Stromal Deletion of the APC Tumor Suppressor in Mice Triggers Development of Endometrial Cancer

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Abstract

The contribution of the stromal microenvironment to the progression of endometrial cancer has not been well explored. We have conditionally expressed a mutant allele of adenomatous polyposis coli (APC) in murine uterine stroma cells to study its effect on uterine development and function. In addition to metrorrhagia, the mice develop complex atypical endometrial gland hyperplasia that progresses to endometrial carcinoma in situ and endometrial adenocarcinoma as evidenced by myometrial invasion. Stromal cells subjacent to the carcinoma cells express alpha-smooth muscle actin (αSMA) with fewer cells expressing platelet-derived growth factor α compared with normal stromal cells, suggesting that the mutant stromal cells have acquired a more myofibroblastic phenotype, which have been described as cancer-associated fibroblasts and have been shown to induce carcinogenesis in other organ systems. Analyses of human endometrial cancer specimens showed substantial αSMA expression in the stroma compared with normal endometrial stroma cells. We also show that APC²⁻ mice have stromal levels of transforming growth factor β and bone morphogenetic protein activities and that the mutant uteri failed to respond to exogenous estradiol stimulation. The mutant stroma cells also had higher levels of vascular endothelial growth factor and stromal derived factor signaling components and diminished expression of estrogen receptor α and progesterone receptor, which is common in advanced stages of human endometrial cancer and is an indicator of poor prognosis. Our results indicate that de novo mutation or loss of heterozygosity in stromal APC is sufficient to induce endometrial hyperplasia and endometrial carcinogenesis by mechanisms that are consistent with unopposed estrogen signaling in the endometrial epithelium. Cancer Res; 71(5); 1584–96. ©2011 AACR.

Introduction

The tissue microenvironment plays an important role in normal organogenesis and tissue homeostasis and can lead to carcinogenesis when pathologically disrupted (1). For example, tissue recombination studies have shown that combining urogenital sinus mesenchyme with embryonic or adult urinary bladder epithelium changes its fate to prostatic epithelium (2). Similarly, combining uterine mesenchyme with vaginal epithelium causes development of uterine epithelium (3). Remarkably, transplantation of neural stem cells or spermatogenic cells into the mammary fat pad is sufficient to reprogram both into mammary epithelial cells (4, 5). These studies highlight the importance of stromal cells during morphogenesis of adjacent epithelial cells and the ability of stromal signals to direct cell fate determination.

The importance of the microenvironment in cancer has been reported in human studies showing tumor development and progression in the presence of tumor-associated fibroblast cells with genetic mutations in tumor suppressor genes (6). For example, mutations in TP53 and PTEN are frequently present in the stroma of breast carcinomas (7). In accordance with these findings, stromal mutations in CTNNB1 and adenomatous polyposis coli (APC) accompanied by nuclear accumulation of β-catenin in stromal cells have been observed in patients with nonmetastasizing breast tumors (8). Finally, fibroblast cells collected from Down syndrome patients, who have a lower incidence of breast cancer, inhibit the proliferation of breast cancer cells in vivo and in vitro (9).

Endometrial cancer is one of the most common gynecologic malignancies of the female reproductive tract with over 40,000 new cases diagnosed and nearly 8,000 deaths every year (10). Mutations and/or alterations in PTEN, TP53, KRAS, mismatch repair genes, β-catenin, and APC have all been observed in human endometrial cancer patients and have been associated with the etiology or progression of this disease (11). Mutations in APC, which are frequently observed in patients with familial colon cancer (12), have been detected in 43% of human endometrial cancer patients (13). APC is a large multidomain protein that interacts with several other...
proteins to regulate various cellular processes including cell proliferation, death, differentiation, migration, and adhesion (12). The best-studied role of APC is its regulation of Wnt signaling by controlling the availability of β-catenin (12). It is still unclear how APC mutations contribute to the development of endometrial cancer.

In vitro, normal human endometrial stromal cells have been shown to decrease proliferation and invasion and to promote differentiation of endometrial adenocarcinoma cell lines (14, 15). Additionally, some human endometrial polyps, which are benign but have been associated with the development of endometrial cancer in an age-dependent manner (16), have genetic mutations in the stromal component (17, 18). However, the tumor-promoting potential of endometrial stroma on endometrial cancer has not been reported. We have examined the effects of expressing a truncated form of APC that lacks β-catenin binding domains in murine endometrial stromal cells and show endometrial hyperplasia and endometrial cancer development, indicating that stromal APC plays an important role in controlling the proliferative potential and plasticity of adjacent endometrial epithelium.

Materials and Methods

Mouse genetics and husbandry

The mice used in this study were maintained under standard animal housing conditions. All protocols involving animal experimentation were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Mice used in this study were maintained on C57BL/6;129/SvEv mixed genetic background. The following parental mice strains: Amhr2-Cre/(Amhr2-Cre) (19), APCfloxflox (20), Gt(ROSA)26Sortm1(EYFP)Cos (Yfpflox/flox) /Gt(ROSA)26Sor tm1Sor (LacZ; both from The Jackson Laboratories) were mated to generate Amhr2-Cre/+; APCfloxflox (APCfloxs), Amhr2-Cre/+; APCfloxs, Amhr2-Cre/+; APCfloxflox, Amhr2-Cre/+; Gt(ROSA)26Sorpm1(EYFP)Cos, Amhr2-Cre/+; LacZ mice. The genotyping of mice was made by PCR with standard PCR protocols using DNA collected from tail biopsies. The gross images were taken with a Nikon SMZ1500 microscope equipped with an attached Spot camera (Diagnostic Instruments) or with a Nikon D60 camera with a macro lens.

Estradiol treatments

To investigate E2 response, control (APCfloxflox; n = 4) and APCfloks (n = 5) mice were injected intramuscularly with 10 μg of a depot formulation of E2 (estradiol valerate; JHP Pharmaceuticals) 1-week postoophorectomy. Animals were euthanized 30 days later and their uteri were collected for further analyses.

Laser capture microdissection and genomic PCR

For laser-cut microdissection (MMI Cellcut from Molecular Devices), 10-μm sections of frozen uterine endometrial cancer tissue from APCfloks mice were mounted on PEP-membrane slides. Genomic DNA from microdissected cells from uterine epithelium and stroma and from ovary, oviduct, uterus, and tails was collected using DNeasy (Qiagen). The recombinant and flox APC alleles (500 and 430 bp, respectively) were detected by PCR with the following primers Apc-Int13F2 (GAGAAACCTGTCTCGAAAAA), Apc-Int13R2 (TTGCA-GACTGTTATATAGC), Apc-Int13R2 (AGTGCCTTTTCTAT-GAGTCAAC) as described (20).

Human endometrial cancer tissue analyses

Paraffin-embedded human endometrial cancer (n = 9) and normal/benign endometrium (n = 4) were obtained from the Department of Pathology, MGH using Institutional Review Board-approved protocols. Sections were cut and stained for alpha-smooth muscle actin (αSMA), progesterone receptor, and estrogen receptor α (ERα), pSmad 1/5/8, and pSmad2.

Histologic analyses, immunofluorescence and immunohistochemistry

Tissues were collected at different developmental stages and fixed by immersion in 4% paraformaldehyde for 10 to 12 hours at 4°C, then transferred to 70% ethanol until processing. hematoxylin and eosin (H&E) staining was carried out using standard protocols. The methods used to conduct immunofluorescence and immunohistochemistry (IHC) are described in our previous studies (21, 22). The primary and secondary antibodies used in this study are β-catenin (BD Transduction Laboratories), pSmad2 1/5/8, pSmad2, LIF1, pMet (Cell Signaling Technology), αSMA-CY3-conjugated (Sigma), Cytokeratin 8 (Developmental Studies Hybridoma Bank), CD133, CD31, platelet-derived growth factor receptor α (PDGFRα), CXCL12, CXCR4 (Santa Cruz Biotechnology), PR, ERα (Dako), vascular endothelial growth factor receptor 2 [VEGFR2/FK1/KDR (Neomarkers)], AlexaFluor secondary antibodies (Invitrogen), and biotinylated donkey secondary F(ab)2 fragments (Jackson ImmunoResearch).

Western analyses

Protein extracts were prepared in RIPA buffer. Protein concentrations were measured by the Bradford assay and an equivalent amount of protein from each sample was analyzed. The following antibodies were used: β-catenin (Sigma), LIF1, TCF1, pSmad 1/5/8, pSmad2, pSmad3, Foxo1, pMET (Cell Signaling Technology), hepatocyte growth factor (HGF; R&D Systems), PR, ERα (Santa Cruz Biotechnology), Cyclin d1, and β-actin (Neomarkers).

Statistical analyses

The unpaired t-test was conducted using Prism (Graphpad Software) to calculate the differences between groups and a P value < 0.05 was considered to be statistically significant.

Results

Uterine stroma-specific expression of truncated APC

In order to generate mice with a floxed allele of APC in uterine stroma, we used mice with the Cre recombinase gene inserted in the Amhr2 locus. Amhr2 is the anti-Müllerian hormone (AMH) type II receptor (also known as Müllerian inhibiting substance type II receptor), which is expressed in
the mesenchyme of fetal Müllerian ducts, the anlagen of the uterus, oviducts, cervix, and upper portion of the vagina. Examination of the receptor is required in fetal males for Müllerian duct regression in response to testicular AMH (23). To confirm that the expression of Amhr2-Cre in mice uteri was limited to cells derived from Müllerian duct mesenchyme and not observed in uterine epithelial cells, we crossed Amhr2-Cre mice with LacZ reporter mice and collected the reproductive tracts of the female progeny for analysis of β-galactosidase activity. Consistent with previous studies (23, 24), strong Cre-induced β-galactosidase activity was detected in the stromal cells but not in endometrial and glandular epithelial cells (Fig. 1A, a). These results were confirmed by crossing Amhr2-Cre and Yellow Fluorescence Protein (YFP) reporter mice. Similar to LacZ activity, YFP expression was observed in stromal cells but not in epithelial cells of mouse uteri (Fig. 1A, b). No expression of β-galactosidase or YFP was observed in control mice (Fig. 1A, inset in a and b). Similar to our previous observations (23), YFP and β-galactosidase staining was also observed in the myometrium (data not shown).

To generate conditional homozygous mutation of APC, Amhr2-Cre mice were crossed with APC
\( ^{\text{floox/flox}} \) mice leading to the deletion of exon 14 of the APC gene, which causes expression of a truncated APC protein in mutant APC
\( ^{\text{cKO}} \) mice (20). To confirm deletion of the APC conditional allele specifically in the stromal compartment but not in the endometrial epithelium, we collected genomic DNA from epithelial and stromal cells of adult mouse uteri using laser capture microdissection (Supplementary Fig. S1) for comparison by PCR (20) with genomic DNA from the tissues where Amhr2-Cre is known to induce recombination (uteri, ovary, and oviduct; ref. 23). We observed bands corresponding to the floxed APC allele (500 bp) in APC
\( ^{\text{cKO}} \) uterine stromal cells, whole uteri, ovaries, and oviducts but not in the uterine epithelial cells and tail (Fig. 1B, a). A band corresponding to the unfloxed APC allele (430 bp) was observed in all the cells and tissues examined for genomic PCR (Fig. 1B, a). Tail DNA was used as negative control for the floxed allele because Amhr2-Cre is not expressed in this tissue (23).

APC plays an important role in canonical Wnt signaling, and its loss leads to the nuclear accumulation of β-catenin (12). We examined the level of β-catenin expression in control and mutant uteri (n = 3 each) by Western blot (Fig. 1B, b). As expected, increased expression of β-catenin was observed in the APC
\( ^{\text{cKO}} \) compared with control APC
\( ^{\text{floox/flox}} \) uteri (Fig. 1B, b). We also observed increased expression of β-catenin transcriptional targets (TCF1, LEF1 and cyclin d1) in uterine lysates of mutant mice (Fig. 1B, b), indicating that the nuclear β-catenin was functionally active. To confirm that Amhr2-Cre induced recombination of the flox APC allele and therefore stabilization of β-catenin only occurred in stromal cells, we analyzed β-catenin expression in 4-week-old and adult uteri by immunofluorescence and observed nuclear accumulation of β-catenin in stromal cells of APC
\( ^{\text{cKO}} \) uteri at both ages (Fig. 1C, b and d). In contrast, mainly membranous expression of β-catenin was observed in the endometrial epithelium of both control and APC
\( ^{\text{cKO}} \) uteri (Fig. 1C, a and c).

Gross abnormalities and metrorrhagia were observed in the reproductive tracts of APC
\( ^{\text{cKO}} \) female mice

Examination of the gross morphology of reproductive tracts from 4-week-old control and APC
\( ^{\text{cKO}} \) mice showed that the oviducts were either absent or smaller with less coiling (100%, 5 of 5), but otherwise looked normal (Fig. 2A). The weight of mutant uteri was also significantly smaller than the controls at 4 weeks of age (Fig. 2B). Abnormal uterine enlargement and blood-filled cysts were also observed in older (>8 months) APC
\( ^{\text{cKO}} \) mice (Fig. 2C). Additionally, these mice displayed abnormal uterine hemorrhagia (metrorrhagia), which could explain the splenomegaly (Fig. 2D), suggesting splenic extramedullary erythropoiesis in response to anemia.

APC
\( ^{\text{cKO}} \) mice progressively develop endometrial hyperplasia and cancer

Histologic examination of APC
\( ^{\text{cKO}} \) and control uteri at different stages of development was conducted to assess the effect of stromal APC loss on the uterine parenchyma. At 4 weeks, control and APC
\( ^{\text{cKO}} \) uteri looked very similar at low magnification (Fig. 3A, a and b); however, closer examination revealed that endometrial glands adjacent to the mutant stroma were hyperplastic compared with controls (Fig. 3A, c–f). By 5 months, we observed complex hyperplasia of both the glandular and luminal endometrial epithelial lining in 100% of mutant mice (Fig. 3A, g and h). At 7 months of age, endometrial epithelium showed complex atypical hyperplasia with polyplike projections into the lumen of uteri (Fig. 3A, j and l), but not in controls (Fig. 3A, i and k). After 7 months, hyperplasia progressed to endometrial carcinoma in situ in some (4 of 7) of the mutant animals (Fig. 3B, a). At 1 year of age, the uteri of some mutant mice (3 of 8) were greatly enlarged and developed endometrial adenocarcinomas (Fig. 3B, b–f). Examination of their uteri revealed that the uterine lumen was occluded by tumors consisting of admixed epithelial and desmoplastic stromal cells (Fig. 3B, b–d). Expression of cytokeratin 8, an epithelial specific marker, confirmed a diagnosis of endometrial adenocarcinoma (Fig. 3B, e and f). No abnormalities were observed in the age-matched control mice (data not shown).

We also analyzed the expression of CD133, which has been associated with “cancer stem cells” in a variety of cancers (25), in control and mutant mice (Fig 4A and B). Similar to human endometrial tumors (25), we found increased expression of CD133 in the epithelial cells of uterine tumors in APC
\( ^{\text{cKO}} \) mice compared with controls, suggesting expansion of these cells with deletion of stromal APC.

The murine uterus has 3 main layers, endometrial epithelium, endometrial stroma, and myometrium, and is covered by a peritoneal serosa layer. Normally all endometrial glands are limited to the stromal compartment and myometrial invasion by endometrial epithelial cells is considered one of the hallmarks of endometrial adenocarcinomas (11). In our study, we found that in some (3/8) of mutant APC
\( ^{\text{cKO}} \) mice with endometrial adenocarcinomas, endometrial glands invaded the myometrium layer (Fig. 4C and D). We conducted colocalization of αSMA (smooth muscle
Figure 1. Analyses of Amhr2-Cre-induced recombination in murine uteri. A, Amhr2-Cre mice were crossed with LacZ and Yfp reporter mice. Amhr2-Cre induced β-galactosidase activity and direct YFP fluorescence was detected in stroma (ES) but not in epithelial cells of uteri (arrowheads and asterisks) or in their respective controls (A, insets). B, PCR of genomic DNA collected from the epithelium and stroma of APC<sub>cko</sub> tumors using laser capture microdissection and from whole uterus, ovary, oviduct, and tail was used to detect the recombined 500 bp floxed allele (a). The unrecombined flox APC allele (430 bp) was present in all tissue examined. B, Western blot analyses of uterine lysates showing increased expression of β-catenin, TCF1, LEF1, and Cyclin d1 in APC<sub>cko</sub> compared with control mice. β-actin was used as a loading control (b). C, Immunolocalization of β-catenin in 4-week-old (a, b) and 5-month old (c, d) uteri of control and APC<sub>cko</sub> mice. Inset in (b) is a higher magnification image of area outlined by dotted lines in same panel. Arrowheads (d) show nuclear accumulation of β-catenin in stroma. Epi, epithelium; ES, endometrial stroma. Scale bar, 50 μm.
marker) and cytokeratin 8 (marker of epithelial cells) on mutant uterine tissue sections to confirm the presence of endometrial glands inside the myometrium (Fig. 4E and F). Control uteri did not show any evidence of endometrial glands in the myometrium (Fig. 4G and H).

**Estrogen receptor α expression and the uterine estrogenic response are suppressed in APCcko mice**

Uterine tissue recombination experiments using estrogen receptor (ER) knockout and control mice have shown that estrogen activates stromal ERα to induce epithelial cell proliferation indirectly (26). We investigated the effects of E2 on uteri of oophorectomized APCcko and control mice maintained on depot estradiol for 30 days. We observed that APCcko mice showed minimal response to E2 treatment and gained significantly less uterine weight compared with controls (Fig. 5A and B). The normal uterine response to E2 also requires ERR-mediated induction of the progesterone receptor (PR), which then induces Foxo1 expression (27). We did Western blot analyses on uterine lysates collected from APCcko and control mice to determine whether the lack of an E2 response in APCcko mice was due to uninduced ERα expression or of its downstream targets, PR and Foxo1 and observed lower expression of ERα, PR, and Foxo1 in APCcko uteri compared with controls (Fig. 5C), suggesting that loss of APC inhibited the response of stromal cells to E2. Of note, loss of Foxo1 expression is a common event in human endometrial cancer (28).

**Loss of APC increases the myofibroblast population in endometrial stroma**

Dysregulated bone morphogenetic protein (BMP), transforming growth factor β (TGFβ), and Wnt signaling has been shown to induce myofibroblast phenotypic changes in stromal fibroblast cells, which is also a characteristic of cancer-associated fibroblast cells (6, 29). Additionally, cross-talk between TGFβ family members and Wnt signaling plays an important role in cancer progression of various organs. For example, compound deletion of Smad4 and APC has a synergistic effect that leads to the formation of highly malignant intestinal tumors (30). To determine whether...
Figure 3. Histologic analyses of uteri collected at different developmental stages from \( APC^{flox/flox} \) and \( APC^{cko} \) mice. A, cross sections (a, b) and longitudinal sections (c, d) of 4-week-old control (a, c) and mutant (b, d) uteri; rectangular areas (d) in shown in higher magnification (e, f); hyperplasia of epithelial lining of 5-month-old \( APC^{cko} \) mice uteri (h) compared with control (g); polyp-like outgrowths (arrow) were observed (i, k) in the endometrium of some 7-month-old mutant uteri (j, l) but not in controls (i, k). B, development of carcinoma in situ in \( APC^{cko} \) uteri; mutant uteri with occlusion of the uterine lumen by carcinogenic growth (b–d); higher-magnification image from (b) showing admixed endometrial epithelial and stroma cells (c); staining of mutant uterus with cytokeratin 8, an epithelial cell–specific marker (e); boxed area is shown at higher magnification (f). Scale bar, 50 \( \mu m \) unless otherwise indicated.
stromal-specific deletion of APC affects TGFβ and BMP signaling between the stromal and epithelial compartments of murine uterus and human endometrial cancer, we examined the expression of the downstream mediators of TGFβ (pSmad2/3) and BMP (pSmad1/5/8) signaling. IHC of pSmad2 and pSmad1/5/8 in mice and human tissues revealed that TGFβ and BMP signaling was suppressed in the stromal cells of APC-cko mice tumor and human endometrial cancer compared with control and normal tissues (Fig. 6A). In contrast, no differences were observed in epithelial-specific expression of pSmad2 and pSmad1/5/8 in both mice and human endometrial cancer compared with controls (Fig. 6A).

Next, we examined whether the mutant endometrial stromal cells were becoming more myofibroblast-like, which is characteristic of cancer-associated fibroblasts (1), by analyzing the expression of αSMA, a myofibroblast marker (31), and PDGFRα, a fibroblast marker (32). We observed more αSMA-positive and
fewer PDGFRα-positive in the stromal compartment of mutant mice compared with controls (Fig. 6B, a–d). In control uteri, most of the αSMA-positive staining was observed in the myometrium and the vascular smooth muscle cells (Fig. 6B, a). Because normal stromal cells maintain the nonmalignant phenotype of adjacent epithelial cells, we predicted that the conversion to a more myofibroblastic stroma we observed in APCcko mice might be contributing to carcinogenesis in human endometrial cancer. To confirm that similar changes also occur in human endometrial cancer patients, we collected and examined human endometrial carcinoma and normal/benign tissues for upregulated αSMA expression in uterine stroma cells, a transformation that has been linked to carcinogenesis in other systems (6). Similar to APCcko mice, αSMA-positive myofibroblast cells were greatly increased in the stroma of endometrial cancer patients [(7 of 9 patients) Fig. 6B, f and h] but only observed in vascular smooth muscle cells in normal/benign human endometrium (n = 4; Fig. 6B, e).

Stromal ERα plays an important role in induction of PR expression, which is thought to subsequently counteract the mitogenic effect of estrogen on epithelium through paracrine mechanisms (26, 33). We examined whether the increase in stromal myofibroblasts in murine and human endometrial cancer affects ERα and PR expression by IHC (Fig. 6C). We
Figure 6. APC deletion suppresses paracrine signaling and increases the myofibroblast population in mutant uterine stroma. A, reduced expression of pSmad2 (a–d) and pSmad 1/5/8 (e–h) in the stroma, but not the epithelium, of mutant uteri and in human endometrial cancer when compared with control uteri and in normal human endometrium was observed by IHC. B, immunofluorescence was carried out for αSMA (a, b) and PDGFRα (c, d) on 7-month-old APC<sup>fl<sup>ox</sup>/</sup>fl<sup>ox</sup> and APC<sup>cko</sup> uteri. In normal/benign human endometrium (n = 4), αSMA is normally detected in vascular smooth muscle cells only (e) but in patients with endometrial cancer (7 of 9), αSMA was detected in stromal cells (f–h). C, suppression of ERα (a–d) and PR (e–h) expression in stroma of APC<sup>cko</sup> uteri and human endometrial cancer patients compared with control or normal tissues was observed by IHC. ES, endometrial stroma; HNormal, normal human endometrium; HEndoCA, human endometrial cancer; M, myometrium. Scale bar, 50 μm.
observed decreased expression of ERα and PR in stromal cells adjacent to the epithelium of both mutant mice and human endometrial cancer compared with controls or normal endometrium, but expression was unchanged in the epithelial cells (Fig. 6C). Loss of stromal ERα and PR expression suggests that unopposed estrogen stimulation of epithelium is a contributing factor in the development of endometrial hyperplasia and cancer in APC<sup>cko</sup> mice.

**Increased expression of intercellular growth factors (VEGF and CXCL12) in APC<sup>cko</sup> mice**

Other intercellular signaling mechanisms are known to be involved in tumorigenesis. For example, expression of VEGF and its receptor VEGFR2 is upregulated in the stroma of human endometrial cancer patients and is correlated with poor outcome (34, 35). In this study, we observed increased expression of VEGFR2 on the leading edge of the APC<sup>cko</sup> mice tumors (Fig. 7A, a and b). VEGF expression itself was assessed by Western blot of uterine lysates and showed increased VEGF expression in mutant uteri compared with controls (Fig. 7B). Additionally, more CD31-positive endothelial cells were observed in murine tumors compared with controls, indicating increased vasculogenesis in mutant tumors in response to increased VEGF signaling (Fig. 7A, c and d). Similarly, stromal derived factor-1 (SDF-1)/CXCL12 is a paracrine factor secreted by cancer-associated fibroblast in various cancers including breast and endometrial cancer (36, 37), whose receptor, CXCR4, has been shown to be overexpressed in the epithelial cells of human endometrial cancer (38). In vitro, CXCL12 increases proliferation and invasion of human endometrial adenocarcinoma cells lines (39). In this study, we observed increased expression of CXCL12 in the stroma and CXCR4 in the epithelium of APC<sup>cko</sup> tumors, suggesting that, similar to human endometrial cancer, disrupted CXCL12/CXCR4 signaling might also play an important role in endometrial carcinogenesis in mutant mice.
Suppression of TGFβ signaling in stromal cells increases HGF secretion and has been associated with prostate and forestomach neoplasia (6). As we observed suppression of TGFβ signaling in stroma of APC^cko mice uteri, we examined expression of HGF and its activated receptor pMET in mutant and control uteri. No difference in the expression of these 2 proteins was observed between control and mutant mice (Fig. 7B). Additionally, pMet was not detected in 8 of 9 human endometrial cancer patients’ tissues examined (Supplementary Fig. S3). These findings suggest that HGF/MET may not play an important role in stromal-epithelial cross-talk in this model system or in human endometrial cancer. Collectively, these data suggest that unopposed estrogen stimulation of epithelium due to suppression of ERα and PR in stroma increases secretion of growth-promoting factors, VEGF and CXCL12, and suppression of growth-inhibitory signals, TGFβ and BMPs, are key mechanisms for the development of endometrial cancer in APC^cko mice and in humans (Fig. 7C).

Discussion

Stromal and epithelial cross-talk plays an important role in organogenesis as well as carcinogenesis of uterus, prostate, mammary glands, and other vital organs (6). Disruption of the signaling pathways responding to cellular communication is believed to be the initial event in cancer development (31, 40). For example, stromal cell only deletion of TGFβ receptor II leads to the formation of aggressive tumors in forestomach and prostate epithelium (40). Similarly, inactivation of BMP receptor II in intestinal stromal cells increases myofibroblast cell number and causes development of hamartomatous polyps (31). Human patients suffering from hamartomatous polyposis syndrome also harbor mutations in Smad4 in mesenchymal cells and show expansion of the stromal component (41). Additionally, loss of Smad4 in the stroma but not in the epithelium of colon, rectal, and stomach cancers suggests that paracrine signaling from the mutated cells in the microenvironment can drive carcinogenesis in the adjacent epithelial cells (42). In the present study, we have shown that APC-deletion in uterine endometrial stromal cells alone is sufficient for endometrial carcinogenesis.

One of the functions of APC is the control of Wnt/β-catenin signaling, which when dysregulated plays an important role in development of various cancers including colon, breast, ovarian, prostate, and uterine cancers. Wnt family members, such as Wnt2 and Wnt5a, are overexpressed in the stroma of human breast and colon cancers (43). Overexpression of Wnt1 in mice causes development of mammary adenocarcinomas, and in coculture experiments, Wnt1 expressing-fibroblasts induce transformation of C57MG epithelial cells without being affected themselves (6, 43). These data suggest that Wnt signaling in stromal cells might be an important therapeutic target for cancer treatment.

Wnt family members also play an important role in normal uterine organogenesis (44, 45). Deletion of Wnt7a, which is only expressed in uterine epithelium, causes abnormal development of both endometrial stroma and myometrium (44). Another Wnt ligand, Wnt5a, is expressed in uterine stroma where it is required for the development of endometrial epithelial glands (45). Similarly, conditional β-catenin knock-out studies have revealed that mesenchymal β-catenin plays an important role in formation of uterine glandular epithelium (23). These studies indicate that Wnt signaling plays an important role in uterine epithelial-stromal cross-talk but to date, there are no reports suggesting that dysregulated Wnt signaling causes endometrial cancer. Recently, we have shown that targeted deletion of exon 3 of β-catenin (Ctnnb1(ex3))^cko^ mice to form a constitutively activated (CA) allele of β-catenin in the Müllerian duct mesenchyme by Amhr2-Cre leads to the development of uterine leiomyomas, endometrial stromal sarcomas and adenomyosis, but not endometrial cancer (22). We also observed uterine leiomyomas in APC^cko^ mice, but endometrial stromal sarcomas were not observed (data not shown). In another study, expression of CA β-catenin in endometrial epithelium using PR-driven Cre causes endometrial hyperplasia, but not endometrial cancer (46). In contrast to these mice with CA β-catenin, we have shown here that stromal-specific APC^cko^ mice develop endometrial cancer. These phenotypic differences between APC^cko^ and Ctnnb1(ex3)^cko^ mice suggest that APC deletion is targeting more functions associated with APC than simply regulating the availability and/or accumulation of nuclear β-catenin and its subsequent transcriptional regulation of target genes.

The proliferation-promoting activity of estrogens and ERα expression in the uterus has been well established (47) as has the risk of endometrial cancer from unopposed estrogens (48), which can occur with the absence of available ERα to mediate E2-induced expression of stromal PR. A recent report has also shown that progesterone inhibits Wnt/β-catenin signaling in endometrial carcinoma cells in vitro, suggesting a mechanism for maintaining tissue homeostasis to counteract the proliferative potential of nuclear β-catenin (49). In human endometrial cancer patients, ERα and PR expression is usually limited to early, moderately or well-differentiated stages of carcinogenesis, but, paradoxically, it is the ERα- and PR-negative tumors that are usually found in relatively undifferentiated malignancies and indicate a poor prognosis (50, 51) as they do in breast cancer patients (52). Here, we showed that APC^cko^ mice have decreased expression of stromal ERα and are nonresponsive to E2 treatment, suggesting the tumor-inhibiting effects of E2 via the induction of the PR are not available in the uteri of APC^cko^ mice. Various epidemiologic studies in postmenopausal women undergoing hormone replacement therapy and investigations with mouse models of colon or intestinal cancer with APC deletions have shown that estrogens inhibit carcinogenesis and tumor progression (53, 54). Deletion of ERα in mice with an APC hypomorphic allele increases intestinal tumor development by increasing activation of the Wnt/β-catenin signaling pathway and its targeted genes (54). Similarly, oophorectomy of APC^+/−^ mice leads to increased tumor formation, whereas treatment with estradiol had an opposite effect (53). Additionally, Western blot analyses of intestinal tumors in APC^min/+^ mice showed decreased expression of ERα (53). Collectively, these observations indicate that estrogen signaling plays a role in regulating Wnt/β-catenin activities.
The current studies, along with our previous report showing that mice with uterine stromal expression of CA β-catenin do not develop endometrial cancer (22), suggest that nuclear accumulation of β-catenin by the expression of a truncated form of APC is that missing its β-catenin binding domains might not be the mechanism involved in carcinogenesis in APC<sup><i>−/−</i></sup> mice. Our data also suggest that deletion of APC activity leads to conversion of the stromal cells to a more myofibroblastic phenotype, which diminishes ERα expression and the ability of the cells to express PR to mediate the suppressive effects of progesterone-induced paracrine signaling on mitogenic E2 activity in the in epithelium. In vitro, estrogen treatment has been shown to increase expression of VEGF and CXCR12/CXCR4 (55, 56). However, the effect of progesterone on secretion of these growth factors is currently unclear. Because cross-talk between estrogen and progesterone plays an important role in uterine functions and endometrial carcinogenesis, deducing the mechanisms used by these ovarian steroids to control secretion of these intercellular growth factors will be critical for our understanding of endometrial tumorigenesis.

In summary, we have shown that deletion of APC activity in murine uterine stroma causes their transdifferentiation to a more myofibroblastic phenotype, which is accompanied by reduced ERα expression and is sufficient to induce endometrial cancer. We speculate that conversion of the stromal cells with perturbed APC activity might be a common mechanism for the effects of unopposed estrogen in endometrial carcinogenesis. Our future goals will be to determine which of the non-β-catenin-related function of APC are mediating the conversion of the stromal fibroblasts to myofibroblasts and the molecular mechanisms subsequently leading to development of endometrial hyperplasia and carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Author Contributions**

P.S. Tanwar provided conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. L. Zhang provided collection and/or assembly of data. D. J. Roberts provided collection and/or assembly of data, data analysis, and interpretation. J.M. Teixeira provided conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval.

**Acknowledgments**

We are grateful to Dr. Richard Behringer for supplying us with the Amhr2-Cre mice and to Drs. James Pru, Patricia K. Donohoe, John O. Schorge, and George L. Mutter for reviewing an early version of this manuscript.

**Grant Support**

These studies were supported in part by a grant from NICHD, HD057201, and by the Vincent Memorial Research Fund to J.M. Teixeira.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 27, 2010; revised December 2, 2010; accepted December 26, 2010; published online March 1, 2011.
disrupts their differentiation and ability to support spermatogenesis. Biol Reprod 2010;82:422–32.


Stromal Deletion of the APC Tumor Suppressor in Mice Triggers Development of Endometrial Cancer

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*Cancer Res* 2011;71:1584-1596.

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