Mig-6LSL mice were bred to PR-Cre mice (25) to generate Mig-6 overexpression in the PR-expressing cells (Mig-6LSL+; Supplementary Fig. S1 and S2).

Loss of Pten (either as a heterozygote or by uterine specific ablation) has been shown to induce endometrial cancer in mice highlighting its important role in endometrial cancer development (7, 26). To assess the effects of the overexpression Mig-6 on endometrial cancer development and progression, Mig-6LSL+ mice were bred to the Pten floxed (PtenΔf) mouse model (26) to generate ablation of Pten and overexpression of Mig-6 in the uterus (Mig-6over PtenΔd mice; Supplementary Fig. S2C and S2D). PtenΔd mice had a decrease survival due to the development of endometrial cancer (26). Therefore, we first examined the survival time of control, Mig-6over PtenΔd, and Mig-6over PtenΔd mice. The survival time of Mig-6over PtenΔd mice was significantly longer than PtenΔd mice (Fig. 1A). Mig-6over PtenΔd mice did not die due to the development of endometrial cancer. However, PtenΔd mice developed vaginal papillomas as well as endometrial cancer. Overexpression of Mig-6 did not have a remarkable effect on the vaginal papilloma phenotype of PtenΔd mice. The mice were euthanized if the mice exhibited immobility, inability to eat, >15% weight loss, and vaginal prolapse.

To address the impact of overexpression of Mig-6 on the development and progression of endometrial cancer, control, Mig-6over, PtenΔd, and Mig-6over PtenΔd mice were sacrificed at 2 and 3 months of age and uterine weight, gross, and histologic morphology were examined. Mig-6over PtenΔd mice showed a significant decrease in uterine weight compared with PtenΔd mice at 3 months of ages (Fig. 1B). Gross morphology at 3 months of age showed that the overexpression of Mig-6 remarkably suppressed the development of endometrial cancer in PtenΔd mice (Fig. 1C). Histologic analysis demonstrated that the uteri of PtenΔd mice exhibited development of endometrial adenocarcinoma at 2 and 3 months of ages as characterized by neoplastic endometrial glands invading through the myometrium (Fig. 1D and Supplementary Fig. S3). Although endometrial hyperplasia was observed in the uteri of Mig-6over PtenΔd mice at 2 months, endometrial adenocarcinoma with invasion into the myometrium was not observed (Supplementary Fig. S3). Surprisingly, Mig-6over PtenΔd mice at 3 months age did not exhibit endometrial hyperplasia (Fig. 1D).
In addition, we could not find any pathologic effects in Mig-6over mice. These results demonstrate that overexpression of Mig-6 suppresses endometrial cancer progression in conditional uterine ablation of Pten.

Overexpression of Mig-6 inhibits epithelial proliferation in Pten-deficient endometrial cancer

To determine whether the suppression of endometrial cancer in Mig-6over Pten<sup>fl/fl</sup> mice is caused by an alteration in cell proliferation, we examined the IHC for phospho-histone H3, a mitotic marker. Immunohistochemical analysis showed significantly lower proliferation in uterine epithelial cells of Mig-6over Pten<sup>fl/fl</sup> mice compared with Pten<sup>fl/fl</sup> mice at 2 months of age (Fig. 2A and B). Estrogen-dependent endometrioid carcinoma is the most common type of endometrial cancer (3). Importantly, one of the major pathologic phenomena of an endometrial cancer is the loss of estrogen and progesterone control over uterine epithelial cell proliferation (27). Estrogen rapidly activates the hypoxia-inducible factor 1 alpha (Hif1α; ref. 28). The expression of Hif1α and its target genes were significantly decreased in Mig-6over Pten<sup>fl/fl</sup> mice compared with the other groups at 3 months of age (Fig. 2C).

The expression of PR is increased in Mig-6over Pten<sup>fl/fl</sup>

Expressions of PR and ERα have been reported prognostic factors for endometrial carcinoma (29). The expression of PR was increased and its target genes (Fst and Il13ra2) were significantly increased in uterus of Mig-6over Pten<sup>fl/fl</sup> mice compared with Pten<sup>fl/fl</sup> mice at 3 months of age (Fig. 3A–C). Expression of ERα itself did not change, but ERα target genes,
Muc-1 and Ltf, were significantly decreased in the uterus of Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice at 3 months of age (Fig. 3D and Supplementary Fig. S4). These results suggest that overexpression of Mig-6 suppress endometrial cancer progression by inducing progesterone signaling and suppressing estrogen signaling.

**ERK1/2 phosphorylation is highly decreased in Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice**

ERK kinases (MEKs) trigger the activation of ERKs by phosphorylating a threonine and a tyrosine in their activation loop. Specificity in the signaling between these molecules is achieved by protein–protein interactions and scaffolding molecules (30). We identified interaction of Mig-6 with ERK2 and demonstrated that ablation of Mig-6 leads to increased phosphorylation of ERK1/2 (19). Abnormal or constitutive phosphorylation of ERK1/2 leads to tumorigenesis (30). To determine whether the decreased tumor formation is related to ERK1/2 signaling in the Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} uteri, we investigated phosphorylation of ERK1/2 by immunohistochemical analysis. The expression of phospho-ERK1/2 was significantly decreased in Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice compared with Pt\textsuperscript{d/d} mice at 2 and 3 months of age (Fig. 4A). To confirm the increase of ERK1/2 phosphorylation observed by immunostaining in the Pt\textsuperscript{d/d} uteri, we examined the expression of phospho-ERK1/2 by Western blot analysis in the uteri of Pt\textsuperscript{d/d} and Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice at 2 and 3 months of age. The level of phospho-ERK 1/2 but not total ERK1/2 was increased in the Pt\textsuperscript{d/d} uteri compared with the Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} uteri, which are consistent with our observations in immunohistochemistry analysis (Fig. 4B). In addition, the expression of ERK1/2 target genes was significantly decreased in Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} uteri compared with Pt\textsuperscript{d/d} mice (Fig. 4C). ERK1/2 phosphorylates the transcription factor C/EBPβ (31). Consistent with the decreased phospho-ERK1/2, phospho-C/EBPβ but not total C/EBPβ was significantly decreased in Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice compared with Pt\textsuperscript{d/d} mice at 2 and 3 months of age (Supplementary Fig. S5A and S5B). PTEN acts as a negative regulator of PI3K signaling, which regulates a number of cellular functions through the activation of AKT (6). The expression of total AKT and phospho-AKT was not altered in endometrium from Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice compared with Pt\textsuperscript{d/d} mice (Supplementary Fig. S5C and S5D). These results suggest that the Mig-6 controlled inhibition of ERK1/2 signaling contributes to the decreased rate of tumor formation in Mig-6\textsuperscript{over} Pt\textsuperscript{d/d} mice.

**MIG-6 directly inhibits phosphorylation of ERK1/2 activity**

To determine whether Mig-6 physically interacts with ERK2, 293T cells, and Ishikawa human endometrial cells were cotransfected with FLAG-MIG-6 and/or ERK2, and then the lysates immunoprecipitated with FLAG antibodies. The immunoprecipitation results showed that Mig-6 physically interacts with ERK2 (Fig. 5A and Supplementary Fig. S6A). Next, we made serial deletions of MIG-6 using PCR amplification of the FLAG-tagged Mig-6 plasmid to determine the ERK2-binding site in Mig-6. The results of in vitro pull-down assays showed that Mig-6 interacts with ERK2 via its SH3-binding domain (Supplementary Fig. S6B).
Both MIG-6 and ERK2 proteins were also colocalized in the cytoplasm of Ishikawa cells transfected with FLAG-tagged MIG-6 and/or V5-tagged ERK2 by immunofluorescence analysis (Supplementary Fig. S6C). To determine whether MIG-6 inhibits phosphorylation of ERK2, we performed in vitro kinase assay and Duolink in situ PLA (32). Overexpression of MIG-6 led to decrease phosphorylation of ERK2 by in vitro kinase assays (Fig. 5B). In addition, we examined the effect of ERK1/2 phosphorylation by MIG-6 overexpression. The overexpression of MIG-6 dose dependently decreased phosphorylation of ERK1/2 (Fig. 5C). Duolink in situ PLA also showed that MIG-6 overexpression decreased phosphorylation levels of ERK1/2 (Fig. 5D and E). These results suggest that MIG-6 directly inhibits phosphorylation of ERK1/2.

ERK1/2 inhibitor significantly reduces endometrial cancer tumorigenesis in Pten\textsuperscript{−/−} mice

To examine whether inhibition of ERK1/2 phosphorylation suppresses tumor progression in endometrial cancer, Pten\textsuperscript{−/−} mice were treated with U0126, an effective inhibitor of MAPK/ERK kinase (33). As assessed by IHC, phospho-ERK1/2 was markedly reduced in U0126-treated Pten\textsuperscript{−/−} mice (Fig. 6A). Pten\textsuperscript{−/−} mice treated with U0126 exhibited a significant reduction in uterine weight (Fig. 6B and C). Histopathologic analysis of the entire animal cohort showed that inhibition of ERK1/2 phosphorylation suppressed endometrial cancer progression, as reflected by the arrest of tumors at the hyperplastic or normal stage, whereas tumors from Pten\textsuperscript{−/−} mice treated with vehicle advanced to endometrial cancer (Fig. 6D). These data suggest that activation of ERK1/2 signaling is critical for endometrial cancer development and progression.

The expression levels between MIG-6 and phospho-ERK1/2 have an inverse correlation in human endometrial cancer

Examination of clinical endometrial specimens showed lower levels of MIG-6 expression in endometrial cancer with grade 1, 2, and 3 in comparison with normal endometrium (Fig. 7A). To assess the clinical relevance between MIG-6 and phospho-ERK1/2 protein levels in human endometrial carcinoma, we next performed RPPA consisting of 109 target genes. Real-time RT-PCR analysis of Il13ra2 and Fst was performed on uteri of control, Mig-6\textsuperscript{−/−}, Pten\textsuperscript{−/−}, and Mig-6\textsuperscript{−/−}Pten\textsuperscript{−/−} mice at 2 and 3 months of age. D, the expression of ERK target genes. Real-time RT-PCR analysis of Muc-1 and Ltf was performed on uteri of control, Mig-6\textsuperscript{−/−}, Pten\textsuperscript{−/−}, and Mig-6\textsuperscript{−/−}Pten\textsuperscript{−/−} mice at 2 and 3 months of age. The results represent the mean ± SEM; *P < 0.05; **P < 0.01; and ***P < 0.001.
endometrioid carcinoma of the uterine corpus. The correlation studies showed an inverse correlation between MIG-6 and phospho-ERK1/2 in human endometrial cancer (Fig. 7B and C).

We validated this reverse correlation in endometrial biopsies from patients with endometrioid carcinoma and normal endometrium by IHC (Fig. 7D).

**Discussion**

The major pathologic phenomenon of endometrial cancer is the loss of ovarian steroid hormone control over uterine epithelial cell proliferation and apoptosis (34). Mig-6, a progesterone signaling mediator, suppresses estrogen signaling in the uterus (16, 35). To determine the tumor-suppressor function of Mig-6 in the development of endometrial cancer, we generated Mig-6 conditional overexpression mice (Mig-6over). To assess the effects of Mig-6 on the PTEN/PI3K/AKT signaling pathway in uterine tumorigenesis, mice with Pten floxed (Ptenfl) and Mig-6over were bred to the PR-Cre mouse model to generate overexpression of Mig-6 and ablation of Pten in the uterus (Mig-6over Ptenfl). Supplementary Figs. S1 and S2. Mig-6over Ptenfl mice suppressed to develop endometrial cancer (Fig. 1). The ablation of both Mig-6 and Pten dramatically accelerated the development of endometrial cancer compared with single ablation of either gene (19). Thus, these results demonstrate the importance of Mig-6 in human endometrial cancer.

Proliferations in epithelial cells were significantly decreased in Mig-6over Ptenfl mice compared with Ptenfl mice (Fig. 2A and B). Regions of hypoxia are reported to exist within many tumors, and the extent of tumor hypoxia correlates with prognosis in number types (36). In addition, increased levels of HIF1α protein have been detected in the cytoplasm and nuclei of 40% to 80% of human carcinoma cases (36). To determine whether overexpressed Mig-6 regulates uterine epithelial proliferation suppressing HIF1α signaling, we determined transcription levels of Hif1α and its target genes. These genes were significantly decreased in Mig-6over Ptenfl mice compared with Ptenfl mice (Fig. 2C). These results indicate that a decrease of proliferation lead to retard the
endometrial cancer development and progression in Mig-6 over Ptend/d mice via regulating HIF1α signaling. We evaluated the expression of PR and ERα by IHC. PR protein level in stromal cells and PR targets (Il13ra2 and Fst) were highly increased in Mig-6 over Ptend/d mice compared with Ptend/d mice at 3 months of age (Fig. 3C). ERα protein level was not changed between Ptend/d and Mig-6 over Ptend/d mice. However, ERα target genes, Muc-1 and Ltf expressions were highly decreased in Mig-6 over Ptend/d mice compared with Ptend/d mice (Fig. 3D and Supplementary Fig. S4). Our results suggest that overexpression of Mig-6 suppresses endometrial cancer progression by inducing P4 signaling and suppressing E2 signaling.

Reduced PR expression was observed in human endometrial cancer (29). Expression of PR is essential for uterine biology (27). In addition, expression of PR and ERα is linked because transcription of the PR gene is induced by estrogen and inhibited by progestins (37). Mig-6 is a downstream target of PR and EGF (16). Conditional ablation of Mig-6 in the uterus leads to the development of endometrial hyperplasia and estrogen-induced endometrial cancer. Interestingly, conditional ablation of Mig-6 in uterus resulted in reduced PR expression in stromal cells as also seen in human endometrial cancer (18) and overexpression of Mig-6–induced PR expression in Ptend mutation (Fig. 3A and B). The regulation of PR expression in the endometrial epithelial and stromal cells by Mig-6 is critical for the ability of P4 to attenuate the E2 regulated proliferation, apoptosis, and expression of ERα target genes. It has been reported that PR is essential for uterine biology as a key regulator of uterine epithelial–stromal crosstalk (38). The expression of PR is significantly decreased by siEGFR in uterine stromal cells (39). It has been reported that EGFR ligands, Hbegf and Areg, as well as Egfr itself, are PR target genes (40). An attractive conjecture would be that a feed-forward amplification loop exists in which PR induces EGFR/ERK signaling that in turn feeds back to maintain PR activation. Such a model would serve as an exquisite sensor of P4 activity.

Figure 5. Mig-6 deregulates ERK1/2 activity. A, the protein interaction between Mig-6 and ERK1/2 by immunoprecipitation and Western blot analysis in FLAG-tagged Mig-6–transfected Ishikawa cells. B, in vitro kinase assays were performed with FLAG-tagged Mig-6–transfected HeLa cell lysates and the GST-fused ERK2 proteins in kinase reaction buffer. Phosphorylated products were eluted and visualized by autoradiography. C, Western blot analysis of phospho-ERK1/2 and ERK1/2 in FLAG-tagged Mig-6–transfected Ishikawa cells. D, for the Duolink in situ PLA analysis, HeLa cells were transfected with GFP-control (a, b, c, and d) and the GFP-tagged Mig-6 (e, f, g, and h) plasmids. The level of ERK1/2 phosphorylation was assessed with ERK1/2 antibody and phospho-ERK1/2 antibody. The positive signal was analyzed using confocal microscopy. E, intensity of PLA analysis was obtained using ImageJ software. Mig-6–overexpressed cells display decreased fluorescence intensity compared with untransfected cells. The results represent the mean ± SEM; *P < 0.05.
Estrogen-mediated induction of the majority of signaling pathways leads to the activation of two key signaling cascades, the PTEN/PI3K/AKT and the ERK pathways (41). The estrogen receptors mediate the effect of estrogen under physiologic and pathologic conditions either by activation of estrogen target genes transcription by binding to specific estrogen response elements (42) or via nongenomic mechanisms, which results in the rapid activation of several signal transduction pathways to regulate different cellular processes, such as proliferation, apoptosis, and differentiation. Estrogen exerts a proliferative effect via nongenomic activation of ERK1/2 and PI3K/AKT (43). To determine whether overexpressed Mig-6 regulate ERK1/2 phosphorylation in PTEN-null endometrial cancer, we assessed the expression of phospho-ERK1/2 by IHC and Western blot analysis. The expression of phospho-ERK1/2 and its target genes was significantly decreased in Mig-6−/− Pten−/− mice compared with Pten−/− mice. However, the PTEN/PI3K/AKT pathways related proteins were not changed between Pten−/− and Mig-6−/− Pten−/− mice (Supplementary Fig. S5).

Mig-6 only decreases ERK downstream genes in Mig-6−/− Pten−/− mice, not in Mig-6+/− mice (Fig. 4). ERK1/2 signaling is known to regulate cell proliferation and apoptosis in uterine endometrial cells. Aberrant activation of ERK1/2 has been implicated in the pathologic processes of endometrial cancer (44). ERK1/2 is critical for decidualization during early pregnancy (45). However, ERK1/2 signaling is not activated in nonpregnant uteri. Mig-6 is a negative regulator
of EGFR–ERK signaling (46). Although there was a slight decrease of Serpine and F3 expression in Mig-6over mice compared with control mice, it was not significant. Therefore, ERK downstream genes are not significantly changed in Mig-6over mice compared with control mice.

We have identified that Mig-6 interacts with ERK2 (19). Therefore, we examined whether Mig-6 directly affects ERK1/2 phosphorylation. Protein interaction between Mig-6 and ERK1/2 was observed by immunoprecipitation and immunofluorescence analysis in transiently cotransfected cells with Mig-6 and/or ERK2 and GST pull-down assay using in vitro–translated Mig-6 proteins and GST-fused ERK2 protein (Supplementary Fig. S6). In vitro kinase assays using GST–ERK2 proteins and Western blot analysis using the phospho-ERK1/2 antibody showed that Mig-6 overexpression decreased phosphorylation of ERK1/2 (Fig. 5B and C). In addition, PLA using the Duolink in situ PLA Kit also showed that Mig-6 overexpression decreased levels of phospho-ERK2 (Fig. 5D and E). These results demonstrate that Mig-6 directly inhibits phosphorylation of ERK1/2. MEKs trigger the activation of ERKs by phosphorylating a threonine and a tyrosine in their activation loop (30). U0126 is highly selective inhibitors of ERK signaling (33). Ptend/d mice showed significantly reduced endometrial tumorigenesis after U0126 treatment (Fig. 6). Ptend/d mice treated with U0126 exhibited a significant reduction in uterine weight. Histopathologic analysis of the entire animal cohort showed that inhibition of ERK1/2 phosphorylation suppressed endometrial cancer progression to hyperplasia or normal stage in Ptend/d mice. These findings suggest that regulation of ERK1/2 phosphorylation is important for the progression of PTEN-mutant endometrial cancer.

To determine the clinical relevance of Mig-6 and ERK1/2 in human, we performed RPPA. RPPA is a recently developed quantitative assay that analyzes nanoliter amounts of sample for potentially hundreds of proteins (20). We revealed the significance of Mig-6 in human endometrial cancer through
sample analysis, in which MIG-6 expression is inversely associated with ERK1/2 phosphorylation (Fig. 7). These results suggest that aberrant overexpression of ERK1/2 phosphorylation is important for a tumor development and progression in mouse as well as human. ERK1/2 is a potential drug target for the intervention of human endometrial cancer.

MIG-6 acts as a negative feedback regulator of EGFR (47). Its transcription is induced by the ERK pathway that is activated by growth factors such as EGF (14). MIG-6 has several functional motifs/domains that are crucial for interaction with other signaling molecules (35, 48). MIG-6 binds to the tyrosine kinase domains of EGFR and ErbB2 and inhibits the tyrosine kinase activity. We have identified a novel interaction of MIG-6 with ERK (Fig. 5 and Supplementary Fig. S6). MEKs trigger the activation of ERKs by phosphorylating a threonine and a tyrosine in their activation loop. Specificity in the signaling between these modules is achieved by protein–protein interactions and scaffolding molecules (30). These results suggest that MIG-6 also acts as a negative feedback regulator of ERK. MIG-6 interaction with ERK may suppress ERK phosphorylation by competing MEK interaction.

PTEN regulates several signaling pathways, such as PI3K/protein kinase B (AKT), JAK/STAT, focal adhesion kinase, and ERK1/2, in which activation of these pathways typically leads to cancer development and progression (49). Our results demonstrate that MIG-6 suppresses tumorigenesis in the uterus of Ptend mutation mice by inhibiting ERK1/2 phosphorylation. PR directly interacts with STAT3 through protein–protein interactions in mouse uterus (50). Our previous study revealed a novel interaction of MIG-6 with ERK (Fig. 5 and Supplementary Fig. S6). MEKs trigger the activation of ERKs by phosphorylating a threonine and a tyrosine in their activation loop. Specificity in the signaling between these modules is achieved by protein–protein interactions and scaffolding molecules (30). These results suggest that MIG-6 also acts as a negative feedback regulator of ERK. MIG-6 interaction with ERK may suppress ERK phosphorylation by competing MEK interaction.

PTEN regulates several signaling pathways, such as PI3K/protein kinase B (AKT), JAK/STAT, focal adhesion kinase, and ERK1/2, in which activation of these pathways typically leads to cancer development and progression (49). Our results demonstrate that MIG-6 suppresses tumorigenesis in the uterus of Ptend mutation mice by inhibiting ERK1/2 phosphorylation. PR directly interacts with STAT3 through protein–protein interactions in mouse uterus (50). Our previous study showed that activated stromal MIG-6 by P4 prevented development of endometrial hyperplasia via PR and STAT3 signaling (18). These data suggest that regulation of STAT3 and PR crosstalk is important for endometrial hyperplasia and cancer development.

Loss of PTEN, and subsequent Akt activation, resulted in the activation of ERK-dependent pathways that play an important role in the tumorigenesis of endometrial cancer. The PTEN/PI3K/AKT signaling pathway can also be activated by E2 and growth factors, suggesting a cross-talk between PTEN/PI3K/AKT and EGFR/MAPK signaling pathways. E2 leads activation of EGFR–ERK signaling (8) and MIG-6 is mainly known to be a negative regulator of EGFR–ERK signaling through direct interaction with the EGFR family (46). Previously, we demonstrated that the absence of MIG-6 in mice results in the inability of P4 to inhibit E2–induced uterine weight gain and expression of E2-responsive target genes (16). MIG-6, a progesterone target gene, directly interacts with ERK2 and inhibits the phosphorylation of ERK1/2 (19). Phospho-ERK1/2 was significantly decreased in the endometrium of Mig-6+/+Ptenfl/fl mice compared with Ptenfl/fl mice (Fig. 4). ERK inhibitor treatment significantly reduced endometrial cancer tumorigenesis in Ptenfl/fl mice (Fig. 6). These data suggest that aberrant activation of ERK signaling is critical for cancer development and progression in PTEN-deficient cancer. Our findings highlight a crucial tumor-suppressor role for Mig-6 in the progression of PTEN-null endometrial cancer by inhibiting ERK1/2 phosphorylation. Mig-6 is a mediator of progesterone signaling, and its activity can suppress unopposed estrogen signaling. Therefore, our studies provide a potential new drug target for the intervention of metastatic human endometrial cancer.

Disclosure of Potential Conflicts of Interest

G.B. Mills received a commercial research grant from Adelson Medical Research Foundation, AstraZeneca, Critical Outcomes Technology, GSK; has ownership interest (including patents) in Catena Pharmaceuticals, PTV Ventures, Spindle Top Ventures; is a consultant/advisory board member for AstraZeneca, Blend, Tau Therapeutics, Critical Outcome Technologies, Hanmi Bio Korea, NuEvolution, Pfizer, Prevista Diagnostics, Roche, SignaChem Lifesciences, Symphogen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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