Deregulation of the *HOXA10* Homeobox Gene in Endometrial Carcinoma: Role in Epithelial-Mesenchymal Transition

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Abstract

Homeobox genes encode transcription factors that control cell differentiation and play essential roles in developmental patterning. Increasing evidence indicates that many homeobox genes are aberrantly expressed in cancers, and that their deregulation significantly contributes to tumor progression. The homeobox gene *HOXA10* controls uterine organogenesis during embryonic development and functional endometrial differentiation in the adult. We investigated whether *HOXA10* expression is deregulated in endometrial carcinomas, and how counteracting this aberrant expression modifies tumor behavior. We found that down-regulation of *HOXA10* expression in endometrial carcinomas strongly correlates with increased tumor grade and is associated with methylation of the *HOXA10* promoter. Enforced expression of *HOXA10* in endometrial carcinoma cells inhibited invasive behavior *in vitro* and tumor dissemination in nude mice. The inhibitory effect of HOXA10 on invasive behavior was attributable at least in part to the ability of HOXA10 to induce expression of the epithelial cell adhesion molecule E-cadherin by down-regulating expression of Snail, a repressor of *E-cadherin* gene transcription. These findings reveal a novel role for *HOXA10* deregulation in the progression of endometrial carcinoma by promoting epithelial-mesenchymal transition. (Cancer Res 2006; 66(2): 889-97)

Introduction

It is widely recognized that many of the molecular pathways that underlie tumor progression are aberrations of processes that control normal embryonic development. Epithelial-mesenchymal transition (EMT) is a dynamic process whereby epithelial cells lose polarity and cell-cell adhesion, acquire a migratory phenotype, and switch to a mesenchymal-like gene expression program. This process is essential for normal morphogenesis, and reactivation of the EMT program is regarded as a primary mechanism underlying carcinoma progression and invasive behavior (1, 2). Homeobox genes have increasingly been thought to also represent important hubs in the intimate relationship between tumor progression and embryogenesis. These genes encode transcription factors that control developmental patterning and specify the unique morphologic identities of adult tissues (3, 4). Several homeobox genes that are normally expressed in embryonic tissues are activated in tumors (4, 5). One example is *PAX2*, which is normally expressed during early urogenital development, and is expressed in renal tumors (6). *PAX2* is thought to promote cell survival, and inhibiting *PAX2* expression in cancer cells induces apoptosis (7). On the other hand, many homeobox genes that are normally expressed in differentiated tissues are lost or down-regulated in tumors. *Nkx3.1* controls prostate morphogenesis and is normally expressed in the prostate during development and in the adult (8). *NKX3.1* is frequently deleted in prostate cancers (9), and enforced expression of *NKX3.1* in prostate cancer cells inhibits cell growth (10). Many other homeobox genes contribute to tumor progression by promoting cell growth and/or survival (4, 5). However, given the important role of homeobox genes in cell differentiation, surprisingly little is known regarding their role in EMT.

Endometrial carcinoma is the fourth most frequently diagnosed cancer in women. Grade 1 (G_1) endometrioid carcinomas of the endometrium are typically well-differentiated, and are mostly detected while still confined to the uterine corpus (11). Grade 3 (G_3) endometrioid carcinomas are mostly solid, whereas uterine papillary serous carcinoma (UPSC) is characterized by papillary patterns of growth. Although histologically distinct, G_3 endometrioid carcinoma and UPSC share several common features including high-grade nuclear atypia and poor responsiveness to chemotherapy, radiation, and progestin therapy (11–13). G_3 endometrioid carcinoma and UPSC have greater propensity for myometrial and lymphatic/vascular space invasion than G_1 tumors (11–13), but the mechanisms underlying the progression of these high-grade tumors are poorly understood.

Several studies indicate that the *HOXA10* homeobox gene controls uterine organogenesis during embryonic development and functional endometrial differentiation in the adult. *HOXA10* expression becomes restricted to the uterine primordium during normal müllerian duct differentiation, and is maintained in the adult uterus (14). Female mice with a targeted disruption of *Hoxa10* produce normal embryos, but their uteri are unable to support embryo implantation (15, 16). Implantation normally occurs during the mid-secretory phase of the menstrual cycle, a period during which the endometrium undergoes extensive glandular differentiation and *HOXA10* expression in the endometrial glands dramatically increases (14). A role for *HOXA10* in endometrial differentiation is also supported by our recent findings that aberrant activation of *HOXA10* in ovarian surface epithelium-derived cells gives rise to the endometrial-like features of endometrioid ovarian carcinoma (17).

Because *HOXA10* plays an essential role in endometrial development, we speculated that its expression is deregulated in endometrial carcinomas. In this study, we found that down-regulation of *HOXA10* expression in endometrial carcinomas correlates with increased tumor grade and is associated with *HOXA10* promoter methylation. Enforced expression of *HOXA10* in endometrial carcinoma cells inhibited invasive behavior, and this inhibitory effect was attributable, at least in part, to the ability of

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doi:10.1158/0008-5472.CAN-05-2828

HOXA10 to up-regulate E-cadherin by suppressing the expression of the Snail transcription factor. These findings reveal that silencing of *HOXA10* expression in the endometrium could contribute to tumor progression by promoting EMT.

Materials and Methods

Cell lines, cDNAs, and antibodies. The endometrial carcinoma cell lines SPEC2 and KLE were obtained from Isaiah Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX) and American Type Culture Collection (Rockville, MD), respectively. The *HOXA10* cDNA clone was provided by Corey Largman (University of California VA Medical Center, San Francisco, CA). The rabbit polyclonal antibody to HOXA10 has been previously described (17). Other antibodies used are as follows: actin, β1 integrin, cadherin-6, Snail (Santa Cruz Biotechnology, Santa Cruz, CA), E-cadherin (Zymed Laboratories, San Francisco, CA), vimentin (Neo-Markers, Fremont, CA), Ki-67 (Vector Laboratories, Burlingame, CA). Secondary antibodies were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Tissue microarrays and immunohistochemistry. Tissue microarrays were constructed from core biopsies of endometrial carcinoma specimens at the University of Texas M.D. Anderson Cancer Center using a Beecher Instruments (Silver Spring, MD) tissue arrayer. Use of specimens was approved by the Institutional Review Board. Cases included the following: normal secretory endometrium ($n = 10$), G_1 ($n = 26$), G_2 ($n = 37$), G_3 ($n = 58$) endometrioid carcinoma, and UPSC ($n = 30$). H&E-stained slides from paraffin blocks of each case were analyzed by a gynecologic pathologist (R. Broaddus) to confirm tumor histotype and grade. For each case, two separate core biopsies were taken from blocks that were representative of the tumor histotype and grade as a whole. Staining with HOXA10 antibody (1:200) was detected with streptavidin peroxidase by using a LSAB staining kit (Dako Corp., Carpinteria, CA). In previous work (17), we found that HOXA10 is expressed in normal mouse endometrial epithelium but not in fallopian tube epithelium. Slides of tissues of mouse endometrium and fallopian tube were stained simultaneously with microarray slides as internal positive and negative controls, respectively. HOXA10 staining intensity was scored independently by two observers (W. Cheng and H. Naora). An average staining score was determined for biopsies of each case. Histopathologic diagnosis of specimens was made independently of immunohistochemical analysis to eliminate bias in scoring staining. Epithelial staining intensity was graded on a 0 to 3 scale, where 0, no staining as assessed by staining with antirabbit secondary antibody alone; 1, weak; 2, moderate; and 3, intense staining.

Cell culture and transfection. SPEC2 and KLE cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and penicillin-streptomycin. The plasmids pCMV-Tag3-HOXA10 and pIRES-EGFP-HOXA10 were generated by subcloning *HOXA10* cDNA into pCMV-Tag3 (Stratagene, La Jolla, CA) and pIRES-EGFP (BD Biosciences Clontech, Palo Alto, CA) vectors, respectively. SPEC2 cells were stably transfected with pCMV-Tag3-HOXA10 and with empty pCMV-Tag vector using FuGENE6 (Roche, Indianapolis, IN). KLE cells were likewise transfected with pIRES-EGFP-HOXA10 and with pIRES-EGFP vector. Stably transfected clones, derived from single colonies, were selected by G418 (400 μg/mL; Invitrogen).

Matrigel culture and invasion chamber assays. Fifty thousand transfected cells, suspended in 500 μL of culture medium containing 5% Matrigel (BD Biosciences, Bedford, MA), were plated per well in 24-well plates. Solidified cultures were maintained for 2 weeks with medium changed every 3 to 4 days. For assaying cell invasion, 6×10^5 transfected cells were seeded into Matrigel invasion chambers (BD Biosciences). Twenty-four hours after seeding, invading cells on the lower surface of the filters were stained by using Giemsa solution, and counted in five different fields per assay under microscopy at ×100 magnification. Each assay was done in triplicate.

Proliferation assays. Cell proliferation was measured by using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). One thousand cells were seeded per well in 96-well plates. Absorbance at 570 nm after MTT addition was measured daily over 5-day time course. The number of trypan blue–excluding cells in replicate plates were also directly counted by using a hemocytometer. Each assay was done in triplicate.

Reverse transcription-PCR. Reverse transcription using 1 μg of DNase I-treated total RNA was done using Superscript II reverse transcriptase (Invitrogen) in a reaction volume of 20 μL. One microliter of the reverse transcription reaction was used for amplification using Platinum Taq DNA polymerase (Invitrogen). Detection of *HOXA10* and *actin* expression has been previously described (17). Other primers are as follows: (Snail) sense, 5′-TGCGCGAATCGGCGACCC-3′; antisense, 5′-CCTAGAGAAGGCCTTCC-CGCAG-3′; (Slug) sense, 5′-GCCTCCAAAAAGCCAAACTACAG-3′; antisense, 5′-GTGTGCTACACAGCAGCC-3′. Amplification was done as follows: denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute and extension at 72°C for 2 minutes for 37 cycles to detect *Snail* expression, and denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1 minute for 40 cycles to detect *Slug* expression. Titrations were done to ensure a linear range of amplification.

Western blot and immunofluorescence staining. Cell lysates were prepared by using M-PER buffer (Pierce, Rockford, IL). Equal amounts of protein (20 μg per lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were probed with antibodies to E-cadherin (1 μg/mL), β1 integrin (1:100), cadherin-6 (1:200), and actin (1:100). For cell staining, cells were fixed in 4% paraformaldehyde and permeabilized for 10 minutes in 0.1% Triton X-100. Cells were stained with antibodies to E-cadherin (2 μg/mL), vimentin (0.5 μg/mL), and Snail (1:50) for 1 hour. Staining was detected using Texas red–conjugated secondary antibodies.

Methylation-specific PCR. Two micrograms of genomic DNA, isolated from cultured cells and tissues, were treated with 0.2 mol/L NaOH for 10 minutes at 37°C, then with 3.75 mmol/L hydroquinone and 2.6 mol/L sodium bisulfite (Sigma, St. Louis, MO) and incubated for 16 hours at 50°C. DNA was purified using the Wizard DNA Purification System (Promega, Madison, WI). Samples were desulfonated with 0.3 mol/L NaOH, precipitated with ethanol, and suspended in 20 μL of distilled water. DNA was analyzed using primer sets located within CpG islands in the *HOXA10* promoter. Primers specific for unmethylated DNA were 5′-TGGAGAGT-GGTTTTGTG-3′ (sense) and 5′-AAATACAATTAACAATCCCC-3′ (antisense; 113 bp product). Primers specific for methylated DNA were 5′-CGGA-GAGCGGTTTCGC-3′ (sense) and 5′-AAACATACTAAATACGATTAA-CAATCCC-3′ (antisense; 122 bp product). PCR amplification of 1 μL bisulfite-treated DNA template was done in a 10 μL reaction containing 2 μmol/L forward and reverse primers, 1.25 mmol/L deoxynucleotide triphosphates, and 0.5 units of HotStarTaq DNA polymerase (Qiagen, Valencia, CA). Amplification conditions were as follows: 15 minutes starting at 95°C, 35 cycles at 95°C for 30 seconds, 60°C (methylated) or 53°C (unmethylated) for 30 seconds, and 72°C for 30 seconds followed by a final extension at 72°C for 10 minutes. PCR products were resolved by electrophoresis on a 6% acrylamide gel.

Propagation of cells in nude mice. Animal studies were approved by the Institutional Animal Care and Use Committee. Five 4-week-old female nude mice per group were inoculated i.p. at one site in the lower abdomen with 3×10^6 cells of each cell line. At 3 months thereafter, mice were killed by CO_2 asphyxiation. Paraffin-embedded tissues collected from mice were stained with H&E, and with antibodies to HOXA10 (1:200), Ki-67 (1:1,000), E-cadherin (2 μg/mL), and Snail (1:100).

Statistical analysis. Statistical significance of differences in HOXA10 staining intensity between groups of normal and malignant cases was calculated by the Mann-Whitney *U* test. Statistical significance of differences in cell proliferation and invasion between vector control and *HOXA10*-transfected cells was calculated by Student's *t* test. $P > 0.05$ was considered not significant.

Results

Down-regulation of *HOXA10* expression correlates with tumor grade. We examined whether *HOXA10* expression is

deregulated in endometrial carcinomas by performing immunohistochemical analysis of tissue microarrays of clinical specimens. Strong HOXA10 staining was observed throughout glands of normal secretory endometrium as likewise reported by others (14). Epithelial staining was reduced in G_1 endometrioid carcinomas as compared with normal secretory endometrium, reduced in G_2 tumors as compared with G_1 tumors, and significantly weaker in G_3 tumors (Fig. 1*A* and *B*). The majority of UPSC cases exhibited little or no HOXA10 staining, as likewise found for G_3 endometrioid carcinomas (Fig. 1*A* and *B*). These results indicate that down-regulation of *HOXA10* expression in endometrial carcinomas correlates with increased tumor grade, and raises the possibility that this down-regulation promotes tumor growth and/or invasive properties.

Enforced expression of *HOXA10* in endometrial carcinoma cells does not affect cell proliferation. To determine the effect of *HOXA10* on the proliferation of endometrial carcinoma cells, we stably expressed *HOXA10* in two model cell lines. The SPEC2 cell line was established from a UPSC tumor (18). The KLE cell line was established from a poorly differentiated endometrial carcinoma (19). These cell lines have been used as models of UPSC and poorly differentiated endometrial carcinoma by others (20, 21), and were ideal for this study because they have almost undetectable levels of endogenous *HOXA10* expression (Fig. 2*A*). KLE and SPEC2 cell lines were each stably transfected with *HOXA10* cDNA and with empty vector. Two clones from each transfection, isolated from single colonies, were used for further study (Fig. 2*A*). Cell proliferation was measured by MTT assays and by cell counts. No significant difference was observed in proliferation rates of vector-transfected SPEC2 cell lines (Vec-6 and Vec-8) and of *HOXA10*-transfected SPEC2 cell lines (A10-4, A10-42; Fig. 2*B*). Similarly, *HOXA10* had no significant effect on the proliferation of KLE cells (Fig. 2*B*). Cells of the stably transfected lines were also inoculated into female nude mice. Tumors that formed in mice were stained with antibodies to

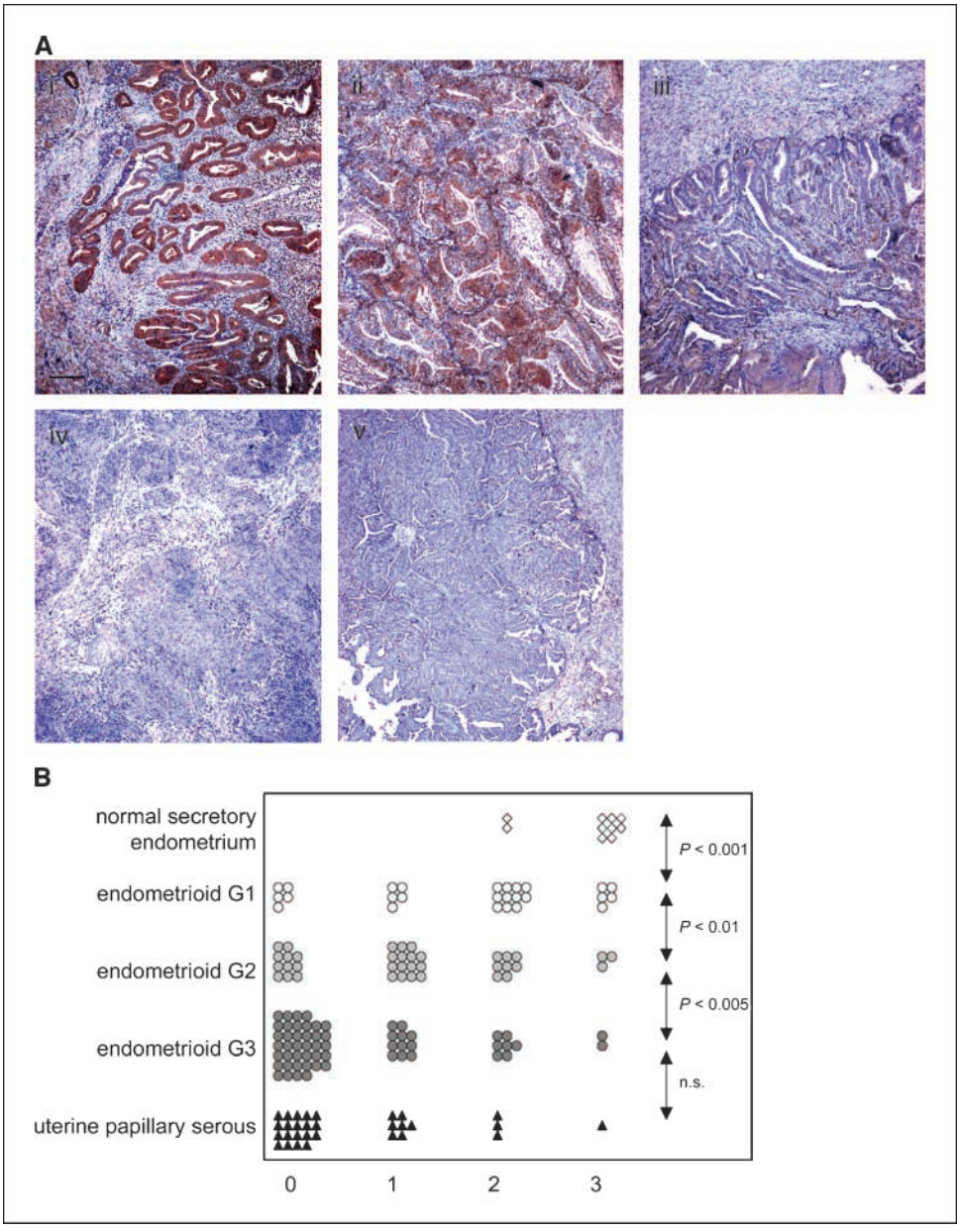

Figure 1. Expression of HOXA10 in endometrial tissues. *A,* immunohistochemical staining of HOXA10 in specimens of (*i*) normal secretory endometrium, (*ii*) G_1, (*iii*) G_2, (*iv*) G_3 endometrioid carcinomas, and (*v*) UPSC; *bar*, 200 μm. *B,* summary of HOXA10 staining in clinical specimens. For each case, epithelial staining intensity was scored as either 0, no staining; 1, weak; 2, moderate; or 3, intense. Each symbol represents an individual case. Statistical significance of differences in staining intensity between the indicated groups of cases was calculated by the Mann-Whitney *U* test. $P > 0.05$ were considered not significant (n.s.).

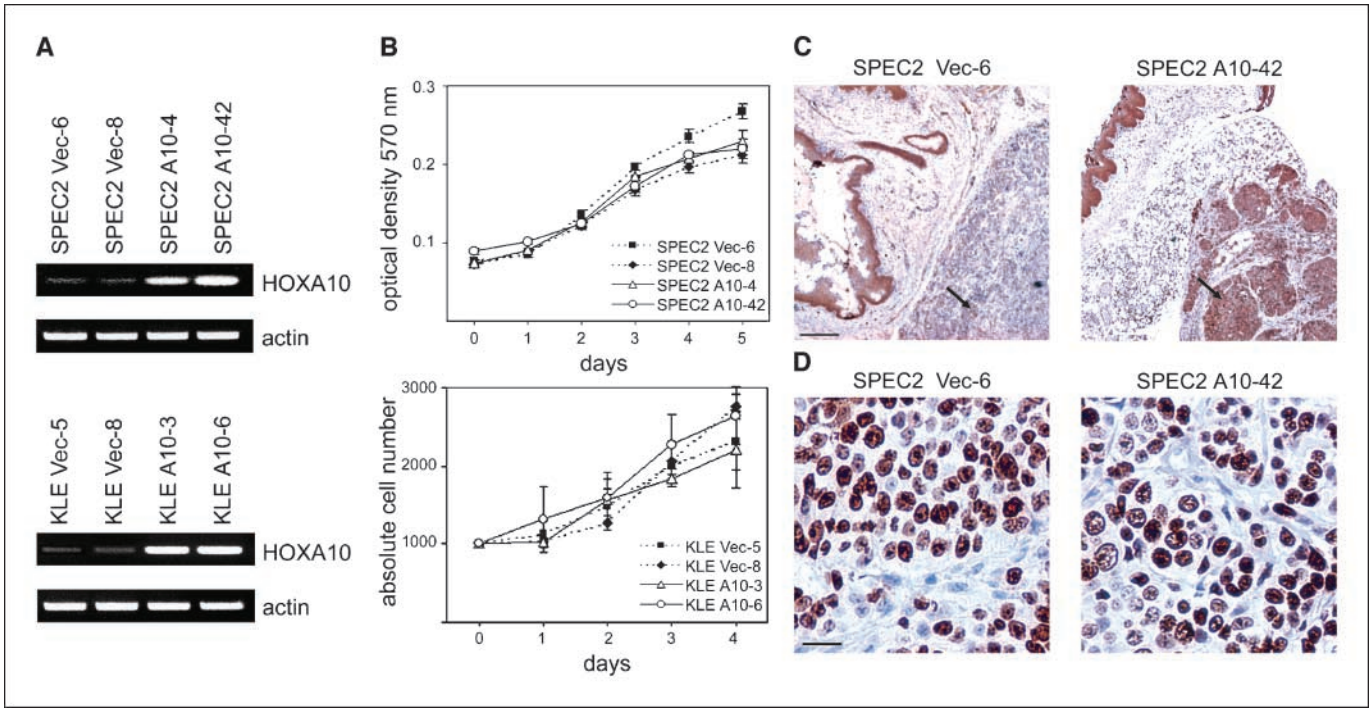

Figure 2. Effect of *HOXA10* on cell proliferation. *A,* RT-PCR analysis of *HOXA10* expression in SPEC2 and KLE cell lines stably transfected with empty vector and with *HOXA10* cDNA. Amplification of actin RT-PCR products was included as a control. *B,* proliferation of stably transfected SPEC2 and KLE cells was measured by MTT assay and by direct cell counts. The results of triplicate assays are shown. *C,* stably transfected SPEC2 cells were inoculated at one site i.p. in the lower abdomen of female nude mice. Sections of tissues collected from sacrificed mice were stained with HOXA10 antibody; *arrows*, tumors. Staining of HOXA10 in normal vaginal epithelium, seen in both panels, served as a positive control; *bar*, 200 μm. *D,* SPEC2-derived tumor tissues were stained with antibody to Ki-67; *bar*, 20 μm.

HOXA10 (Fig. 2*C*) and to the proliferation marker Ki-67 (Fig. 2*D*). No difference was observed between the proportion of Ki-67-positive cells in tumors derived from vector control cells and in tumors derived from *HOXA10*-transfected cells (Fig. 2*D*).

Enforced expression of *HOXA10* promotes cell adhesion and inhibits invasive behavior. We initially investigated the possibility that *HOXA10* regulates the adhesive properties of endometrial carcinoma cells by examining the growth of transfected cells in semisolid Matrigel cultures. Vector control SPEC2 cells grew as small clusters of loosely aggregated cells (Fig. 3*A*). In contrast, *HOXA10*-transfected SPEC2 cells grew as large, well-formed spheroids. *HOXA10*-transfected KLE cells, but not vector control KLE cells, also formed tightly aggregated spheroids (Fig. 3*A*). These findings indicated that *HOXA10* expression in endometrial carcinoma cells promotes cell-cell adhesion. *HOXA10*-transfected SPEC2 and KLE cells exhibited a decreased ability to invade in Matrigel invasion chamber assays as compared with vector control cells (Fig. 3*B*). The possibility that the decreased number of invading *HOXA10*-expressing cells could be due to reduced proliferation can be eliminated because *HOXA10* was found not to significantly affect cell proliferation (Fig. 2*B*).

To confirm our findings, we investigated whether *HOXA10* alters the invasive behavior of tumor cells propagated in female nude mice. UPSCs behave similarly to serous ovarian carcinomas in their propensity for i.p. spread (13). To mimic this behavior, transfected SPEC2 cells were inoculated i.p. at one site in the lower abdomen. Both vector- and *HOXA10*-transfected SPEC2 cells formed solid, bulky tumors at the injection site (Fig. 3*C*). However, vector control tumors were more locally invasive and extensively involved the ovaries (Fig. 3*C*). Animals inoculated with vector control cells (Vec-6 and Vec-8) developed tumors at sites distant from the injection site such as the omentum and diaphragm within 3 months (Fig. 3*D*). In contrast, diaphragmatic implants were not observed in any of the mice inoculated with cells of the *HOXA10*-transfected lines (A10-4 and A10-42) within the same period (Fig. 3*D*). Only one of five animals injected with A10-4 cells developed omentum implants. Expressing *HOXA10* therefore seems to modify the behavior of endometrial carcinoma cells by inhibiting their ability to disseminate.

***HOXA10* up-regulates E-cadherin expression.** Down-regulation of E-cadherin expression plays a central role in the loss by epithelial cells of polarity and cell-cell adhesion, concomitant with the acquisition of migratory properties (1, 2). Because HOXA10 inhibited invasive behavior of endometrial carcinoma cells, we investigated whether HOXA10 up-regulates E-cadherin expression. Western blot analysis revealed that vector control SPEC2 and KLE cells expressed very little E-cadherin, whereas E-cadherin levels were markedly elevated when *HOXA10* was expressed (Fig. 4*A*). *HOXA10*-transfected cells exhibited strong E-cadherin staining on cell surfaces and also increased intracellular staining. In contrast, very little staining was observed in vector control cells (Fig. 4*B*). *HOXA10*-transfected SPEC2 cells formed tumors in mice that highly expressed E-cadherin and exhibited more nesting than tumors that formed from vector control cells (Fig. 4*C*). Expression of the mesenchymal marker vimentin was strongly detected in vector control SPEC2 and KLE cells, but was abolished when *HOXA10* was stably expressed in these cells (Fig. 4*B*). Inhibition of invasive behavior of endometrial carcinoma cells by progesterone is associated with down-regulation of β1 integrin and cadherin-6 (22). Expression of these cell adhesion molecules was not affected by HOXA10 (Fig. 4*A*). Enforced expression of *HOXA10* in endometrial carcinoma cells therefore seems to inhibit invasive

behavior by up-regulating E-cadherin expression and promoting mesenchymal-to-epithelial transition.

***HOXA10* inhibits *Snail* expression.** Snail is a zinc finger protein that induces EMT and represses *E-cadherin* gene transcription (23, 24). Because HOXA10 induced E-cadherin expression, we investigated whether HOXA10 down-regulates expression of *Snail*. As shown in Fig. 5*A*, *Snail* transcripts were detected in vector control SPEC2 and KLE cells, but were almost undetectable in *HOXA10*-transfected cells. These findings were confirmed by staining cultured cells and tumors derived from transfected cells with antibody to Snail. Nuclear staining was detected in vector control KLE cells but not in *HOXA10*-transfected cells (Fig. 5*B*). Nuclear staining was also observed in cells of tumors that formed from vector control SPEC2 cells, but not from *HOXA10*-transfected SPEC2 cells (Fig. 5*C*). We also examined expression of Slug, a zinc finger protein that is closely related to Snail and also represses *E-cadherin* gene transcription (25). In contrast to *Snail*, levels of *Slug* transcripts were very similar in vector- and *HOXA10*-transfected cells (Fig. 5*A*). These findings indicate that HOXA10 induces E-cadherin expression by down-regulating expression of the Snail repressor but not of Slug.

Down-regulation of *HOXA10* expression is associated with promoter methylation. Methylation of CpG islands in the promoter region is an important mechanism that silences expression of many genes in cancers, including various homeobox genes (26, 27). The *HOXA10* promoter contains a CpG island–rich region that extends from −20 to −210 bp upstream of the transcription start site. We determined the methylation status of this promoter region in normal and malignant endometrial tissues by using methylation-specific PCR (MSP). As a control, we used the cervical cancer cell line SiHa that constitutively expresses HOXA10 (17). Only unmethylated and no methylated DNA was detected in SiHa cells (Fig. 6). In contrast, only methylated and no unmethylated DNA was detected in parental SPEC2 and KLE cells that do not express *HOXA10* (Fig. 6). Only methylated and no unmethylated DNA were detected in five of eight G_3 tumors (Fig. 6). Methylated DNA was more strongly detected than unmethylated DNA in the three other G_3 tumors. In contrast, only partial methylation was detected in six of seven G_1 tumors, and a seventh case exhibited only unmethylated DNA. Partial methylation was detected in all four specimens of normal endometrium, with unmethylated DNA being more strongly detected than methylated DNA in two of these cases (Fig. 6). The prevalence of *HOXA10* promoter methylation in G_3 tumors is strongly consistent with the lack of HOXA10 expression seen in the majority of G_3 tumors, whereas the lower frequency of promoter methylation in G_1 tumors

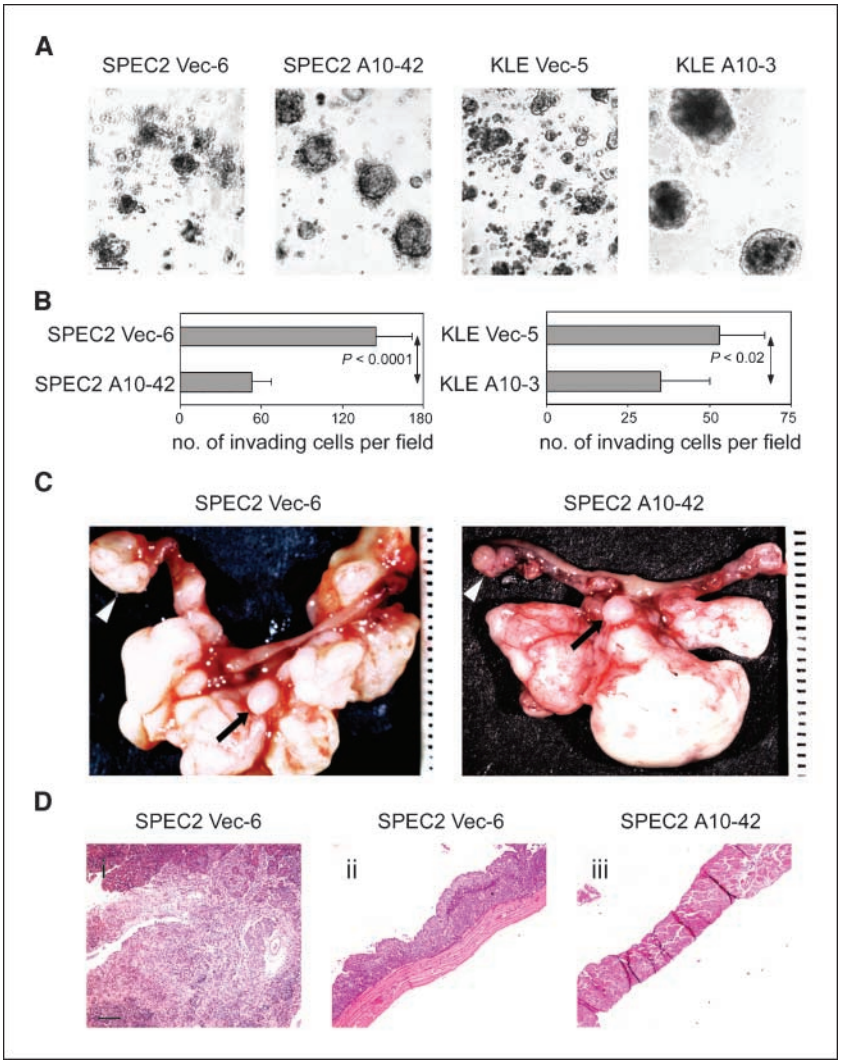

Figure 3. *HOXA10* promotes cell adhesion and inhibits invasive behavior. *A,* morphology of empty vector- and *HOXA10*-transfected SPEC2 and KLE cells in semisolid Matrigel culture; *bar*, 100 μm. *B,* 6×10^5 transfected cells were seeded per well in Matrigel invasion chambers. Twenty-four hours thereafter, invading cells were counted in five different fields per assay under microscopy at ×100 magnification. The results of triplicate assays are shown. Statistical significance of differences in the number of invading cells was determined by Student's *t* test. *C,* tumor tissues and urogenital organs excised from female mice inoculated with equivalent numbers (3×10^6) of stably transfected SPEC2 cells as described in Fig. 2 and sacrificed 3 months thereafter. *Black arrows*, bladder; *white triangles*, ovaries. *D,* H&E-stained sections of tumors derived from vector control cells that involved the (*i*) omentum (*top*, pancreas) and (*ii*) diaphragm. In contrast, mice inoculated with *HOXA10*-transfected cells did not develop diaphragmatic implants (*iii*); *bar*, 200 μm.

and normal endometrium is consistent with the higher HOXA10 expression in these tissues (Fig. 1). These findings indicate that loss of *HOXA10* expression in endometrial carcinomas, particularly high-grade tumors, is attributable at least in part to methylation of the *HOXA10* promoter.

Discussion

A hallmark of EMT is loss of E-cadherin expression that is mediated by a battery of transcriptional repressors, the best-characterized of which is Snail (1, 2). E-cadherin plays a central role in the differentiation and histotypic organization of all types of epithelial cells, and an inverse correlation between E-cadherin expression and tumor grade has been documented in a wide variety of carcinomas (1, 2). On the other hand, the specialized differentiation patterns that are unique to each type of epithelial cell and, indeed of all cell types, are governed by specific repertoires of homeobox genes (3, 4). Our study reveals that *HOXA10*, a homeobox gene that controls endometrial differentiation during embryogenesis and in adulthood, regulates E-cadherin expression, and that down-regulation of *HOXA10* expression could contribute to the progression of endometrial carcinoma by promoting EMT. There is evidence that other homeobox genes induce expression and/or activity of E-cadherin in a tissue-specific manner (28, 29). However, this is the first report indicating that a regulatory interaction exists between a homeobox gene and the transcriptional machinery that represses *E-cadherin* gene expression. This regulatory interaction seems to be highly specific, as HOXA10 was found to inhibit expression of *Snail* but not of *Slug*. SIP1 and Twist also repress *E-cadherin* gene transcription via E boxes that are targeted by Snail and Slug (30), and it has yet to be determined whether HOXA10 regulates the expression of these repressors. Nevertheless, our findings are consistent with increasing evidence that the various repressors of *E-cadherin* gene transcription are regulated by distinct signaling pathways in different cell types (1, 2, 30). One example of differential regulation is that mediated by metastatic tumor antigen 3 (MTA3) in breast epithelial cells. MTA3 is a component of the Mi-2/NuRD complex that has been found to repress transcription of *Snail*, but not of *Slug* (31). It has yet to be determined whether HOXA10 directly represses *Snail* gene transcription or inhibits *Snail* expression by an indirect mechanism.

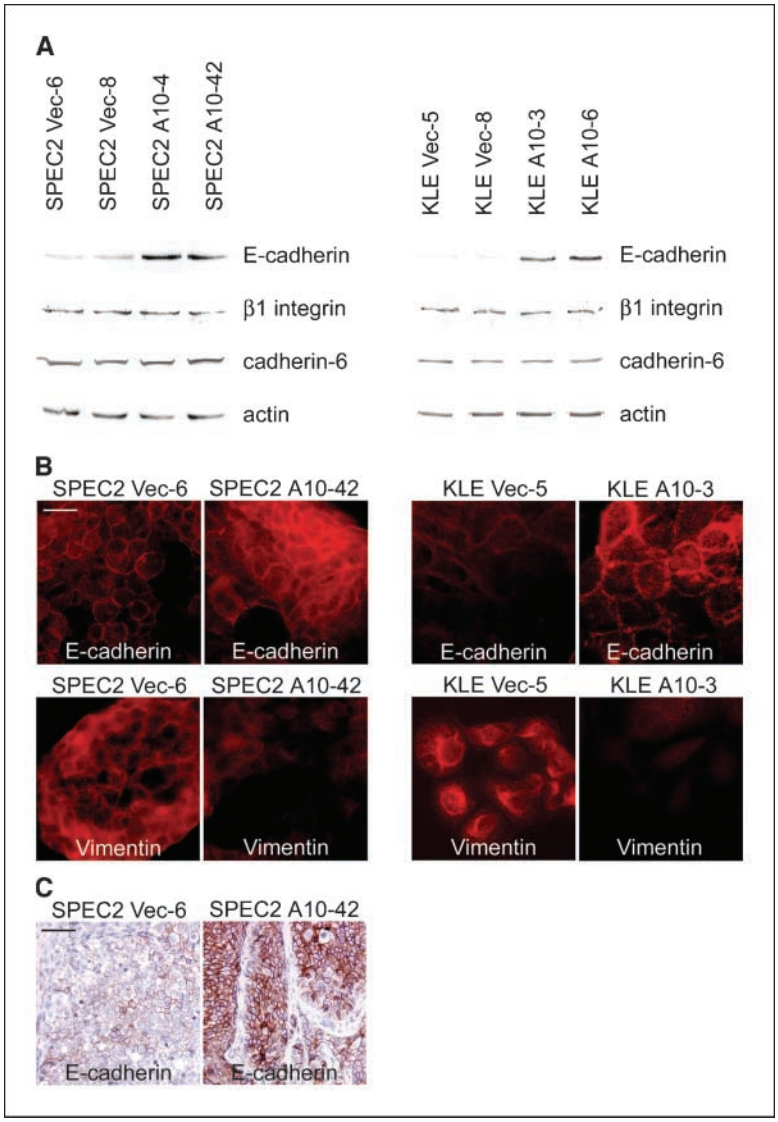

Figure 4. HOXA10 down-regulates vimentin and induces E-cadherin expression. *A,* Western blot analysis of E-cadherin, β1 integrin, cadherin-6, and actin expression in stably transfected SPEC2 and KLE cell lines. *B,* expression of E-cadherin and vimentin in transfected SPEC2 and KLE cell lines as detected by immunofluorescence staining of cultured cells; *bar*, 25 μm. *C,* immunohistochemical staining of E-cadherin in tumor tissues collected from nude mice inoculated with vector control and with *HOXA10*-transfected SPEC2 cells; *bar*, 50 μm.

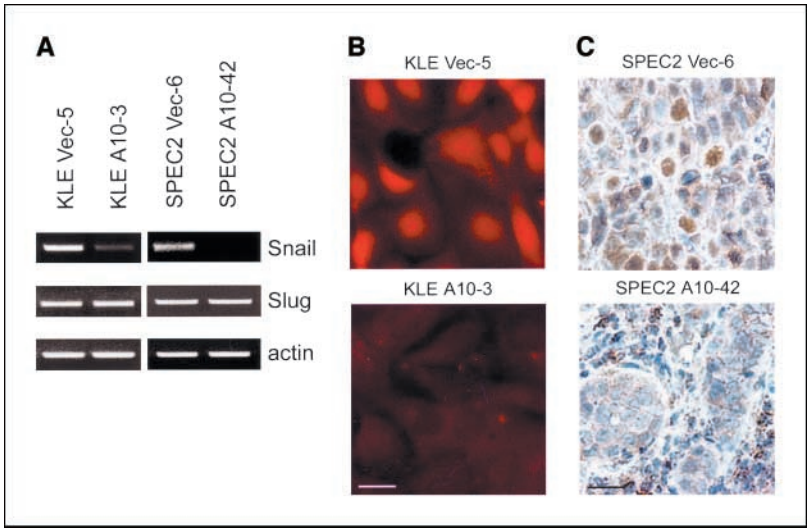

Figure 5. HOXA10 inhibits *Snail* expression. *A, Snail* expression in vector- and *HOXA10*-transfected SPEC2 and KLE cells detected by RT-PCR. *B,* Snail expression in transfected KLE cells detected by immunofluorescence staining; *bar*, 20 μm. *C,* immunohistochemical staining of Snail in tumor tissues derived from transfected SPEC2 cells. Nuclear staining was observed in cells of vector control-derived tumors, whereas in sections of tumors that formed from *HOXA10*-transfected cells, some staining was observed in stromal cells, but almost no staining was detected in epithelial cells; *bar*, 20 μm.

The tissue-specificity of homeobox gene expression and function, and the tissue specificity of the regulatory pathways that control *Snail* expression prompt the question as to what receptor-mediated signal(s) could regulate HOXA10 in endometrial epithelial cells. One possible candidate is the progesterone receptor (PR). Endometrial differentiation during the secretory phase of the menstrual cycle is driven by progesterone-stimulated induction of *HOXA10* expression (32). The induction by progesterone of the differentiated, secretory phenotype is primarily mediated through the PR-B isoform (22). Loss of PR-B in high-grade, poorly differentiated endometrial carcinomas is thought to be a driving force of tumor progression (33, 34). Progesterone can inhibit the invasive behavior of endometrial carcinoma cells that express PR-B (22). It is possible that loss of PR-B and progesterone-responsiveness in endometrial carcinoma cells leads to methylation of the *HOXA10* promoter, and that the silencing of *HOXA10* expression in turn leads to activation of Snail expression, inhibition of E-cadherin expression and increased invasive behavior.

This possible model has an interesting analogy to the study by Fujita et al. (31), which revealed that loss of functional estrogen receptors (ER) in aggressive breast cancers results in decreased expression of MTA3, a component of a transcriptional complex that normally represses *Snail* transcription. The implication of the study of Fujita et al. is that ER status controls EMT in breast cancers. It is possible that PR-B status could likewise control EMT in endometrial carcinomas. It should be pointed out that loss of E-cadherin expression alone does not constitute EMT, and that multiple mechanisms contribute to invasive behavior. Inhibition of invasive behavior of endometrial carcinoma cells by progesterone is associated with down-regulation of several cell adhesion molecules such as β1 integrin and cadherin-6 (22). However, HOXA10 had no effect on β1 integrin and cadherin-6 levels in KLE and SPEC2 cells (Fig. 4*A*). This suggests that HOXA10 cannot repress all of the pathways that mediate invasive behavior and that are inhibited by progesterone. Progesterone not only inhibits the invasive behavior of endometrial carcinoma cells, but also inhibits cell proliferation (22). The notion that not all signaling pathways emanating from PR-B are regulated by HOXA10 is supported by our findings that *HOXA10* expression did not significantly affect the proliferation of endometrial carcinoma cells. This observation is consistent with findings that the rate of epithelial cell proliferation in the uteri of *Hoxa10*-deficient mice in response to estrogen is very similar to the rate observed in wild-type mice (35).

Several parallels exist between our findings and those of Taylor and colleagues. As likewise found by these investigators (36), we detected intact HOXA10 expression in a subset of G_1 and G_2 endometrioid carcinomas. However, we observed that HOXA10 expression significantly decreases with tumor grade, such that G_3 endometrioid carcinomas and UPSCs had lower HOXA10 expression than G_1 and G_2 tumors. Taylor et al. did not examine G_3 endometrioid carcinomas and UPSCs. In our study, 86% of the cases that extended beyond the uterine corpus (stages II-IV) were G_3 tumors and UPSCs. Sixty-one percent of G_3 and UPSC cases were stages II to IV, as compared with only 7% of G_1 tumors. The association of *HOXA10* down-regulation with progression of endometrial carcinoma is consistent with our findings that

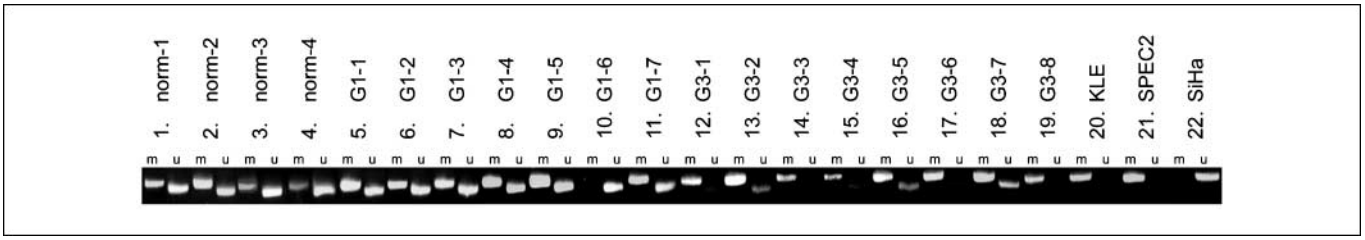

Figure 6. Methylation status of the *HOXA10* promoter. Detection of unmethylated (113 bp product) and methylated (122 bp product) DNA corresponding to the CpG island–rich region in the *HOXA10* promoter was done by MSP in tissues of normal endometrium (*n* = 4; *lanes 1-4*), G_1 endometrioid carcinoma (*n* = 7; *lanes 5-11*), and G_3 endometrioid carcinoma (*n* = 8; *lanes 12-19*), and on cells of the parental KLE (*lane 20*), SPEC2 (*lane 21*), and SiHa (*lane 22*) cell lines.

expressing *HOXA10* inhibits invasive behavior. Interestingly, Taylor and colleagues also found that HOXA10 inhibits the invasive behavior of breast cancer cells (37), but it is unclear whether this inhibition occurs by the same mechanism as in endometrial carcinoma cells. The same group reported that the normal midsecretory increase of *HOXA10* expression is abolished in the endometrium of women with endometriosis (38). Endometriosis is a benign condition, but exhibits many of the characteristics of malignancy. A hallmark of endometriosis is acquisition of the ability of endometrial tissue to invade other normal tissues. Epithelial cells derived from endometriotic lesions have been found to be as invasive as carcinoma cells in Matrigel assays (39), and to have reduced expression of PR-B and of E-cadherin (40, 41). Down-regulation of *HOXA10* expression could therefore be responsible for the acquisition of invasive behavior of endometrial epithelial cells in endometriosis as well as in endometrial carcinoma.

HOXA11, a neighbor of *HOXA10* in the *HOXA* gene cluster, also plays an important role in regulating uterine development. As likewise observed in *Hoxa10*-deficient mice, female mice with a targeted disruption of *Hoxa11* have implantation defects (42). *HOXA10* and *HOXA11* have similar patterns of expression during the menstrual cycle and both genes are up-regulated by progesterone (32, 43). Whereas *Hoxa10* is expressed uniformly throughout the uterus, *Hoxa11* is more highly expressed in the posterior than in the anterior uterine segment (17). The *HOXA11* promoter has been found to be frequently methylated in endometrial carcinomas, and the rate of *HOXA11* promoter methylation is significantly higher in recurrent than in nonrecurrent tumors (27). These observations are strikingly similar to the correlations that we found between *HOXA10* promoter methylation, down-regulation of *HOXA10* expression, and aggressive clinical behavior, and raise the possibility that down-regulation of *HOXA11* in addition to *HOXA10* could promote the progression of endometrial carcinoma.

The findings that *HOXA10* expression is down-regulated in endometriosis as well as in endometrial carcinomas implies that loss of *HOXA10* expression alone is insufficient to induce endometrial malignancy. This notion is supported by the fact that *Hoxa10* mutant mice do not develop endometrial tumors. Furthermore, this implication is consistent with several studies which indicate that loss of expression of a homeobox gene that is normally expressed in differentiated tissues is alone insufficient to induce malignancy. Inactivation in mice of *Nkx3.1*, a regulator of prostate morphogenesis, leads to the development of prostatic intraepithelial neoplasia, however, these lesions do not progress to carcinoma (8). *CDX2* controls the differentiation of intestinal epithelium and is down-regulated in colorectal cancers, particularly high-grade tumors (44). Although *Cdx2* heterozygous mutant mice do not develop spontaneous malignant tumors, they develop adenomatous intestinal polyps and have increased sensitivity to chemically induced colon carcinogenesis as compared with wild-type mice (45). This suggests that loss of homeobox gene expression can collaborate with other oncogenic events to induce malignancy. Indeed, inactivation of *Nkx3.1* cooperates with loss-of-function of *Pten* to induce prostate carcinoma (46). *PTEN* mutations are the most frequently detected genetic alterations in endometrioid carcinomas followed by microsatellite instability, and mutations of K-*ras* and β-catenin (47). In contrast, *p53* mutations occur in 10% to 20% of endometrioid tumors, but occur in almost 90% of UPSCs (47). It is possible that loss of *HOXA10* expression could cooperate with one or more of these genetic alterations to induce endometrial carcinoma, and that the histologic type of tumor depends on the specific combination of these molecular events.

Acknowledgments

Received 8/9/2005; revised 10/7/2005; accepted 10/25/2005.

Grant support: NIH grant R01 CA101826 (H. Naora), faculty start-up funds from The University of Texas M.D. Anderson Cancer Center (H. Naora), and NIH grant P50 CA098258 (R. Broaddus).

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We thank Dr. Yutaka Kondo (University of Texas M.D. Anderson Cancer Center) for generously assisting in designing MSP primers, the M.D. Anderson Cancer Center Gynecologic Tumor Bank for providing tissue specimens, Angela Thornton for tissue microarray construction, Kimberly Head for technical assistance, and Dr. Brian Slomovitz for providing additional slides of whole sections to confirm staining of tissue microarrays.

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Cancer Res 2006;66:889-897.