The Transcription Factor ZEB1 Is aberrantly expressed in Aggressive Uterine Cancers

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Abstract

The transcription factor ZEB1 (Zfhx1a, Zfhep, AREB6, OEB1) in mice has been implicated in cellular processes during development and tumor progression including epithelial to mesenchymal transition. ZEB1 null mice die at birth, but heterozygotes expressing a LacZ reporter inserted into the ZEB1 gene live and reproduce. Using these mice, we observed ZEB1 promoter activity in the virgin myometrium, stroma and myometrium of the pregnant uterus. ZEB1 protein is up-regulated in the myometrium and endometrial stroma after progesterone or estrogen treatment of ovariecтомized mice. In the normal human uterus, ZEB1 protein is increased in the myometrium and stroma during the secretory stage of the menstrual cycle. ZEB1 is not expressed in the normal endometrial epithelium. In malignancies of the uterus, we find that ZEB1 (a) is overexpressed in malignant tumors derived from the myometrium (leiomyosarcomas), (b) is overexpressed in tumor-associated stroma of low-grade endometrioid adenocarcinomas, and (c) is aberrantly expressed in the tumor epithelial cells of aggressive endometrial cancers. Specifically, in grade 3 endometrioid adenocarcinomas and uterine papillary serous carcinomas, ZEB1 could be expressed in the epithelial-derived carcinoma cells as well as in the stroma. In malignant mixed Müllerian tumors, the sarcomatous component always expresses ZEB1, and the carcinomatous component can also be positive. In summary, ZEB1 is normally regulated by both estrogen and progesterone receptors, but in uterine cancers, it is likely no longer under control of steroid hormone receptors and becomes aberrantly expressed in epithelial-derived tumor cells, supporting a role for ZEB1 in epithelial to mesenchymal transitions associated with aggressive tumors. (Cancer Res 2006; 66(7): 3893-902)

Introduction

The transcription factor ZEB1 (Zfhx1a, Zfhep, AREB6, oEF1) is up-regulated by estrogen in the chick oviduct and is responsible for estrogen-mediated regulation of the ovalbumin gene (1–3). We sought to investigate its regulation and expression in vivo in the uterus, where both the myometrial layer and the endometrium are responsive to estrogen and progesterone.


dEF1/ZEB1 LacZ homozygote mice develop to term, but do not survive postnatally. In addition to severe T cell deficiency of the thymus, dEF1 null mice exhibit skeletal defects of various lineages (17). We used heterozygote dEF1/ZEB1 LacZ mice to study ZEB1 expression in the mouse uterus. We have determined that ZEB1 is up-regulated by both estrogen and progesterone in the mouse myometrium and endometrial stroma. Additionally, we find that in human surgical resection specimens, ZEB1 protein expression is normally confined to the myometrium and endometrial stromal cells, with no expression in the glandular epithelium. However, ZEB1 is overexpressed in leiomyosarcomas, an aggressive smooth muscle–derived malignancy of the myometrium. In low-grade endometrioid cancers, it is overexpressed in tumor-associated stroma as compared with normal stroma. Furthermore, in grade 3 endometrioid adenocarcinomas, and other aggressive types of uterine cancers including uterine papillary serous carcinomas (UPSC) and malignant mixed Müllerian tumors (MMMT), ZEB1 can be aberrantly expressed in the epithelial-derived tumor cells.

We conclude that the abnormal expression of ZEB1 in a subpopulation of tumor cells is a marker of biologically more aggressive uterine malignancies.

Materials and Methods

Reagents. An expression vector for ZEB1 (pCS2MT-ZEB1) was kindly provided by Dr. Douglas C. Dean (Washington University, St. Louis, MO). A rabbit polyclonal anti-ZEB1 antibody directed against the homeodomain

in mammalian target tissues. Hormonal regulation or expression of ZEB1 has not previously been studied in mammalian target tissues.

ZEB1 contains two clusters of Kruppel-type zinc fingers, one at the NH2 terminus and the other at the COOH terminus, by which it binds E-box-like sequences (CACCTG) on DNA. Between the zinc finger clusters, ZEB1 contains a homeodomain that resembles members of the POU homeodomain family, thought to play a role in protein-protein interactions (5). ZEB1 can act as a transcriptional repressor or activator depending on several poorly understood conditions, including its expression levels, conformation, and the gene promoter on which it is acting (6, 7). In developing mesodermal and neural tissues, ZEB1 expression levels change dynamically during differentiation (8, 9). In skeletal muscle, ZEB1 may act as a repressor of differentiation (10–12), but it has not yet been studied in vascular or uterine smooth muscle. In lung and mammary gland tumor cells, ZEB1 has been implicated in epithelial to mesenchymal transition (EMT; refs. 13–15), a process associated with tumor metastasis (16). Because of previous indications that female steroid hormones regulate ZEB1 (2–4), we sought to investigate its regulation and expression in vivo in the uterus, where both the myometrial layer and the endometrium are responsive to estrogen and progesterone.

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region (amino acids 557-663) was used for immunohistochemistry. This region is 84% identical between the human and mouse and ZEB1/EF1 (8). This antibody works well on paraffin-embedded sections (8) and specifically detects ZEB1 and not ZEB2 (18).

Cell culture. Clonetics normal myometrial smooth muscle cells were obtained from Cambrex Bio Science (Copenhagen, Denmark). They are derived from a 49-year-old woman, have a finite life span in culture, and are maintained in SMGM-2 medium with growth factor supplements provided by Cambrex Bio Science. Normal myometrial smooth muscle cells were washed, counted, and plated at 2.5 million cells per 150 mm dish in phenol red–free medium supplemented with 5% dextan-coated charcoal–striped serum for 24 hours before treatment with 10 nmol/L estradiol or the synthetic progestin R5020 or both (E for 24 hours, followed by R5020 for 12 hours). RNA was isolated using Oligotex mRNA Maxi Kit (Qiagen, Valencia, CA).

Real-time quantitative PCR. The UCHSC Cancer Center Quantitative PCR Core Facility uses an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) for continuous measurement of the fluorescence spectra in 96 wells of a thermal cycler during PCR amplification. Forward and reverse primers (Invitrogen Life Sciences, Carlsbad, CA) and probes were designed following the recommendations of the TaqMan PCR chemistry and optimized using the Primer Express software (Applied Biosystems). A TaqMan probe specific for ZEB1 5’-labelled with 6-carboxyfluorescein (FAM) and 3′-labelled with 6-carboxy-tetramethylrhodamine (TAMRA) was purchased from Applied Biosystems. The primer and probe sequences used were hZEB1, Fwd-TCCATGCTTAAG AGCGCTAGCT; hZEB1, Rev-ACCGTAGTTGAGTAGGTGTATGCCA; hZEB1, probe-6-carboxy-aminem (TAMRA) was purchased from Applied Biosystems. A TaqMan probe specific for ZEB1 5′-labeled with 6-carboxyfluorescein-CCAATAAGCAAACGATTCTGATTCCCCAG-6-carboxy tetramethyl rhodamine. Amplification reactions and thermal cycling conditions were done according to the manufacturer’s recommendations. A standard curve was generated using the fluorescence data from the 10-fold serial dilutions of known quantities of control plasmid for human ZEB1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the amount of ZEB1 was normalized to the amount of GAPDH.

Animals and hormone treatments. C57BL/6 mice in which the lacZ reporter gene encoding β-galactosidase (β-gal) was inserted into exon 1 of the gene encoding βEF1/EF1-ZEB1 were provided by Dr. Yujiro Higashi (17). Animals were housed in the University of Colorado Health Sciences Center for Laboratory Animal Care.

Short-term steroid hormone injection. Wild-type C57BL/6 mice were ovariectomized at 5 to 6 weeks and rested for 2 weeks. A single s.c. injection of 17β-estradiol (1 μg) or 1 mg progesterone (Sigma, St. Louis, MO) or sesame oil, the vehicle control, was administered and mice were euthanized after 6 or 24 hours. Two mice were used for each treatment group. At necropsy, mice were anesthetized with Avertin and blood was collected from the retro-orbital sinus. After this, mice were killed with CO2 and uteri were excised. Estradiol and progesterone levels in sera were assayed using a RIA kit (Diagnostic Products Corporation, Los Angeles, CA) in duplicate for each mouse. The levels of estradiol and progesterone in the sera of the mice 6 hours after injections were 50 to 60 pg/mL and 20 to 30 ng/mL, respectively (data not shown). These are physiologic levels similar to those observed in late pregnancy in mice. By 24 hours after injection, the hormone levels had dropped to ~20 pg/mL of estradiol (comparable to diestrous) and 2 ng/mL of progesterone (similar to proestrus).

β-Galactosidase activity assay. Mice (three per time point) were euthanized at 10 weeks of age (virgin), days 5, 12, and 19 of pregnancy, days 1 and 9 of lactation, and at day 2 of involution. The uterus was prepared as detailed previously (19). Briefly, uteri were fixed in 2% paraformaldehyde, washed, immersed in X-Gal stain, washed, fixed in 4% paraformaldehyde and paraffin embedded. Cut sections were counterstained with Nuclear Fast Red, rinsed, dehydrated in a series of graded ethanol and xylene, mounted, and coverslipped with Permount.

Immunohistochemistry. Mouse tissues were fixed in 10% neutral buffered formalin for 24 hours then embedded in paraffin. Sections were cut at 4 μm and heat-immobilized at 60 °C for 60 minutes on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). After deparaffinization, antigens were retrieved using Citra Plus 10+ Retrieval Solution (BioGenex, San Ramon, CA) with a Biocare Medical Decloaker (Concord, CA) followed by 3% hydrogen peroxide for 5 minutes. After rinsing in PBS-T (0.1%), sections were blocked with 10% normal goat sera in PBS-T (0.1%), and were incubated with primary rabbit anti-ZEB1 (1:1,500) for 1 hour at room temperature. Rabbit IgG (Invitrogen) was used as an isotype-negative control. Following three washes in PBS-T (0.1%), sections were incubated with biotinylated goat anti-rabbit IgG (1:500; Dako-Cytomation, Carpinteria, CA) for 30 minutes. Following washing, sections were incubated with streptavidin-conjugated horseradish peroxidase (1:1,000; Dako-Cytomation) for 30 minutes, washed, and then incubated with 3,3′-diaminobenzidine (Dako-Cytomation) for 10 minutes, rinsed in water, counterstained with dilute hematoxylin, dehydrated, and coverslipped for bright-field microscopy.

Immunohistochemistry of human surgical resection specimens. Sections from archival paraffin-embedded blocks of normal uterine biopsies and uterine cancers were obtained via Institutional Review Board–approved protocols from the University of Colorado Health Sciences Center and The University of Texas M.D. Anderson Cancer Center. Immunohistochemistry was done as described above with the following exceptions: slides were stained using the Autostainer Universal Staining System (Dako-Cytomation) along with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for detection. ZEB1 antibody was used at an optimal dilution of 1:6,000, and TBS-T (0.05%) was used for all washes. Negative controls included substitution of the primary antibody with a subclass-matched concentration-matched rabbit immunoglobulin. ZEB1 stain intensity was scored on a scale of 0 (negative) to + (intense staining) and the proportion of positive cells was determined by review of one representative section from each case.

Dual immunofluorescence. Uteri from estrogen-treated mice were formalin fixed, paraffin embedded, and processed for antigen retrieval. Sections were blocked with 10% normal goat sera in PBS-T (0.1%) and primary rabbit anti-ZEB1 (1:1,000) was added overnight at room temperature. Rabbit IgG (Invitrogen) was used as an isotype-negative control. Following three washes in PBS-T (0.1%), biotinylated goat anti-rabbit IgG (1:500; Dako-Cytomation) was added and incubated for 1 hour. Following washing, rhodamine red-X-conjugated to streptavidin (1:200; Jackson Immunoresearch Laboratories Inc., West Grove, PA) was incubated on sections for 1 hour. Sections were washed again and blocked with 10% normal goat sera, drained, and then incubated with anti–estrogen receptor (ER) monoclonal antibody 1D5 (1:50; Dako-Cytomation) overnight at room temperature. Sections were washed again and incubated with Alexa 488 conjugated goat anti-mouse IgG (1:500; Invitrogen) for 1 hour at room temperature. Following washing, slides were rinsed in water and mounted with Biomeda M01 gel mount (Foster City, CA) and stored in the dark at 4 °C.

Results

ZEB1 in the mouse uterus. The spatiotemporal regulation of ZEB1 promoter activity was monitored in the normal uterus during postnatal development using βEF1/βEF1lacZ heterozygote mice, which are viable and reproduce (17). Mice were euthanized at 10 weeks of age, at days 5, 12 and 19 of pregnancy, at days 1 and 9 of lactation, and at day 2 of involution. Uterine horns were processed for β-gal activity staining (Fig. 1). In 10-week-old virgin mice, ZEB1-lacZ reporter activity was detected only in the myometrium (Fig. 1A). In addition, ZEB1 promoter activity was detected in smooth muscle cells of blood vessels (red arrows) associated with the uterus (example in Fig. 1B is from a pregnant, day 5, mouse). At day 5 of the pregnancy, the degree of myometrial staining increases and some stromal staining is evident (Fig. 1C and D). At pregnancy (day 12), continued LacZ expression is evident in the stroma (Fig. 1E), but during lactation, coincident with decreasing hormone levels, stromal staining recedes (Fig. 1F). These results show ZEB1 promoter activity in the myometrial and stromal compartments of the uterus and suggest that the promoter may be stimulated by pregnancy hormones.
We next asked whether promoter activity is associated with protein production in response to hormonal stimuli. To this end, we tested an antibody (rabbit anti-ZEB1) for specificity on immunoblots containing protein extracts from HeLa cells transfected with either an expression vector encoding the 180 kDa ZEB1 protein tagged with an 83 amino acid 6-myc epitope, or an empty expression vector (Fig. 2A). The antibody detected a protein of the appropriate size only in cells transfected with the ZEB1 expression vector, similar to previous results with COS cells (20). The same protein band was also detected with an anti-myc antibody (data not shown). This rabbit anti-ZEB1 antibody was then used for immunohistochemistry on sections of formalin-fixed, paraffin-embedded uteri taken from ovariectomized wild-type C57BL/6 mice rested for 2 weeks then treated with either vehicle control, estrogen, or progesterone for 6 or 24 hours (Fig. 2B). Up-regulation of ZEB1 protein in the myometrium (black arrows) and stroma (red arrows) is evident after 6 hours of hormone treatment and is more intense by 24 hours. The ZEB1 protein expression data differ somewhat from the promoter activity data using the heterozygous αEF1/ZEB1lacZ mice, in that immunohistochemistry shows more stromal staining. There are many possibilities for this difference as they are measuring two different things, the former detects ZEB1 protein expression, whereas the latter indicates active promoter activity. In addition, the X-gal staining is done on heterozygous αEF1/ZEB1lacZ mice, which have one allele encoding the LacZ gene and the other, ZEB1, so they should have, at most, half as much ZEB1 as wild-type mice (on which the immunohistochemistry was done). Indeed, in the heterozygous αEF1/ZEB1lacZ mice, the ZEB1 immunohistochemistry results in much weaker staining in both the stroma and myometrium than in wild-type mice (data not shown).

To determine if steroid hormone receptors are expressed in the same cells as ZEB1, we did dual immunofluorescence using the anti-ZEB1 antibody and the 1D5 anti-ER monoclonal antibody. This shows that ER and ZEB1 are nearly 100% colocalized in the nuclei of myometrial and stromal cells in the estrogen-treated uterus as indicated by orange or yellow cells (Fig. 2C). Estradiol-treated mice were used because ovariectomized mice poorly express both ER and ZEB1 (data not shown). PR, which are

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**Figure 1.** ZEB1 promoter activity is observed in the myometrium and stroma of the mouse uterus. X-Gal staining of uteri from ZEB1/αEF1LacZ female mice shows ZEB1 promoter activity in uteri from a 10-week-old (virgin) mouse (A), uterine-associated blood vessels in a pregnant day 5 mouse (B), pregnancy day 5 mice (C and D), pregnancy day 12 (E), and day 2 of lactation (F). Black arrows, stromal cells positive for X-gal staining; red arrows, blood vessels; bars, 100 μm.
ER-regulated proteins, are coexpressed with ER in the myometrium and stroma. Having determined that ZEB1 is up-regulated by estrogen and progesterone in the mouse uterus, we next asked if it is also hormonally regulated in human cells.

**ZEB1 in the normal human uterus.** We examined expression of the ZEB1 protein in the normal human uterus, postulating that it would be expressed in the endometrial stroma and myometrium as it was in the mouse, and that its expression levels might be affected by the menstrual cycle.

We did immunohistochemistry with ZEB1 antibody on eight endometrial biopsies, each from the proliferative and secretory phases of the menstrual cycle. Average expression scores for ZEB1 were calculated by multiplying the intensity (on a scale of 1-3) by the percentage of cells staining. In both the stroma and myometrium, ZEB1 protein expression is significantly higher in the secretory phase than in the proliferative phase (Fig. 3A). Representative pictures of ZEB1 immunostaining in the endometrium during the proliferative and secretory stages from Fig. 3A are shown (Fig. 3B). There is no ZEB1 staining in the normal endometrial epithelium.

Expression of ZEB1 transcripts was assayed by real-time quantitative reverse transcription-PCR in normal human myometrial smooth muscle cells treated with vehicle, 10 nmol/L of the synthetic progestin R5020 for 12 hours, 10 nmol/L estradiol for 12 hours, or estradiol for 24 hours with addition of R5020 for the last 12 hours. Primers were designed for human ZEB1 and GAPDH, and control plasmids contained ZEB1 and GAPDH cDNAs. The ZEB1 transcript was up-regulated by either R5020 (7.4-fold) or estradiol (8.3-fold). Estradiol pretreatment, intended to induce PR, followed by R5020, had little additional effect (Fig. 3C).
ZEB1 in uterine tumors. We compared normal myometrium to leiomyomas (benign tumors that arise from the myometrium) and to leiomyosarcomas (rare malignant smooth muscle tumors). ZEB1 was expressed in the normal human myometrium (2+ staining) in 70% to 80% of cells and there was no apparent difference between this and its expression in leiomyomas (Fig. 4A). However, we found more intense ZEB1 staining (3+ in 90% of nuclei) in 10 of 10 leiomyosarcomas, than in adjacent normal myometrium (2+ in 70-80% of cells; Fig. 4B). Expression of ZEB1 in the malignant areas was up-regulated compared with adjacent normal areas. In addition, the intensity of ZEB1 protein expression correlated with regions of nuclear atypia and in multinucleated tumor cells (red arrows, Fig. 4B).

Because myometrial-derived malignant tumors overexpressed ZEB1, we next assessed its expression in the normal endometrium and various malignancies derived from this tissue. We examined both low-grade and high-grade endometrioid adenocarcinomas and two other aggressive types of endometrial cancer, UPSCs and MMMTs. All of these tumors arise from endometrial epithelial cells; however, the MMMTs have both stromal (sarcomatous) and epithelial (carcinomatous) components in the tumors.

We did ZEB1 immunostaining on eight low-grade and seven high-grade endometrioid adenocarcinomas. We found that in low-grade tumors, ZEB1 expression is more intense in the tumor-associated stroma than in adjacent normal stroma (Fig. 5A-C). The average intensity of ZEB1 immunostaining in the stroma of all 15 endometrioid adenocarcinomas was 3.6 versus 2.3 in adjacent normal endometrial stroma. Figure 5A shows a tumor in the right corner, and adjacent normal tissue in the same optical field on the top left corner. Figure 5B and C show an additional sample with adjacent normal tissue (B) and tumor (C) shown in different fields. Red arrows indicate tumor-associated stroma and black arrows

Figure 3. ZEB1 regulation during the human menstrual cycle and by estrogen and progesterone in human myometrial cells. A, eight endometrial biopsies each from the proliferative and secretory stages of the menstrual cycle were immunostained with ZEB1 antibody. Average expression scores for ZEB1 are plotted on the y-axis and proliferative (white columns) and secretory (dark columns) phases were examined separately for the stroma and myometrium. Bars, SD. In both the stroma and the myometrium, ZEB1 protein expression is significantly higher in the secretory phase than in the proliferative phase. There is no ZEB1 staining in the normal endometrial epithelium. B, representative pictures of ZEB1 immunostaining in the endometrium during the proliferative and secretory stages from (A). Black arrows, myometrium; red arrows, stroma; bar, 100 μm. C, normal uterine smooth muscle cells were treated with ethanol vehicle or 10 nmol/L R5020 for 12 hours, or 10 nmol/L estradiol for 12 hours, or estradiol for 24 hours with the addition of R5020 for the last 12 hours in phenol red-free medium with 5% dextran-coated charcoal-stripped serum. cDNA synthesis reactions were done from 1 μg of RNA. Subsequent real-time analysis of the ZEB1 PCR product was done using the TaqMan system with primers designed for human ZEB1 and GAPDH. Control plasmids containing ZEB1 and GAPDH cDNAs were used for quantification.
represent adjacent normal stroma. Note that in the normal endometrium and the low-grade tumors (Fig. 5A–C), ZEB1 is not expressed in the epithelium (open arrows). We have started a more extensive examination of ZEB1 expression in endometrial cancers using tissue microarrays constructed in the Department of Pathology, University of Colorado Health Sciences Center. ZEB1 expression in tumor-associated stroma was statistically significantly higher in carcinomas than in hyperplasia or normal endometrium ($P = 0.0001$).\(^6\)

In contrast to the low-grade [Federation Internationale des Gynaecologistes et Obstetristes (FIGO) grades 1 and 2] endometrioid adenocarcinomas, some high-grade (FIGO grade 3, four out of seven) expressed ZEB1 in the epithelial-derived carcinoma cells (examples from two cases are shown in Fig. 5D and E). Thus, we observe an interesting trend of heightened ZEB1 expression, first in the stromal compartment, then, in the high-grade tumors, in the epithelium where it is not normally expressed.

UPSCs are another histologic type of aggressive endometrial cancer. Of 12 cases examined, 4 showed extensive ZEB1 staining (2–3+) in the carcinoma cells (Fig. 6A, a–d). Within the same tumors, poorly differentiated foci (Fig. 6A, a and c), had more ZEB1-positive cells than other areas (Fig. 6A, b and d).

Lastly, we examined ZEB1 expression in endometrial cancers known as MMMT, also called “carcinosarcomas.” These tumors have both sarcomatous and carcinomatous components. ZEB1 immunostaining of 29 MMMTs showed high levels of ZEB1 protein expression in the sarcomatous compartment (black arrows) of all the tumors (Fig. 6B, a–c). In 5 of the 29, ZEB1 was also present in the carcinomatous component (red arrows, Fig. 6B, a–c). When MMMTs metastasize, it is generally the carcinomatous component that is observed at distant sites. In two cases of MMMT metastases to the omentum, the carcinoma cells showed intense ZEB1 staining in the majority of cells (red arrows, Fig. 6B, d and e).

**Discussion**

The transcription factor ZEB1. In addition to roles in lymphopoiesis (21) and skeletal patterning (17), ZEB1 is likely involved in chondrogenesis (22, 23), neurogenesis, neural crest cell development (24), and tumorigenesis (13–15). Some of these roles seem to involve regulation of events at the crossroad between proliferation and differentiation (6, 24). It is known that ZEB1 contributes to maintaining the cell cycle because ZEB1 antisense RNA abrogates proliferation (24), apparently due to

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\(^6\) N.S. Spoelstra, J.K. Richer, and M. Singh, unpublished data.
regulation of the p73 gene in muscle and neuronal cells (6, 25). It is frequently observed that ZEB1 represses genes that are markers of terminal differentiation in skeletal muscle (10, 12) and represses α1-collagen and collagen type II genes in osteoblasts (22, 23) and vascular smooth muscle cells (26). ZEB1 has been suggested to function as a repressor of skeletal muscle differentiation based on the repression of myogenesis in cell lines in vitro (10–12); however, in vivo, it is highly expressed in actively differentiating myoblasts and myofibers (6) and there is no skeletal muscle defect in ZEB1-null embryos (19). ZEB1 has not previously been studied in uterine smooth muscle. ZEB1 is up-regulated by estrogen in the chick oviduct (1), where it regulates the estrogen-mediated induction of the ovalbumin gene (2). Our observation that ZEB1 is up-regulated by progesterone in human breast cancer cells (4) was the first suggestion that this transcription factor is regulated by steroid hormones in a mammalian tissue. One study includes a tissue blot demonstrating that ZEB1, but not ZEB2, mRNA is expressed in the human uterus (27).

We observe that both estrogen and progestins up-regulate ZEB1 in the stroma and myometrium. Progesterone is usually thought of as opposing estrogen-induced proliferation. Indeed, both estradiol and progesterone regulate mitosis in the epithelial and stromal cells in the uteri of mature cycling rodents. However, estradiol induces mitosis in both cell types, whereas progesterone inhibits it in the epithelium, but stimulates it in stromal cells (28, 29).

**ZEB1 in the normal mouse uterus.** Using ΔEF1/ZEB1LacZ heterozygote mice, we observe ZEB1/ΔEF1 promoter activity in the normal mouse uterus in the myometrium of virgin mice and in the stroma during pregnancy, a time when both estrogen and progesterone levels increase. ZEB1 is up-regulated in both the myometrium and stroma in vivo within 6 to 24 hours of progesterone or estrogen treatment, and it colocalizes with ER in smooth muscle cells in the myometrium and stroma. These studies clearly document sex steroid hormone regulation of this protein. We also observe ZEB1 promoter activity in vascular smooth muscle cells, which may shed light on the mechanisms by which steroid hormones influence the vasculature.

![Figure 5](image-url)
Figure 6. ZEB1 expression in tumor cells in MMT and UPSCs. **A**, in UPSCs, poorly differentiated regions (a and c) have higher ZEB1 expression than foci of papillary differentiation (b and d). Bottom, higher magnifications of (a) and (b). **B**, ZEB1 immunostaining in MMTs in the sarcomatous portion (black arrows) of the tumors and in the carcinomatous portion (red arrows, a-c). Negative control with isotype-matched IgG substituted for the ZEB1 primary antibody (inset). Two MMT metastases to the omentum (d and e) show intense ZEB1 staining (3+) in 90% of the carcinoma cells (red arrows). Bars, 100 μm.
ZEB1 in the normal human uterus. Endometrial biopsies from the proliferative and secretory stages of the menstrual cycle show that ZEB1 expression is significantly higher in both the stroma and the myometrium in the secretory phase (when estrogen and progesterone are present) than in the proliferative phase when estrogen is high, but progesterone levels are low. As in the mouse, ZEB1 protein is present in the stroma and myometrium, but not in the normal endometrial epithelium. In primary myometrial cells in culture, treatment with estrogen or progesterone increased expression of ZEB1 mRNA by >7-fold over no hormone vehicle control. The above studies show in mouse model systems and normal human cells and both in vitro and in vivo, that estradiol and progesterone affect expression levels of the ZEB1 gene and protein. However, there are no canonical estrogen or progesterone response elements 1,000 bp upstream of the translational start site and the regulation may be indirect because we previously observed that up-regulation of the ZEB1 transcript by progesterone was abolished by the use of cycloheximide to inhibit new protein synthesis (4).

ZEB1 in leiomyosarcomas. As it is in the mouse, ZEB1 is expressed in the normal human myometrium. We find that ZEB1 protein is overexpressed in leiomyosarcomas and the degree of nuclear atypia correlates with the degree of ZEB1 overexpression. ZEB1 may function as a repressor of differentiation in the myometrium, as it does in skeletal muscle, and its overexpression in leiomyosarcomas may lead to dedifferentiation. Leiomyosarcomas typically arise in postmenopausal women, but they do still often retain their steroid receptors (30, 31). Interestingly, overexpression of the steroid receptor coactivator SRC-3/AIB1 in transgenic mice leads to the development of leiomyosarcomas (32).

Relationship between steroid hormones and endometrial cancers. Endometrial cancer is the most common invasive neoplasm of the female genital tract and the fourth most frequently diagnosed cancer in the U.S. There is a strong association between the risk of endometrial cancer and estrogen exposure (reviewed in ref. 33). In general, it is thought that the type I endometrial cancers, specifically, the low grade endometrioid adenocarcinomas, are more associated with unopposed estrogen exposure and are more hormonally responsive than the type II endometrial cancers such as grade 3 endometrioid adenocarcinomas, MMMTs, and UPSCs (reviewed in ref. 33). The type I cancers tend to occur in younger perimenopausal women and tend to be low grade, whereas the type II cancers occur in postmenopausal women and tend to be more aggressive. However, both types of endometrial cancers often retain ER and PR. Also, a recent study of 39,451 patients diagnosed with breast cancer and treated with tamoxifen found that the overall risk of subsequent uterine cancer was substantially higher for MMMTs (considered a type II endometrial cancer) than for endometrioid adenocarcinomas (34). Among those who survived for 5 years or longer, there was an 8-fold relative risk for MMMTs and a 2.3-fold risk for endometrioid adenocarcinomas. In addition, the MMMT patients had a worse prognosis. This study, and others (34–36), indicate that hormonal exposure to the estrogenic properties of tamoxifen may have delayed effects particularly in these more rare, but aggressive type II uterine tumors. We find that in the normal uterus, ZEB1 protein expression levels are influenced by steroid hormones, and that its expression is confined to the myometrium and stroma, but not the epithelium. However, as discussed in detail below, in tumors, particularly in grade 3 endometrioid adenocarcinomas and other type II, aggressive tumors, ZEB1 can be aberrantly expressed in epithelial-derived carcinoma cells.

ZEB1 in endometrial cancers. In the normal human endometrium, ZEB1 is expressed in stromal cells, but not in glandular epithelial cells. In low-grade, endometrioid adenocarcinomas, ZEB1 protein remains confined to the stroma, but it is overexpressed in tumor-associated stroma as compared with normal stroma. There is increasing evidence that the microenvironment exerts major effects on epithelial-derived tumors, and that the stroma makes an important contribution to the process of tumor progression (37, 38).

In high-grade endometrioid adenocarcinomas, we find that ZEB1 expression is no longer confined to the stroma, but can be anomalously expressed in the malignant epithelium. Expression of ZEB1 in the carcinoma cells may relate to the loss of differentiation markers, increased proliferation, or EMT (13–15). EMT is still controversial as a potential mechanism for carcinoma progression. A major reason for this is the inability to track EMT in time and space in human tumors (16). The closest picture of what this process might look like during tumor progression is in MMMTs. MMMTs are the best-known type of carcinosarcoma. In these tumors, we observe that both the carcinomatous (epithelial-derived) and the sarcomatous (mesenchymal, usually stromal or muscle derived) components can express ZEB1, which may indicate that the carcinoma cells are undergoing EMT. Loss of E-cadherin expression is characteristic of epithelial cells undergoing EMT. E-cadherin is normally expressed in epithelial cells and is involved in maintaining polarity and cell-cell contact (39, 40). The loss of E-cadherin expression has been observed during tumor progression of most carcinomas (reviewed in refs. 39, 40). E-cadherin can be down-regulated in epithelial cells by transcription factors that bind E-box elements in its promoter such as Snail, Slug, E12/E47, and importantly, ZEB1/Snail1 (13–16, 39, 40). Specifically, ZEB1 has been shown to bind to E-boxes in the E-cadherin promoter in lung and breast cancer cells (13–15). We observe an interesting pattern of ZEB1 expression in MMMTs. ZEB1 is expressed in fewer epithelial cells when there are recognizable glandular structures as compared with poorly differentiated, metastatic disease in which ZEB1 is expressed in a high percentage of cells. Additionally, it is the carcinomatous portion of MMMTs that metastasizes, and in two metastatic MMMTs, nearly all of the carcinoma cells highly express ZEB1. In addition, grade 3 endometrioid adenocarcinomas and a UPSC metastasis also showed 3+ intensity ZEB1 staining in a high percentage of tumor cells. In summary, ZEB1 is up-regulated by steroid hormones in the normal uterus, but its expression is confined to the myometrium and to endometrial stromal cells, with no expression in glandular epithelium. In low-grade, less aggressive type 1 uterine cancers, tumor-associated stroma has elevated ZEB1 expression, but the tumor cells do not express ZEB1. In contrast, in aggressive type II endometrial cancers, including grade 3 endometrioid adenocarcinomas, not only the stroma, but also the epithelial-derived (carcinoma) cells can express this protein. We speculate that inappropriate expression of ZEB1 causes EMT, contributing to the metastatic potential of these aggressive tumors. The mechanism by which ZEB1 becomes aberrantly expressed in malignant endometrial epithelium remains unknown. Hormonal regulation of ZEB1 may also be aberrantly acquired in the malignant epithelium, and this is a possible explanation for the increase in cases of MMMTs with tamoxifen use.
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