Critical Tumor Suppressor Function Mediated by Epithelial Mig-6 in Endometrial Cancer

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Abstract

Endometrial cancer is preceded by endometrial hyperplasia, unopposed estrogen exposure, and genetic alterations, but the precise causes of endometrial cancer remain uncertain. Mig-6, mainly known as a negative regulator of the EGF receptor, is an important mediator of progesterone signaling in the uterus, where it mediates tumor suppression by modulating endometrial stromal–epithelial communications. In this study, we investigated the function of Mig-6 in the uterine epithelium using a tissue-specific gene knockout strategy, in which floxed Mig-6 (Mig-6fl/fl) mice were crossed to Wnt7a-Cre mice (Wnt7aCre+Mig-6fl/fl). Wnt7aCre+Mig-6fl/fl mice developed endometrial hyperplasia and estrogen-dependent endometrial cancer, exhibiting increased proliferation in epithelial cells as well as apoptosis in subepithelial stromal cells. We documented increased expression of NOTCH1 and BIRC3 in epithelial cells of Wnt7aCre+Mig-6fl/fl mice and decreased expression of the progesterone receptor (PR) in stromal cells. Progesterone therapy controls endometrial growth and prevents endometrial cancer, but the effectiveness of progesterone as a treatment for women with endometrial cancer is less clear. We noted that the hyperplasic phenotype of Wnt7aCre+Mig-6fl/fl mice was prevented by progesterone treatment, whereas this treatment had no effect in PRcre/+Mig-6fl/fl mice where Mig-6 was deleted in both the epithelial and stromal components of the uterus. In contrast, activation of progesterone signaling in the stroma regulated proliferation and apoptosis in the epithelium via suppression of ERα signaling. In summary, our results establish that epithelial Mig-6 functions as a critical tumor suppressor that mediates the ability of progesterone to prevent the development of endometrial cancer. Cancer Res; 73(16); 1–10. ©2013 AACR.

Introduction

Endometrial carcinoma, commonly referred to as uterine cancer, is the most common malignancy of the female genital tract. In the United States, approximately 49,560 cases are diagnosed and approximately 8,190 women die from the disease each year (1). The most common type of endometrial carcinoma, approximately 85% of cases, is endometrioid carcinoma (2). Endometrial hyperplasia, which is a proliferative process in the epithelium, is associated with endometrioid carcinoma. This process is commonly associated with unopposed estrogen stimulation (3).

The ability of ovarian steroids to regulate uterine cell proliferation depends upon the ability of hormonal stimulation to regulate growth factor communication networks between the uterine stroma and epithelium. E2 stimulates proliferation of uterine epithelial cells while P4 is inhibitory to estrogen-mediated proliferation of the epithelium. P4 achieves this inhibition of proliferation by coordinating stromal–epithelial crosstalk (4–6). Elucidating the molecular mechanisms by which the steroid hormones control uterine physiology is paramount to understanding female infertility and tumorigenesis of endometrial cancer.

Progestin therapy has been used in the conservative endocrine treatment of endometrial complex atypical hyperplasia—the direct precursor lesion to endometrial cancer in women in order to preserve their fertility—as well as in palliative treatment to advanced-stage patients who are poor surgical candidates (7–9). Expression of PR has been positively correlated with good prognosis and response to progestin treatment (10). However, more than 30% of patients do not respond to progestin due to de novo or acquired progestin resistance (8, 11–14). The mechanism of progestin resistance is still unknown.

Mitogen inducible gene 6, Mig-6 (Errf1, RALT, or gene 33), is an immediate early-response gene that can be induced by various mitogens and commonly occurring chronic stress
stimuli. It contains a CRIB domain, a src homology 3 (SH3) binding domain, a 14-3-3 binding domain, and an EGF receptor-binding domain as an adaptor protein, but no domain with enzymatic activity (15). Ablation of Mig-6 in mice has led to the development of animals with epithelial hyperplasia, adenoma, and adenocarcinomas in organs such as the uterus, lung, gallbladder, and bile duct (16–18). We identified Mig-6 as a target of progesterone receptor (PR) action using a genome-wide gene expression profiling (19, 20), and showed that targeted ablation of Mig-6 gene in the mouse uterus led to the development of endometrial hyperplasia and estrogen-induced endometrial carcinoma (18, 20). Using this mouse model, we further showed that Mig-6 suppresses estrogen signaling in the presence of progesterone and that Mig-6 regulates PTEN phosphoinositide 3-kinase (PI3K)–AKT signaling (21). In searching for relevance of the findings from the animal study to human endometrial cancer, we found a significantly lower Mig-6 expression in human endometrioid carcinoma cells compared with normal uterine cells, suggesting a similar Mig-6-regulated system present in the human uterus (20). Tumor suppressor function of Mig-6 coordinates endometrial stromal–epithelial communication. The expression of Mig-6 in these cellular compartments is under tight temporal and endocrine control. However, the function of Mig-6 in endometrial cancer has remained elusive.

In this study, we used endometrial epithelial–cell-specific Mig-6 knockout (Wnt7acre/+; Mig-6fl/fl) mice to assess the role of epithelial Mig-6 in steroid hormone responsiveness and tumorigenesis (18, 22). We observed that Wnt7acre/+; Mig-6fl/fl mice display normal P4 attenuation of E2-mediated uterine hyperplasia. However, Wnt7acre/+; Mig-6fl/fl mice develop endometrial hyperplasia as well as estrogen-induced endometrial cancer. The development of endometrial hyperplasia in Wnt7acre/+; Mig-6fl/fl mice is prevented by P4 treatment. These data suggest that P4-induced stromal Mig-6 prevents hyperplasia seen in Wnt7acre/+; Mig-6fl/fl mice by regulating estrogen signaling.

Materials and Methods

Animals and tissue collection

Mice were cared for and used in the designated animal care facility according to the Michigan State University institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. We generated uterine epithelial-specific Mig-6 knockout mice using the Wnt7a-cre mouse model (23, 24). Control mice and Wnt7acre/+; Mig-6fl/fl mice were ovariectomized at 6 weeks of age. Two weeks later, ovariectomized control and Wnt7acre/+; Mig-6fl/fl mice were injected with one of the following: vehicle (sesame oil), P4 (1 mg/mouse), E2 (0.1 µg/mouse), and P4 plus E2. Five mice from each group were injected with one of these treatments every 24 hours and uteri were collected at 72 hours. For the determination of the development of endometrial hyperplasia and estrogen/progesterone effects, either vehicle (beeswax), E2 (20 µg/pellet), or P4 (40 µg/pellet) pellets were placed subcutaneously into ovariectomized control and Wnt7acre/+; Mig-6fl/fl mice for every 1 month and aged for 3 months before euthanization. Uterine tissues were flash-frozen at the time of dissection and stored at −80 °C for RNA isolation or fixed with 4% (vol/vol) formaldehyde for histologic analysis.

Quantitative real-time PCR

RNA was extracted from the uterine tissues using the RNeasy total RNA isolation kit (Qiagen). Expression levels of mRNA transcripts for Mig-6, estrogen receptor α (ERα) target genes (Mac-1, Clea3, Lif, Birc1a, and Birc1b), PR target genes (Mig-6, Fst, and Il13ra2), and 18S RNA (for normalization) were measured by quantitative real-time PCR (qRT-PCR) analysis using the Applied Biosystems StepOnePlus qRT-PCR systems according to the manufacturer’s instructions (PE Applied Biosystems). Prevalidated TaqMan probes and primers were purchased from Applied Biosystems. cDNA was produced from 1 µg of total RNA using random hexamers and MMLV Reverse Transcriptase (Invitrogen Corp.). RT-PCR was carried out using RT-PCR Universal Master Mix reagent (Applied Biosystems) according to the manufacturer’s instructions. All RT-PCR was done by using five independent RNA sets. The relative expression of each transcript was normalized to 18S rRNA using ABI rRNA control reagents. Statistical analyses were conducted using Student t test and one-way ANOVA. Differences between multiple groups were determined by Tukey post hoc multiple comparisons test. Statistical analyses were conducted using the Instat package from GraphPad.

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 incubated with 10% normal goat 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. The following day, sections were washed in PBS and incubated with a secondary antibody (1:100 for anti-phospho-STAT3 (cat. no. 3977, Cell Signaling Technology), 1:1,000 for anti-STAT3 (cat. no. 4904, Cell Signaling Technology), 1:200 for anti-BIRC3 (sc-96557, Millipore), 1:500 for anti-cleaved caspase 3 (cat. no. 9661, Cell Signaling Technology), 1:200 for anti-ERT2 (M-7047, DAKO Corp.), 1:50 for anti-MIG-6 (PE-16, Sigma), 1:1,000 for antiphosphohistone H3 (06-570, Millipore), 1:500 for anti-cleaved caspase 3 (cat. no. 9661, Cell Signaling Technology), 1:200 for anti-NOTCH1 (sc-6014-R, Santa Cruz Biotechnology), 1:200 for anti-BIRC3 (ab32059, Abcam Inc.), 1:1,000 for anti-STAT3 (cat. no. 4904, Cell Signaling Technology), 1:100 for anti-phospho-STAT3 (cat. no. 9131, Cell Signaling Technology). On the following day, sections were washed in PBS and incubated with a secondary antibody (5 µl/ml; Vector Laboratories) for 1 hour at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB kit (Vector Laboratories).

Isolation of the uterine epithelium

Isolated uteri were placed into Hanks balanced salt solution (HBSS; Ca2+-free and Mg2+-free), and cut into 1-mm segments. The cut uteri were placed into 1% trypsin/HBSS solution for 1.5 hours at room temperature and then washed with cold HBSS. The uteri were then incubated with DNase I solution for 1 minute to breakdown DNA. The uterine luminal epithelium was gently removed from the uterine stroma under a dissecting microscope.
Results

Generation of epithelial Mig-6 ablation in the murine uterus

Previously, we generated conditional ablation of Mig-6 in all compartments of the uterus (PR\textsuperscript{cre/+, Mig-6\textsuperscript{-/-}}), which leads to the development of endometrial hyperplasia and estrogen-induced endometrial cancer (18, 20). Mig-6 is a critical mediator of stromal–epithelial communication in steroid hormone regulation and tumor suppressor function. In order to investigate the role of epithelial Mig-6 in the uterus, we bred Mig-6\textsuperscript{-/-} mice to Wnt7a-Cre mice (22–24). Ablation of epithelial Mig-6 in Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice was confirmed by qRT-PCR and immunohistochemical analysis. After isolating epithelium from control (PR\textsuperscript{cre/+} and Mig-6\textsuperscript{-/-}) mice and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice, Mig-6 mRNA expression was detected in control epithelium, whereas it was not detected in Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} epithelium (Fig. 1A). In control mice, Mig-6 was expressed in the luminal epithelium, glandular epithelium, and stroma. However, Mig-6 was only detected in stroma but not the epithelial cells of Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice (Fig. 1B). These results suggested that we successfully generated epithelial Mig-6 ablation in the uterus of mice.

Steroid hormone regulation and tumor suppressor function of epithelial Mig-6

We showed that PR\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice result in the inability of P\textsubscript{1} to inhibit E\textsubscript{2}-induced uterine weight gain and expression of E\textsubscript{2}-responsive target genes (20). To examine the effect of ovarian steroid hormone regulation on epithelial Mig-6 expression, ovariectomized control and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice were injected daily with either vehicle (sesame oil), P\textsubscript{4}, E\textsubscript{2}, and E\textsubscript{2} + P\textsubscript{4} for 3 days (n = 5 per genotype per treatment). Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice displayed a normal P\textsubscript{4} attenuation of E\textsubscript{2}-mediated uterine hypertrophy (Supplementary Fig. S1). These results suggest that stromal Mig-6 has an important role in acute steroid hormone responsiveness. Wnt7acre analysis of ovarian steroid hormone regulation on epithelial Mig-6 mice developed endometrial hyperplasia and cancer in a hormone-dependent manner (20). We examined the development of endometrial hyperplasia and steroid hormone-dependent endometrial cancer in the Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice. To investigate the impact of epithelial Mig-6 ablation on endometrial hyperplasia development, control and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice were sacrificed at 5 months of age. Uterine weight as well as gross and histologic morphology were examined (n = 5 per genotype). Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice showed an increased gross morphology when compared with control mice (Fig. 1C). Uterine weight was significantly increased in Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice when compared with control mice (Fig. 1D). Histologic analysis revealed that Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice developed endometrial hyperplasia (Fig. 1E). In addition, Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice developed estrogen-dependent endometrial cancer (Supplementary Fig. S2). To address whether endometrial hyperplasia in Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice is caused by an alteration in endometrial epithelial cell proliferation, we conducted immunohistochemical analysis for phosphohistone H3, a mitotic marker, in endometrium from mice at 3 and 5 months of age. The levels of phosphohistone H3 were significantly increased in epithelial cells of Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice.

Figure 1. Generation of epithelial Mig-6 ablation in the murine uterus and the development of endometrial hyperplasia. A, quantitative RT-PCR analysis of Mig-6 transcripts levels in whole uteri and isolated epithelium from control mice and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice. The results represent the mean ± SE of five independent RNA sets. Mig-6 transcripts are significantly reduced in the epithelium of Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice compared with controls; *** P < 0.001. B, immunohistochemical analysis of Mig-6 in control mice (a) and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice (b). Arrowheads indicate luminal and glandular epithelium. C, gross morphology of control mice (a) and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice (b) at 5 months of age. D, Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice show an increase in uterine weight compared with control mice at 5 months of age. E, hematoxylin and eosin staining of control mice (a) and Wnt7a\textsuperscript{cre/+}, Mig-6\textsuperscript{-/-} mice (b) at 5 months of age. The results represent the mean ± SE (n = 5 per genotype). * P < 0.05.
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Figure 2. Regulation of proliferation and apoptosis by epithelial Mig-6. A, immunostaining of phosphohistone H3 was significantly increased in the endometrial epithelial cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice (b and d) compared with control mice (a and c) at 3 months of age (a and b) and 5 months of age (c and d). B, quantification of phosphohistone H3-positive in endometrial stroma and epithelial cells. C, immunohistochemical analysis of NOTCH1 in the uteri of control mice (a and c) and Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice (b and d) at 3 months of age (a and b) and 5 months of age (c and d). D, immunohistochemistry of cleaved caspase-3 was increased in epithelial cells and subepithelial stromal cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice (b and d) compared with control mice (a and c) at 3 months of age (a and b) and 5 months of age (c and d). E, quantification of cleaved caspase-3-positive in endometrial stromal and epithelial cells. F, immunohistochemical analysis of BIRC3 in the uteri of control mice (a and c) and Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice (b and d) at 3 months of age (a and b) and 5 months of age (c and d). Arrowheads indicate positive-cleaved caspase-3 cells. The results represent the mean ± SE. *P < 0.05; **P < 0.01; ***P < 0.001.

compared with controls at both 3 and 5 months of age (Fig. 2A and B). However, proliferation in stromal cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice was not changed. Notch pathway activation leads to increased proliferation and tumor progression in endometrial cancers (25). Because ablation of Notch1 in PR-positive cells showed decreased cellular proliferation (26), we examined NOTCH1 expression in highly proliferative epithelial cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice. Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} uteri showed a robust expression of NOTCH1 in the luminal and glandular epithelium, while the Mig-6\textsuperscript{-/-} mice display NOTCH1 expression only in the stroma (Fig. 2C).

The number of cleaved caspase-3-positive cells was significantly increased in epithelial cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice compared with controls. Interestingly, apoptosis in subepithelial stromal cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice was significantly increased compared with control mice at 3 and 5 months of age (Fig. 2D and 2E). BIRC3 contributes to the survival of endometrial cancer cells against apoptosis mediated by inhibition of AKT (27). Therefore, it was determined whether Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice altered regulation of BIRC3 during endometrial hyperplasia development. The expression of BIRC3 was increased in the luminal and glandular epithelium of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice compared with Mig-6\textsuperscript{-/-} mice, whereas it was not observed in the subepithelial stromal cells of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice (Fig. 2F).

Expression of PR has been reported as a prognostic factor for endometrial carcinoma (28–30). We evaluated the expression of PR by immunohistochemistry in mice at 3 and 5 months of age. Immunostaining of PR showed a significant decrease of stromal PR expression in the endometrium of Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice when compared with control mice at 5 months of age (Fig. 3A and B). These results indicate that Wnt7a\textsuperscript{cre+} Mig-6\textsuperscript{-/-} mice exhibited development and progression of endometrial cancer as observed in humans. Activation of STAT3 interacts with PR

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for decidualization in the uterus. The expression of stromal P4 was decreased during decidualization and the preimplantation period in PR<sup>Cre/+/Stat3<sup>−/−</sup></sup> mice and PR target genes were significantly downregulated after progesterone treatment (31). Therefore, we examined the expression of STAT3 by immunohistochemistry in endometrial hyperplasia from Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice at 3 and 5 months of age. The immunostaining results showed decreased STAT3 protein in endometrial stroma of Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice when compared with control mice at 3 and 5 months of age (Fig. 3C).

Prevention of the development of endometrial hyperplasia by progesterone treatment

P<sub>4</sub> has been used as a therapeutic agent for the treatment of early-stage endometrial cancer in patients (11). However, the effectiveness of P<sub>4</sub> for women with endometrial cancer is less clear. To assess the effect of P<sub>4</sub> on epithelial ablation of Mig-6, we placed P<sub>4</sub> or vehicle pellets subcutaneously into the control, PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup>, and Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice at 6 weeks of age and treated for 3 months (n = 8 per genotype per treatment). There was no reduction in the development of endometrial hyperplasia with P<sub>4</sub> treatment in the PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice. However, the development of endometrial hyperplasia in the Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice was prevented by P<sub>4</sub> treatment (Fig. 4A).

To determine whether the prevention of endometrial hyperplasia in Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice was caused by an alteration in cell proliferation and apoptosis, we examined the immunohistochemical samples for phosphohistone H3 and cleaved caspase 3 following P<sub>4</sub> treatment. Immunostaining of phosphohistone H3 showed a significant decreased expression in the endometrial epithelium of Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice compared with PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment (Fig. 4B and C). This indicates that P<sub>4</sub> is decreased in epithelial proliferation in Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice but not PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice. Immunostaining of cleaved caspase 3 showed that apoptosis was significantly increased in the endometrial epithelium of Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice compared with PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice (Fig. 4B and D). In addition, apoptosis of stromal cells was not observed in Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment. These results suggest that activation of P<sub>4</sub> signaling including Mig-6 in stroma induces epithelial cell apoptosis.

To determine whether the suppression of the hyperplastic phenotype observed was due to altered ovarian steroid hormone signaling, we examined the expression of ERα and PR using immunohistochemistry. The expression of ERα was significantly decreased in the endometrium of Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice as compared with control and PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment (Fig. 5A). Transcript levels of the ERα target genes, Muc-1, Clca3, and Ltf, were also significantly decreased in the Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice as compared with the PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment (Fig. 5B). It is known that E<sub>2</sub> can tip this balance toward cell survival in uterine epithelial cells by inducing the expression of baculoviral inhibitors of apoptosis repeat-containing 1 (Birc1), a family of antiapoptotic proteins (32). To determine if P<sub>4</sub> treatment suppresses uterine epithelial apoptosis by suppressing Birc1 expression, the expression of Birc1a and Birc1b was determined in control, PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup>, and Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice treated with P<sub>4</sub> for 3 months by real-time RT-PCR. Interestingly, the expression of Birc1a and Birc1b was significantly decreased in the Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice as compared with the PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment (Fig. 5B). These results suggest that P<sub>4</sub> treatment induces uterine epithelial apoptosis via downregulation of Birc1 expression.

The expression of PR was not significantly different between control and Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment. However, transcript levels of the PR target genes Mig-6, Fst, and Il13ra2 were increased in the Wnt7a<sup>Cre+</sup>-Mig-δ<sup>−/−</sup> mice as compared with the PR<sup>Cre/−</sup>-Mig-δ<sup>−/−</sup> mice after P<sub>4</sub> treatment (Supplementary Fig. S3). Interestingly, the levels of BIRC3 and NOTCH1...
endometrial hyperplasia in the mutant mice by estrogen (39, 40). Here, it is shown that epithelial Birc3 and Notch1 may play an important role in the development of hyperplasia in the uterus, we generated ablation of uterine epithelial Birc3 mice using Wnt7aCre mice (Fig. 1). Ablation of epithelial Birc3 in the murine uterus did not show any alterations in ovarian morphology, ovulation, or fertilization. In addition, there were normal levels of P4 and E2 in the serum of Wnt7aCre/Mig-6f/f mice (data not shown). One of the endocrine risk factors for developing endometrial cancer and endometriosis is exposure to E2; conversely, a lower incidence of these diseases in women is associated with decreased endogenous E2 production (36). Although Wnt7aCre/Mig-6f/f mice have normal acute steroid hormone responsiveness (Supplementary Fig. S1), Wnt7aCre/Mig-6f/f mice developed endometrial hyperplasia (Fig. 1). Endometrial hyperplasia is defined as an increased proliferation of the endometrial glands relative to the stroma, resulting in an increased gland-to-stroma ratio when compared with normal proliferative endometrium (37). Endometrial hyperplasia deserves special attention because of its relationship with endometrial carcinoma. Clinicopathologic and epidemiologic studies have supported the malignant potential of endometrial hyperplasia and the concept of a continuum of proliferative glandular lesions culminating, in some cases, in carcinoma. However, details of molecular signaling during development of endometrial hyperplasia have remained elusive. Our mouse models are invaluable for further study of endometrial tumorigenesis.

Proliferation in epithelial cells and apoptosis in subepithelial stromal cells were significantly increased in Wnt7aCre/Mig-6f/f mice compared with control mice in epithelial cells at 5 months of age (Fig. 2). These results indicate that these increases lead to the development of endometrial hyperplasia. Notch signaling plays an important role in the regulation of cellular proliferation, differentiation, and apoptosis (38). Deregulation of Notch signaling was found in a variety of cancers (38). Moreover, Notch signaling is prominently regulated by estrogen (39, 40). Here, it is shown that epithelial Birc3 inhibits epithelial proliferation through its regulation of Notch1 protein (Fig. 2C). The inhibitor of apoptosis proteins (IAP) are negative key regulators of apoptosis (41). Alterations in IAPs are found in many types of human cancer and are connected with chemoresistance, disease progression, and poor prognosis (42, 43). IAPs have important roles in suppression of estrogen-mediated apoptosis in the uterine epithelium (32). Because the cellular inhibitor of apoptosis genes including Birc1 and Birc3 can tip toward cell survival in uterine epithelial cells (27, 32), the expression of Birc1 and Birc3 was significantly increased in Wnt7aCre/Mig-6f/f mice (Figs. 2 and 5). These

Discussion

P4 and E2, acting through their cognate nuclear receptors, play critical roles in uterine functions associated with the establishment and maintenance of pregnancy (33, 34). E2 is required for proliferation and differentiation of the uterine epithelium whereas the coordinated action of E2 and P4 promotes stromal cell differentiation (35). Elucidating P4-regulated pathways in the uterus is thus critical for understanding the impairments that underlie disruption of steroid hormone control of uterine cell proliferation and differentiation. The progesterone-induced gene Mig-6 suppresses E2 signaling as a tumor suppressor by regulating proliferation and apoptosis in endometrial cancer (20, 21). The expression of Mig-6 in these cellular compartments is under tight temporal and endocrine control (15). However, the in vivo role of Mig-6 in uterine epithelium has remained elusive.

To understand the role of epithelial Mig-6 in the uterus, we generated ablation of uterine epithelial Mig-6 using Wnt7aCre mice (Fig. 1). Ablation of epithelial Mig-6 in the murine uterus did not show any alterations in ovarian morphology, ovulation, or fertilization. In addition, there were normal levels of P4 and E2 in the serum of Wnt7aCre/Mig-6f/f mice (data not shown). One of the endocrine risk factors for developing endometrial cancer and endometriosis is exposure to E2; conversely, a lower incidence of these diseases in women is associated with decreased endogenous E2 production (36). Although Wnt7aCre/Mig-6f/f mice have normal acute steroid hormone responsiveness (Supplementary Fig. S1), Wnt7aCre/Mig-6f/f mice developed endometrial hyperplasia (Fig. 1). Endometrial hyperplasia is defined as an increased proliferation of the endometrial glands relative to the stroma, resulting in an increased gland-to-stroma ratio when compared with normal proliferative endometrium (37). Endometrial hyperplasia deserves special attention because of its relationship with endometrial carcinoma. Clinicopathologic and epidemiologic studies have supported the malignant potential of endometrial hyperplasia and the concept of a continuum of proliferative glandular lesions culminating, in some cases, in carcinoma. However, details of molecular signaling during development of endometrial hyperplasia have remained elusive. Our mouse models are invaluable for further study of endometrial tumorigenesis.

Proliferation in epithelial cells and apoptosis in subepithelial stromal cells were significantly increased in Wnt7aCre/Mig-6f/f mice compared with control mice in epithelial cells at 5 months of age (Fig. 2). These results indicate that these increases lead to the development of endometrial hyperplasia. Notch signaling plays an important role in the regulation of cellular proliferation, differentiation, and apoptosis (38). Deregulation of Notch signaling was found in a variety of cancers (38). Moreover, Notch signaling is prominently regulated by estrogen (39, 40). Here, it is shown that epithelial Mig-6 inhibits epithelial proliferation through its regulation of Notch1 protein (Fig. 2C). The inhibitor of apoptosis proteins (IAP) are negative key regulators of apoptosis (41). Alterations in IAPs are found in many types of human cancer and are connected with chemoresistance, disease progression, and poor prognosis (42, 43). IAPs have important roles in suppression of estrogen-mediated apoptosis in the uterine epithelium (32). Because the cellular inhibitor of apoptosis genes including Birc1 and Birc3 can tip toward cell survival in uterine epithelial cells (27, 32), the expression of Birc1 and Birc3 was significantly increased in Wnt7aCre/Mig-6f/f mice (Figs. 2 and 5). These

Figure 4. The endometrial hyperplasia in the Wnt7aCre/Mig-6f/f, but not the PRcre/Mig-6f/f, mice was prevented with P4 treatment. A, hematoxylin and eosin staining of control mice (a and d), PRcre/Mig-6f/f mice (b and e), and Wnt7aCre/Mig-6f/f mice (c and f) before (a, b, and c) and after (d, e, and f) P4 treatment. B, immunostaining for phosphohistone H3 (a, b, and c) and cleaved caspase-3 (d, e, and f) in the uteri of control mice (a and d), PRcre/Mig-6f/f mice (b and e), and Wnt7aCre/Mig-6f/f mice (c and f) after P4 treatment. Arrowheads indicate positive-phosphohistone H3 (b and c) and positive-cleaved caspase-3 cells (e and f). C, quantification of phosphohistone H3 and cleaved caspase-3-positive cells in epithelial cells. The results represent the mean ± SE. **, P < 0.01, * P < 0.05.

were decreased in endometrial epithelium of Wnt7aCre/Mig-6f/f mice compared with PRcre/Mig-6f/f mice after P4 treatment (Fig. 6). Therefore, BIRC3 and NOTCH1 may play an important role in the development of hyperplasia in Wnt7aCre/Mig-6f/f mice. These results suggest that activated stromal P4 signaling including Mig-6 prevents the development of endometrial epithelial hyperplasia seen in the Wnt7aCre/Mig-6f/f mice by regulating estrogen signaling.
results suggest that an increase of epithelial proliferation in Wnt7a	extsuperscript{cre-1}Mig-6	extsuperscript{f/f} mice leads to the development of endometrial hyperplasia through BIRC3 and NOTCH1. The mechanism by which this is achieved is suggested to be through the EGFR–ERK and PI3K–Akt signaling pathways (27). Therefore, future studies are needed to determine whether it is through these pathways or others that epithelial Mig-6 regulates proliferation and apoptosis.

As P	extsubscript{4} attenuates E	extsubscript{2} regulation of proliferation and gene expression by regulating the expression of a yet-to-be-identified paracrine signal from the stromal to the epithelial cells, the regulation of the expression of PR in the endometrial stromal cells by epithelial Mig-6 is critical for the ability of P	extsubscript{4} to attenuate the E	extsubscript{2}-regulated proliferation, apoptosis and expression of E	extsubscript{R}α target genes. Wnt7a	extsuperscript{cre-1}Mig-6	extsuperscript{f/f} mice exhibited reduced PR expression in stromal cells (Fig. 3), as observed in human endometrial cancer (30, 44). It has been reported that PR is essential for uterine biology as a key regulator of uterine epithelial–stromal crosstalk (45, 46). P	extsubscript{4} was unable to stimulate the expression of its epithelial target genes and inhibit neonatal endometrial glandular development in conditional ablation of epithelial PR in the uterus of Wnt7a	extsuperscript{cre-1}PR	extsuperscript{f/f} mice (24). PR directly interacts with STAT3 through protein–protein interactions (31, 47). STAT3 signaling pathways are activated by cytokines and growth factors. The expression of stromal PR was decreased during decidualization and the preimplantation period in PR	extsuperscript{cre-1}Sta	extsuperscript{f/f} mice, and PR target genes were significantly downregulated after progesterone induction (31). The expression of PR and STAT3 was decreased during the development of endometrial hyperplasia in the stroma of Wnt7a	extsuperscript{cre-1}Mig-6	extsuperscript{f/f} mice (Fig. 3). There are differential roles in uterine stromal and epithelial compartments, and stromal–epithelial communication is critical in pregnancy, steroid hormone regulation, and in tumor suppressor function. Therefore, these results suggest that dysregulation of STAT3 and PR crosstalk is important for endometrial hyperplasia development.

In contrast, a negative risk factor for these endometrial diseases is exposure to P	extsubscript{4} (48). It is well known that endometrial cancer is an estrogen-dependent disease and...
protein and ERγ transcription levels of Wnt7acre was highly increased in Wnt7acre and apoptosis is highly increased in Wnt7acre after P4 treatment (Fig. 6). It suggested that the induction of Mig-6 expression of PTEN contributes to the prevention of endometrial hyperplasia, and activated stromal suppressor and/or mediator of PR-P4 signaling, although the significance of P4 therapy. It is sufficient that baculoviral inhibitors of apoptosis observed in some women with endometrial carcinoma (27).

Table 1. The comparison between whole uterine and epithelial-specific Mig-6 ablation

<table>
<thead>
<tr>
<th>Steroid hormone response</th>
<th>PRcre/+·Mig-6fl (total uterine KO)</th>
<th>Wnt7acre/+·Mig-6fl (epithelial KO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia</td>
<td>No P4 inhibition</td>
<td>Normal P4 inhibition</td>
</tr>
<tr>
<td>Hormone dependence of hyperplasia</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cancer development by chronic E2 treatment</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>P4 treatment</td>
<td>No effect</td>
<td>Suppression of hyperplasia</td>
</tr>
</tbody>
</table>

Abbreviation: KO, knockout.

In conclusion, our results show the role of epithelial Mig-6 in steroid hormone regulation and endometrial cancer. Ablation of epithelial Mig-6 in the murine uterus resulted in development of endometrial hyperplasia and P4 treatment prevented the occurrence of the endometrial hyperplastic phenotype, which occurs via Mig-6 regulation of ERα activity (Table 1). The Wnt7acre/+·Mig-6fl model is useful for studying new targets during cancer progression and can be exploited therapeutically to identify new therapies for the prevention and treatment of endometrial cancer. Determining the role of Mig-6 in stromal–epithelial crosstalk will be critical in understanding the role of steroid hormone signaling in endometrial function and dysfunction associated with infertility and endometriosis as well as in developing therapy for both of these common uterine diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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