Hormone control and expression of androgen receptor coregulator MAGE-11 in human endometrium during the window of receptivity to embryo implantation

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Introduction

The androgen receptor (AR) is a ligand-activated transcription factor of the male and female reproductive tracts whose activity is modulated by coregulator binding. We recently identified melanoma antigen gene protein-11 (MAGE-11) of the MAGEA gene family that functions as an AR coregulator by binding the AR N-terminal FXXLF motif. Here we report that MAGE-11 is expressed in a temporal fashion in endometrium of normally cycling women. Highest levels of MAGE-11 mRNA and protein occur in the mid-secretory stage, coincident with the window of uterine receptivity to embryo implantation. Studies in human endometrial cell lines together with the hormone profile of the menstrual cycle and pattern of estrogen receptor-α expression in cycling endometrium suggest the rise in MAGE-11 mRNA results from down-regulation by estradiol during the proliferative phase and up-regulation by cyclic AMP signaling in the early and mid-secretory stage. In agreement with its coregulatory function, MAGE-11 localizes with AR in glandular epithelial cell nuclei in the mid-secretory stage. The increase in AR protein in the mid-secretory endometrium without an increase in AR mRNA suggests MAGE-11 stabilizes AR in glandular epithelial cell nuclei. This was supported by expression studies at low androgen levels indicating AR stabilization by MAGE-11 dependent on the AR N-terminal transactivation domain. The results suggest that MAGE-11 functions as a coregulator that increases AR transcriptional activity during the establishment of uterine receptivity in the human female.

Keywords: androgen receptor; human endometrium; melanoma antigen gene protein 11 (MAGE-11); estrogen receptor; cyclic AMP

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Understanding the mechanisms of transcriptional regulation by AR is an area of active investigation. AR transcriptional activity depends on two critical domains, activation function 1 (AF1) in the N-terminal region and activation function 2 (AF2) in the ligand-binding domain. Both AF1 and AF2 serve as interaction sites for coregulator proteins that bridge to the transcriptional machinery (Heinlein and Chang, 2002). One AR coregulator identified recently is the X chromosome-linked melanoma antigen gene protein-11 (MAGE-11 or MAGEA-11) of the MAGEA gene family. MAGE-11 was identified as an AR interacting protein in a yeast two-hybrid screen of a human testis library using the AR N-terminal FXXLF motif as bait (Bai et al., 2005). MAGE-11 is one of the so-called cancer-testis antigens and is expressed in primates, but not in rats, mice or other mammals. MAGE-11 binds the AR FXXLF motif and increases androgen-dependent AR transcriptional activity (Bai et al., 2005). Binding of MAGE-11 to the AR FXXLF motif inhibits the androgen-dependent AR N- and C-terminal (NC) interaction between the AR FXXLF motif and AF2. Transcriptional activity derived from AF2 is established by competitive binding by AF2 of the AR FXXLF motif,
 SRC1/p160 coactivator LXXLL motifs and FXLLF motifs in putative AR coregulators (He et al., 2002; Hsu et al., 2003).

Our studies on MAGE-11 in human endometrium arose from observations that AR is required for female reproductive function (Shiina et al., 2006) and that MAGE-11 is expressed in tissues and cell lines of the human female reproductive tract in a manner that correlates with AR expression (Bai et al., 2005). Based on its ability to increase AR transcriptional activity through increased AR stabilization and SRC/p160 coactivator recruitment, we considered whether MAGE-11 provides an androgen signal amplification mechanism in the human female. In this report, we show that MAGE-11 is expressed in a temporal fashion in human endometrium during the menstrual cycle. Highest levels of MAGE-11 mRNA and protein coincide with the window of uterine receptivity to embryo implantation. MAGE-11 expression is regulated in human endometrial cell lines by steroids and second messengers consistent with the endometrial expression and hormone profile in the menstrual cycle.

Materials and Methods

Antibodies

MAGE-11 protein expression was determined using antibodies raised in rabbits against human MAGE-11 N-terminal peptides 9ITIQIFPTVRPADLTR108, 3DLPRQVQPW6RFDPSPR87 and 13SPASIKKKKRED86 (Ponco Rabbit Farm & Laboratory, Inc., Canadensis, PA). Each peptide is specific to MAGE-11 and is coded by a separate 5’ exon (Bai et al., 2005). MAGE-11 antibodies were purified by peptide affinity chromatography using antigen-coupled Affi-Gel 10 (Bio-Rad) (Bai et al., 2005). Antibodies were prepared from endometrial cell lines by steroids and second messengers consistent with the endometrial expression and hormone profile in the menstrual cycle.

Endometrial tissue sampling

Endometrial biopsies were obtained under approved IRB protocols with informed consent at different stages of the menstrual cycle from 21 healthy cycling women volunteers 18–35 years of age with 25–35 day intermenstrual intervals. Women were excluded who used hormone contraception or medications that alter reproductive hormone levels or had infertility or upper reproductive tract disease. Cycle day was determined by the first day of menstruation. Urinary LH was determined by a home test kit (Ovuquick One Step, Conception Technologies, San Diego, CA). Endometrial biopsies were obtained from proliferative, early, middle and late secretory stages and excluded for evidence of inflammation, hyperplasia or neoplasia. Study participants were randomized for endometrial sampling on a specific predetermined day after the onset of menstruation for proliferative phase samples, and a specific day after the urinary LH surge for post-ovulatory samples. Endometrium samples were collected by patient reported cycle day and number of days after the LH surge. Histological staging of hematoxylin and eosin stained fixed sections according to Noyes et al. (1988) served to confirm cycle stage, but no changes were made to cycle day or phase based on histological criteria. Samples for immunostaining were placed in 10% neutral buffered formalin in the clinic within 10 min of surgical removal. Samples for RNA extraction were flash frozen and stored at −80°C.

Immunostaining

Paraffin embedded serial sections (8 μm) of normal human endometrium were immunostained using deparaffinized fixed sections treated with 83% methanol and 5% H2O2 at room temperature to reduce endogenous peroxidase activity. Tissue sections were not treated further for rabbit polyclonal MAGE-11 antibody MagAb94–108 (9 μg/ml) and progesterone receptor (PRL) H-190 antibody (Santa Cruz sc-7208, 2 μg/ml). For MAGE-11 antibodies MagAb59–79 (4 μg/ml) and MagAb13–26 (4 μg/ml), sections were further treated with 0.05 mg/ml trypsin for 5 min at room temperature followed by washing in cold phosphate-buffered saline (PBS). For preadsorption studies, antibodies were pre-incubated with 0.1 or 0.2 mg/ml respective peptide antigens for 2 days at 4°C, centrifuged for 10 min at 4°C at 12.600 g and used under identical conditions as untreated antibody. For rabbit polyclonal AR antibody (Abcam ab3510, 0.38 μg/ml) and mouse monoclonal human estrogen receptor α (ERα) antibody (NovoCastra NCL-ER-6F11, 1:500 dilution), tissue sections were exposed to 0.01 M sodium citrate, pH 6.0 for 15 min in a microwave at high setting (Balaton et al., 1993). Sections were blocked with 2% normal goat serum, incubated overnight at 4°C in a humidified chamber with primary antibody, and blocked with 2% normal goat serum followed by a 1 h incubation at room temperature with biotinylated secondary antibody (Vector Labs). Slides were incubated with avidin DH-biotinylated horse-radish peroxidase–H complex (Vectorstain Standard ABC kit, Vector Labs) for 1 h at room temperature followed by immersion in 3,3-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co.) at 150 mg/200 ml 0.05 M Tris–HCl buffer containing 0.02% hydrogen peroxide for 10 min with constant stirring. Sections were exposed to osmium vapors and counterstained with 0.05% toluidine blue in 30% ethanol, dehydrated, cleared in xylene and mounted with Permount (Fisher). Photographs were taken using a SPOT-4 Megapixel Digital Camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Nikon ECLIPSE E600 microscope and prepared using SPOT image processing software.

RNA extraction, first-strand cDNA synthesis and quantitative real-time RT–PCR

Total RNA was extracted from frozen endometrial biopsies using RNeAqueous–4 PCR kit (Ambion) and from endometrial-epithelial cell line 1 (ECC-1) and Ishikawa cells using RNeasy Plus Mini Kit (Qiagen). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). For human endometrium biopsies, real-time PCR was performed on a Stratagene Mx3000 (Stratagene, La Jolla, CA) using Taqman chemistry and the delta delta Ct relative quantitation method using peptidylprolyl isomerase A (PIA, cyclophilin A) as a constitutive housekeeping control gene. PPIA expression was constant in endometrial biopsies taken across the menstrual cycle (S.L.Y., unpublished observations). Samples were analyzed in triplicate at the efficiency of the primer probe sets confirmed for each run using serial dilutions of a standardized sample. For ECC-1 and Ishikawa cell lines, real-time PCR was performed on a LightCycler using LightCycler TaqMan Master mix (Roche) in a final reaction volume of 20 μl.

PCR primers and fluorogenic probes included Hs009999994-m1 (PPIA), Hs00377815-m1 (MAGE-11), Hs00907244-m1 (AR) and Hs174860-m1 (ERα) from Assays-On-Demand (Applied Biosystems, Foster City, CA). PPIA primers amplify a 98 bp 317–414 nucleotide (nt) fragment coding for amino acid residues 102–133 in exon 4 (GenBank Y00052). MAGE-11 primers amplify a 63 bp 123–185 nt DNA fragment coding for amino acid residues 24–43 with the probe centered at 154 nt (GenBank AY747607.1) (Bai et al., 2005) overlap the exon 2 and 3 and 24 nt. AR primers amplify a 99 bp 2197–2295 nt DNA fragment coding for AR amino acid residues 612–644 with the probe centered at 2244 nt (Lubahn et al., 1988) overlapping the exon 3 and 4 junction. ERα primers amplify a 62 bp 1093–1154 nt DNA fragment corresponding to amino acid residues 245–264 at assay location 1124 nt (GenBank NM-000125) spanning the exon 3 and 4 boundary. β-glucuronidase (GusB) forward primer 5’-ggtgctggaggtgga-3’ and reverse primer 5’-tagctgccacacc-3’ amplify a 65 bp region coding for amino acid residues 120–140 and the probe 5’-gcacccctaatcagttg-3’ overlaps the exon 3 and 2 boundary. PCR reactions (20 μl) contained cDNA from 0.4 μg total RNA, 4 μl LightCycler TaqMan Master mix (Roche) and 0.5 μl 20× TaqMan Mix (Applied Biosystems) for AR and MAGE-11, and 0.5 μM primer and 0.2 μM probe for GusB. Thermal cycler conditions were one cycle at 95°C for 10 min followed by 55 cycles of 95°C for 15 s, 60°C for 25 s and 72°C for 1 s. A non-template RNase-free water control was included in each run. Four serial 10-fold dilutions of cDNA or plasmid DNA were amplified in duplicate to construct standard curves for each gene using LightCycler software. Sample mRNA levels were extrapolated based on standard curves and Ct values and normalized to GusB, which except for dihydrotestosterone (DHT), was constant under the test conditions and expressed as ratios of target gene to GusB. GusB is constitutively expressed without significant variation due to hormonal stimulation in multiple endometrial cell lines including ECC-1 and Ishikawa (S.L.Y., unpublished observations).
Immunoblot analysis

MAGE-11, ER\(\alpha\) and PR protein levels in ECC-1 (4 × 10^6/10 cm dish) and Ishi-kawa cells (4 × 10^6/10 cm dish) were determined by immunoblot. Cells were plated in medium without phenol red containing 5% charcoal stripped serum after culturing in the same media for 4 days. Cells were treated, washed and harvested in cold PBS containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml pepstatin A and 5 \(\mu\)g/ml aprotinin, and solubilized in buffer containing 1% Triton X-100, 0.15 M NaCl, 0.5 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.4 and protease inhibitors listed above. AR and MAGE-11 protein interactions were performed in COS cells (1.8 × 10^6 cells/10 cm dish) transfected using DEAE dextran with 2 \(\mu\)g wild-type or mutant pCMVhAR and 5 \(\mu\)g pCMV-FLAG-MAGE-11. Cells were washed, harvested in cold PBS and solubilized as above. Protein concentration was determined by BioRad assay using bovine serum albumin as standard. Extracts were separated on 10% acrylamide gels containing SDS and probed with MagAb94–108 immunoglobulin G (8 \(\mu\)g/ml at 4\(^\circ\)C), rabbit polyclonal AR32 (1 \(\mu\)g/ml), PR H-190 (Santa Cruz Biotechnology, 1:500 dilution), mouse monoclonal human ER\(\alpha\) antibody (NovoCastra NCL-ER-6F11, 1:150 dilution) and mouse monoclonal \(\beta\)-actin antibody (Abcam, 1:5000). COS cell expression of pSG5-MAGE-11, pSG5-PR-B, pSG5-PR-A and pCMVhER\(\alpha\) served as positive controls.

Transcription assays

Human endometrial Ishikawa cells were maintained in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 2 mM l-glutamine. Human endometrial ECC-1 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin, streptomycin and 2 mM l-glutamine. ECC-1 and Ishikawa cells (7.5 × 10^4 well of 12-well plates) were transfected using FuGENE-6 (Roche Applied Science) as described (Askew et al., 2007) with 0.1 \(\mu\)g PSA-Enh-Luc reporter vector, 2 ng pCMVhAR, pCMVhAR-FXXAA or 25 ng pCMVhAR1-660 and 50–250 ng pSG5-MAGE-11. After 24 h, cells were placed in serum-free medium with and without 1 nM DHT and assayed the next day for luciferase activity using a Lumistar Galaxy (BMG Labtech) multiwell plate luminometer.

Results

Menstrual cycle dependence of MAGE-11 protein in human endometrium

Timing of MAGE-11 expression relative to several steroid receptors was determined in serial sections of endometrial biopsies from normally cycling women at different stages of the menstrual cycle using MAGE-11 antibodies raised against three MAGE-11 N-terminal peptides and antibodies to AR, PR and ER\(\alpha\). We found similar MAGE-11 immunostaining in endometrial sections for each MAGE-11 anti-peptide antibody and loss of immunoreactivity after preadsorbing with the respective peptide immunogens (data not shown). The survey of MAGE-11 expression in endometrial sections was performed using MAGE-11 antibody MagAb94–108.

The majority of proliferative stage endometrial specimens lacked prominent immunostaining for MAGE-11 in the small symmetrical glands that characterize this stage, with some immunoreactivity evident in stromal cell nuclei (Fig. 1A). Immunostaining for AR and...
PR was similarly weak in glandular epithelial nuclei throughout the proliferative stage, and more prominent in stromal cell nuclei (Fig. 1B and C). In contrast, ERα immunostaining was intense in glandular epithelial and stromal cell nuclei throughout the proliferative phase (Fig. 1D).

Early secretory stage endometrium is characterized by elongating glands with prominent subnuclear vacuoles (Noyes et al., 1950, 1975; Murray et al., 2004). Immunostaining for MAGE-11 increased in glandular epithelial and stromal cell nuclei in the early secretory stage LH+5 and paralleled increased immunostaining for AR (Fig. 1E and F). PR was evident in the early secretory stage but ERα immunostaining declined (Fig. 1G and H). MAGE-11 and AR persisted in the mid-secretory stage LH+9, and ERα and PR were heterogeneous between the glands (Fig. 1I–L). By the late secretory stage LH+14 (cycle day 24), MAGE-11, AR, PR and ERα immunostaining declined (Fig. 1M–P).

The results suggest a parallel increase in MAGE-11 and AR during the early and mid-secretory endometrial glandular epithelium when ERα levels declined.

**MAGE-11 mRNA expression in normal cycling human endometrium**

Timing of MAGE-11 mRNA expression in the cycling endometrium was investigated by quantitative real-time PCR using RNA extracted from frozen biopsies obtained at different stages of the cycle. MAGE-11 mRNA levels measured in individual samples were low during the proliferative phase between menstrual cycle Days 5 and 10 (Fig. 2A). MAGE-11 mRNA levels increased after LH+1, were elevated between LH+5 and LH+10, and declined at LH+11. There was a 30–70-fold increase in MAGE-11 mRNA during the early and mid-secretory phase relative to the proliferative and late secretory stages (Fig. 2B). Timing of maximal MAGE-11 mRNA expression coincided with the window of receptivity to embryo implantation between LH+6 and LH+10 (Fig. 2E).

In contrast, AR mRNA levels were higher in individual biopsy samples from the proliferative and late secretory endometrium, and lower in the early and mid-secretory phase (Fig. 2C). However, these differences were not significant when patients were grouped (Fig. 2D) by the classical cycle stage shown in Fig. 2E. PR mRNA levels were relatively constant through the cycling endometrium (data not shown) and ERα mRNA declined during the early and mid-secretory stage as summarized in Fig. 3. The relative levels of MAGE-11 mRNA exceeded AR and ERα mRNA by 10–100-fold in the early and mid-secretory phase, whereas AR mRNA exceeded MAGE-11 mRNA by 2–20-fold in the proliferative and late secretory stages (Fig. 3). In untreated ECC-1 and Ishikawa human endometrial cell lines, AR exceeded MAGE-11 mRNA by 50–100-fold (∼100 MAGE-11 mRNA copies/μg total RNA, data not shown) indicative of the proliferative or late secretory endometrial cell stage.

The data suggest a reciprocal relationship between AR and MAGE-11 mRNA expression in human endometrium through the menstrual cycle, with increased MAGE-11 mRNA expression coincident with the window of receptivity to embryo implantation. The greater AR and MAGE-11 immunostaining during the early to mid-secretory stage raised the possibility that MAGE-11 stabilizes AR in the secretory endometrium.

**Hormone regulation of MAGE-11**

We investigated the hormone regulation of MAGE-11 in ECC-1 and Ishikawa human endometrial cell lines that express AR, ERα and PR (Tabibzadeh et al., 1990; Nishida et al., 1996; Lovely et al., 2000; Hanifi-Moghaddam et al., 2005; Mo et al., 2006). MAGE-11 mRNA levels increased in ECC-1 cells during 72 h of culture in phenol red-free, charcoal stripped serum medium in the absence of added hormone (Fig. 4A). The increase in MAGE-11 mRNA was blocked by 10 nM estradiol (E2) in a dose-dependent manner, with partial inhibition by 0.01 nM E2 (Fig. 4A and B). The inhibitory effect of E2 required >6 h (data not shown) and was ERα mediated since the ICI-182780 antagonist increased MAGE-11 mRNA levels in the absence and presence of E2 (Fig. 4B). The increase in MAGE-11 mRNA over time in cultures containing charcoal stripped serum appeared to result from depletion of estrogenic activity. This was supported by a ∼7-fold increase in MAGE-11 mRNA in ECC-1 cells cultured in serum-free medium (data not shown). In contrast, AR mRNA levels increased ∼3-fold in ECC-1 cells in response to 10 nM E2 (Fig. 4C). The increase in AR mRNA was detected at 1 nM E2 and was blocked by ICI-182780 (Fig. 4D).

MAGE-11 mRNA levels increased 8–12-fold in ECC-1 cells in response to 2 mM dibutyryl-cyclic AMP (cAMP) (Fig. 5A) and ∼4-fold by 50 μM forskolin, but were unchanged for up to 48 h after treatment with 100 units/ml human chorionic gonadotropin, 10 nM progesterone, 10 nM DHT or 10 ng/ml epithelial growth factor (EGF) (data not shown). The lack of response to progesterone was not due to absence of PR since pretreatment of ECC-1 cells with 10 nM E2 for 48 h increased PR-B levels as shown in Fig. 6A below, yet MAGE-11 mRNA was not increased by progesterone with or without pretreatment with E2 (data not shown). The cAMP induced increase in MAGE-11 mRNA in ECC-1 cells was inhibited by 10 nM E2 (Fig. 5B). AR mRNA levels decreased transiently between 0.5 and 6 h in ECC-1 cells in response to dibutyryl-cAMP (Fig. 5C) but were otherwise stable over 72 h (Fig. 5D).

Similar induction of MAGE-11 mRNA by dibutyryl-cAMP was seen in Ishikawa cells (Fig. 7). As in ECC-1 cells (data not shown), MAGE-11 mRNA levels increased in a dose-dependent manner in response to dibutyryl-cAMP (Fig. 7A), but the cAMP-dependent increase in MAGE-11 mRNA was inhibited to a lesser extent by 10 nM E2 in Ishikawa cells (Fig. 7A) than in ECC-1 cells (Fig. 5B). This may reflect lower ERα levels in Ishikawa cells compared with ECC-1 cells as shown in Fig. 6B below. The increase in AR mRNA by 10 nM E2 was inhibited by dibutyryl-cAMP in Ishikawa cells (Fig. 7B).

Hormone dependent changes in MAGE-11 protein were more difficult to assess. The level of the ~67 kDa MAGE-11 protein was lower in Ishikawa cells than in ECC-1 cells (Fig. 6C) and MAGE-11 protein levels declined in Ishikawa cells in response to 10 nM E2 (Fig. 6D). However, an increase in MAGE-11 protein by cAMP was not detected within the 48-h culture period (data not shown).

The data indicate cAMP-dependent up-regulation and E2-dependent down-regulation of MAGE-11 expression in the ECC-1 and Ishikawa endometrial cell lines. E2 had opposing effects on AR and MAGE-11 by increasing AR mRNA and reducing MAGE-11 mRNA.

**MAGE-11 increases AR transcriptional activity and stability**

Expression studies were performed to determine the effect of MAGE-11 on AR transactivation in the endometrial-epithelial cell lines. Transient expression of MAGE-11 increased the androgen-dependent transcriptional activity of full-length AR in ECC-1 and Ishikawa cells (Fig. 8A and B) and the constitutive activity of AR1-660, an AR N-terminal and DNA binding domain fragment (shown for Ishikawa cells, Fig. 8C). The MAGE-11-dependent increase in AR transcrip-tional activity in Ishikawa cells was reduced by the AR FXXAA mutation (Fig. 8B), providing further evidence that the AR N-terminal FXXLF motif is the primary interaction site for MAGE-11. Androgen independent AR activation increased slightly with the expression of MAGE-11 in Ishikawa cells (Fig. 8B) as observed in other cell lines.
The results support the coregulatory function of MAGE-11 in endometrial cells and suggest that MAGE-11 increases AR transcriptional activity dependent on the AR N-terminal FXXLF motif and transactivation domain.

We found that MAGE-11 can stabilize AR through mechanisms that require the AR N-terminal AF1 transactivation domain (amino acid residues 142–337) in a manner that complements the stabilizing effects of the AR N/C interaction in the presence of DHT. At a...
saturating DHT concentration (10 nM), AR was stabilized in an FXXLF motif manner (Fig. 9A and B, lanes 1–6), which reflects the effects of the AR N/C interaction (Kemppainen et al., 1992; Langley et al., 1995; He et al., 2000). AR stabilization by MAGE-11 in the absence or at low levels of DHT (Fig. 9B, lanes 1 and 2) was also largely dependent on the FXXLF motif (lanes 4 and 5), with less of an increase in the AR-FXAA mutant by MAGE-11 (Fig. 9B, lane 4). The AR stabilizing effect of MAGE-11 required, in addition, the AR N-terminal AF1 transactivation domain, but was not strictly linked to AR transcriptional potential. Transcriptionally inactive AR nuclear transport mutant AR-4KM (lanes 10–12) and DNA binding mutant AR-C576A (lanes 13–15) were stabilized by the AR N/C interaction in the presence of DHT, and by MAGE-11 in the absence and presence of DHT. However, transcriptionally inactive ARΔAF1, which deletes N-terminal residues 142–337, was stabilized by the N/C interaction, but only weakly by MAGE-11 (lanes 7–9). At 10 nM DHT, AR levels increased as a result of the N/C interaction, and MAGE-11 protein levels declined depending on the transcriptional status of AR. MAGE-11 levels declined in the presence of DHT-bound wild-type AR (Fig. 9, lanes 1–3) but not with transcriptionally inactive mutant ARΔAF1 (lanes 7–9), nuclear transport mutant AR-4KM (lanes 10–12) or the DNA binding mutant AR-C576A (lanes 13–15).

The results suggest that the AR N-terminal AF1 transactivation domain is required for AR stabilization by MAGE-11 and that MAGE-11 may increase AR transcriptional activity in the relatively low circulating androgen environment of the early and mid-secretory endometrium by stabilizing AR.

**Discussion**

MAGE-11 belongs to a 12 member MAGEA gene family encoded on a 3.5 Mb segment at Xq28 of the human X chromosome (Rogner et al., 1995). Before its identification as an AR coregulator, the function of MAGE-11 in the absence or at low levels of DHT (Fig. 9B, lanes 1 and 2) was also largely dependent on the FXXLF motif (lanes 4 and 5), with less of an increase in the AR-FXAA mutant by MAGE-11 (Fig. 9B, lane 4). The AR stabilizing effect of MAGE-11 required, in addition, the AR N-terminal AF1 transactivation domain, but was not strictly linked to AR transcriptional potential. Transcriptionally inactive AR nuclear transport mutant AR-4KM (lanes 10–12) and DNA binding mutant AR-C576A (lanes 13–15) were stabilized by the AR N/C interaction in the presence of DHT, and by MAGE-11 in the absence and presence of DHT. However, transcriptionally inactive ARΔAF1, which deletes N-terminal residues 142–337, was stabilized by the N/C interaction, but only weakly by MAGE-11 (lanes 7–9). At 10 nM DHT, AR levels increased as a result of the N/C interaction, and MAGE-11 protein levels declined depending on the transcriptional status of AR. MAGE-11 levels declined in the presence of DHT-bound wild-type AR (Fig. 9, lanes 1–3) but not with transcriptionally inactive mutant ARΔAF1 (lanes 7–9), nuclear transport mutant AR-4KM (lanes 10–12) or the DNA binding mutant AR-C576A (lanes 13–15).

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MAGE-11 was unknown. Androgen binding to AR initiates a sequence of events that involves AR stabilization by the N/C interaction (Kemppainen et al., 1992; Langley et al., 1995; He et al., 2000) and AR interaction with coregulatory proteins (He et al., 2001; Bai et al., 2005). MAGE-11 selectively activates AR by binding the AR N-terminal FXXLF motif sequence (Bai et al., 2005). By binding the AR FXXLF motif, MAGE-11 competitively inhibits the AR N/C interaction, because the same AR FXXLF motif that binds MAGE-11 also binds AF2 in the AR ligand-binding domain in the presence of bound androgen. Binding of MAGE-11 therefore interrupts the AR N/C interaction and increases coactivator recruitment by AF2. Thus, one mechanism for increased AR transcriptional activity by MAGE-11 is increased AR recruitment of the SRC/p160 coactivators SRC1, TIF2 and AIB1 (SRC3) (Bai et al., 2005).

These coactivators, along with p300 and pCAF, are expressed in human endometrium during the menstrual cycle and are known to increase AR transcriptional activity (Mertens et al., 2001; Gregory et al., 2002).

In the present report, we demonstrate that MAGE-11 is expressed in a temporal fashion in nuclei of the endometrial glandular epithelium of normally cycling women. Highest levels of MAGE-11 mRNA and protein occur between LH+5 through LH+10 of the menstrual cycle and coincide with the window of receptivity at LH+6 through LH+10 (Psychoyos, 1973; Lessey, 2000). Localization of MAGE-11 with AR in epithelial cell nuclei suggests that AR and MAGE-11 have a transcriptional role in preparing the uterus for implantation and pregnancy.

The original report of Noyes et al. (1950, 1975) provided a histological classification scheme for the developing human endometrium during the secretory (luteal) phase. Histological landmarks included epithelial mitoses, nuclear pseudostratification and subnuclear vacuoles. The staging scheme of Noyes et al. placed subnuclear vacuoles...
Methods indicated. Cells were extracted as described in the section Materials and Methods. In (C), Ishikawa cells were transfected with 25 ng pCMV5 empty vector (p5) or pCMVhAR1-660 (coding for the AR N-terminal and DNA binding domains) with and without 50, 100 or 250 ng pSG5-MAGE-11 as indicated. Luciferase activity expressed as average ± SEM. is representative of three independent experiments.

Figure 8: Increase in AR transcriptional activity by MAGE-11 in endometrial cells. ECC-1 (A) and Ishikawa cells (B) were transfected in 12-well plates with 0.1 µg PSA-Enh-Luc, 2 ng pCMVhAR or pCMVhAR-FXXAA (AF1FWNLNF27) with and without 100 ng pSG5-MAGE-11 per well using FuGENE 6 as described in the section Materials and Methods. In (C), Ishikawa cells were transfected with 25 ng pCMV5 empty vector (p5) or pCMVhAR1-660 (coding for the AR N-terminal and DNA binding domains) with and without 50, 100 or 250 ng pSG5-MAGE-11 as indicated. Luciferase activity expressed as average ± SEM. is representative of three independent experiments.

Figure 9: Modulation of AR and MAGE-11 protein levels. (A) Schematic of full-length human AR amino acid residues 1–919 including AR FXXLF motif 23FQNLF27, AF1 N-terminal activation domain, DNA binding domain (DBD), nuclear localization signal (NLS), ligand binding domain (LBD) and AF2. (B) Immunoblots of protein extracted from COS cells transfected with 2 µg wild-type pCMVhAR (WT) or pCMVhAR mutants AR-FXXAA with 23FQNLF27 changed to FQNAA (He et al., 2000), ARΔAF1 with the Δ142–337 deletion (Zhou et al., 1995), nuclear transport mutant 4KM with R617M, K618M, K632M and K633M mutations (Zhou et al., 1994) and DNA binding mutant C576A (Zhou et al., 1995) in the absence (upper panel) and presence (lower 2 panels) of 5 µg pCMV-Flag-MAGE-11. Cells were incubated for 24 h in 10% charcoal-stripped serum medium in the absence and presence of 2 and 10 nM DHT as indicated. Cells were extracted as described in the section Materials and Methods and total protein (10 µg/lane) separated in 10% acrylamide gels containing SDS. AR was detected using AR32 rabbit polyclonal antibody (1:100, upper two panels) and Flag-MAGE-11 with anti-Flag M2 mouse monoclonal antibody (Sigma, 1:2000, lower panel). The lower portion of the blot was probed with a mouse monoclonal β-actin antibody as a loading control.
endometrial stromal cells is promoted by prostaglandin-E2, LH and relaxin (Telmann et al., 1997), each of which increases adenyate cyclase activity and cAMP levels during the transition from proliferative to secretory stage in the human endometrium (Bergamini et al., 1985; Tanaka et al., 1993).

The delay in maximal MAGE-11 mRNA and protein expression until 5 days after the LH surge during the mid-luteal estrogen surge might be explained by the decline in ERα that occurs in the early to mid-secretory phase. Mid-luteal depletion of ERα would abrogate E2-induced suppression of MAGE-11 mRNA, allowing MAGE-11 levels to increase. ERα is down-regulated by progesterone in epithelial cells, suggesting that increasing progesterone during the mid-secretory phase suppresses ERα at a time when MAGE-11 increases and uterine receptivity is established (Fazleabas et al., 1999; Lessey et al., 2006). MAGE-11 expression appears to be coordinately regulated by the menstrual cycle dependent timing of hormone and receptor levels through the combined actions of cAMP, E2 and progesterone and the mid-luteal decline in ERα. Changing levels of MAGE-11 provide a mechanism to modulate AR transcriptional activity during endometrial maturation. Additional studies are needed to establish the mechanisms whereby AR and MAGE-11 influence endometrial receptivity and whether alterations in MAGE-11 expression have a role in polycystic ovarian syndrome and endometriosis.

Compared with the established roles for ERα and PR as transcriptional regulators in the cyclic function of the human endometrium (Lessey et al., 1988), relatively little is known about AR. The endometrium is influenced by androgens that are reported to circulate near constant low levels during the menstrual cycle (Jabbour et al., 2006). Evidence that AR signaling is required for embryo implantation derives from fertility defects in female AR knockout mice (Hu et al., 2004). In agreement with previous studies in the primate endometrium during the menstrual cycle (Fujimoto et al., 1994; Adesanya et al., 1999; Slayden et al., 2001; Apparao et al., 2002; Slayden and Brenner, 2004), we found that AR mRNA levels are up-regulated by estrogen in the human endometrial cell lines and only transiently reduced by cAMP. AR levels were reported higher in endometrial stromal cells compared with glandular epithelial cells during the proliferative phase, were persistent in stromal cells in the mid-secretory stage (LH+7 to LH+10), and present at lower levels in luminal and glandular epithelial cells late in the cycle (Lessey et al., 1988; Horne et al., 1992; Mertens et al., 1996, 2001; Slayden et al., 2001; Apparao et al., 2002; Burton et al., 2003; Narvekar et al., 2004; Villicivicció et al., 2006). The opposing actions of cAMP and E2 on AR and MAGE-11 appear to provide a closely linked regulatory mechanism for controlling AR transcriptional activity in the cycling endometrium. The actions of these signaling molecules occur within a context of inhibitory progesterone effects on estrogen and androgen signaling and the influence of growth factors and hormones in a dynamic interrelationship between the stroma and epithelium (Lessey, 2003; Jabbour et al., 2006).

We presented evidence that MAGE-11 increases AR levels in the absence and at low levels of androgenic contingent on the AR N-terminal AF1 transcriptional activation domain (residues 142-337) that lies outside the 23FQNLF27 AR FXXLF interaction site for MAGE-11. AR stabilization by MAGE-11 is also supported by the FXXLF motif and MAGE-11-dependent increase in AR half-life in the absence of androgen (Bai et al., 2005). The loss of ARΔAF1 stabilization by MAGE-11 was not linked to its lack of androgen-dependent transcriptional activity because other transcriptionally inactive AR mutants were stabilized by MAGE-11. Rather, it appears that the AF1 region is part of an extended AR N-terminal interaction region for MAGE-11. The inability of the AR-FXXAAMutant to completely eliminate the AR-dependent transcriptional effects of MAGE-11 provides further evidence that regions outside of the FXXLF motif contribute to the AR interaction with MAGE-11. Alternatively, there may be an AF1 interacting protein that facilitates AR stabilization by MAGE-11 in the absence or at low levels of androgen.

The menstrual cycle dependent expression of MAGE-11 could modulate AR levels and account for the increase in AR immunostaining during the early and mid-secretory endometrium when circulating androgen levels are low. The timing of MAGE-11 expression in the mid-secretory endometrium in response to hormone signals of the menstrual cycle may enhance AR transcriptional activity during the window of uterine receptivity to embryo implantation. However, the mechanisms whereby MAGE-11 controls AR transcriptional activity are only beginning to be fully understood. Recent studies have shown that EGF increases site-specific phosphorylation and ubiquitylation of MAGE-11 which modulates the interaction between AR and MAGE-11 (Bai and Wilson, 2008). EGF signaling may be an additional important control mechanism relevant to the human endometrium that modulates the AR and MAGE-11 interaction. Additional studies are needed in biopsies, endometrial cell lines and primary cell cultures, to establish the androgen-dependent transcriptional events important for developing and maintaining the window of receptivity to embryo implantation.

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