Published in final edited form as:
*Mol Hum Reprod*. 2008 February ; 14(2): 107–116. doi:10.1093/molehr/gam080.

# 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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## Abstract

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 F*XX*LF 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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## Introduction

The requirement for androgen receptor (AR) mediated gene regulation in male sex development is demonstrated by individuals with the androgen insensitivity syndrome. In this X chromosome-linked disorder, 46XY genetic males with single missense mutations in the AR gene are born with ambiguous or completely female external genitalia. However, identical loss-of-function mutations have relatively little phenotypic effect in 46XX carrier females (Quigley *et al.*, 1995) due in part to the double allele status of the AR gene and to random and partial inactivation of the X chromosome. Female mice homozygous for AR inactivating mutations have reproductive abnormalities that implicate a role for AR in fertility (Shiina *et al*., 2006). Reduced fertility in homozygous female AR knockout mice is associated with prolonged estrous cycles, fewer oocytes after super-ovulation, follicular atresia and diminished endometrial growth (Hu *et al*., 2004). The AR knockout mouse demonstrates that AR plays an important role in normal female reproductive physiology, but the mechanisms underlying these functions remain poorly understood.

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 inter-acting protein in a yeast two-hybrid screen of a human testis library using the AR N-terminal F*XX*LF 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 F*XX*LF motif and increases androgen-dependent AR transcriptional activity (Bai *et al*., 2005). Binding of MAGE-11 to the AR F*XX*LF motif inhibits the androgen-dependent AR N- and C-terminal (N/C) interaction between the AR F*XX*LF motif and AF2. Transcriptional activity derived from AF2 is established by competitive binding by AF2 of the AR F*XX*LF motif, SRC/p160 coactivator L*XX*LL motifs and F*XX*LF 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 $^{94}$ITQIFPTVRPADLTR$^{108}$, $^{59}$DLPRVQVFREQANLEDRSPRR$^{79}$ and $^{13}$SPASIKRKKKREDS$^{26}$ (Pocono 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 anti-bodies were purified by peptide affinity chromatography using antigen-coupled Affi-Gel 10 (Bio-Rad) (Bai *et al*., 2005). Antigens were coupled in 0.2 M ethanolamine, pH 8.0, and antibodies were eluted in 0.1 M glycine, pH 3.0, neutralized with 0.1 volume of 1 M Tris–HCl, pH 8.0 (He *et al*., 2002) and amended to 0.05 M NaCl and 5% glycerol. Antibody specificity was verified by preadsorption and immunoblot analysis of human MAGE-11 (pSG5-MAGE-11) expressed in COS cells which migrates on SDS polyacrylamide gels as 67±3 kDa.

## 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 classified 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*. (1950, 1975) 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% $H_2O_2$ 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 (PR) H-190 anti-body (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 (Vectastain 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 MTris–HCl buffer containing 0.002% 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 RNAqueous-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 (PPIA, 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 and 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 Hs99999904-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) overlapping the exon 2 and 3 junction. 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′-tggtgctgaggattggca-3′ and reverse primer 5′-tagcgtgtcgaccccattc-3′ amplify a 65 bp region coding for amino acid residues 120–140 and the probe 5′-tgcccattcctatgccatcgtgtg-3′ overlaps the exon 2 and 3 boundary.

PCR reactions (20 μl) contained cDNA from 0.4 μg total RNA, 4 μl Light- Cycler 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 nontemplate 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, ERα and PR protein levels in ECC-1 ($2 \times 10^6$/6 cm dish) and Ishikawa cells ($4 \times 10^6$/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 μg/ml leupeptin, 5 μg/ml pepstatin A and 5 μg/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 ($1.8 \times 10^6$ cells/10 cm dish) transfected using DEAE dextran with 2 μg wild-type or mutant pCMVhAR and 5 μg 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 (8μg/ml at 4°C), rabbit polyclonal AR32 (1 μg/ml), PR H-190 (Santa Cruz Biotechnology, 1:500 dilution), mouse monoclonal human ERα antibody (NovoCastra NCL-ER-6F11, 1:150 dilution) and mouse monoclonal β-actin antibody (Abcam, 1:5000). COS cell expression of pSG5-MAGE-11, pSG5-PR-B, pSG5-PR-A and pCMVhERα 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 ($7.5 \times 10^4$/ well of 12-well plates) were transfected using FuGENE-6 (Roche Applied Science) as described (Askew *et al*., 2007) with 0.1 μg PSA-Enh-Luc reporter vector, 2 ng pCMVhAR, pCMVhAR-F*XX*AA 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 ERα. 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 ($E_2$) in a dose-dependent manner, with partial inhibition by 0.01 nM $E_2$ (Fig. 4A and B). The inhibitory effect of $E_2$ 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 $E_2$ (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 $E_2$ (Fig. 4C). The increase in AR mRNA was detected at 1 nM $E_2$ 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 epidermal 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 $E_2$ 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 $E_2$ (data not shown). The cAMP

induced increase in MAGE-11 mRNA in ECC-1 cells was inhibited by 10 nM $E_2$ (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 $E_2$ 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 $E_2$ 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 $E_2$ (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 $E_2$-dependent down-regulation of MAGE-11 expression in the ECC-1 and Ishikawa endometrial cell lines. $E_2$ 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 transcriptional activity in Ishikawa cells was reduced by the AR F*XX*AA mutation (Fig. 8B), providing further evidence that the AR N-terminal F*XX*LF 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 (Bai *et al.*, 2005). 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 F*XX*LF 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 AR F*XX*LF ($^{23}$FQNLF$^{27}$) 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 F*XX*LF motif (lanes 4 and 5), with less of an increase in the AR-F*XX*AA 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 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 F*XX*LF motif sequence $^{23}$FQNLF$^{27}$ (Bai *et al*., 2005). By binding the AR F*XX*LF motif, MAGE-11 competitively inhibits the AR N/C interaction, because the same AR F*XX*LF 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 woman. 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 between LH+2 and LH+4. In agreement with a recent report of normally cycling women (Murray *et al*., 2004), we observed persistence of subnuclear vacuoles into endometrial stage LH+5 and LH+6 and later into the mid-secretory phase. With these latter criteria, the temporal alignment between MAGE-11 mRNA expression and the window of receptivity to embryo implantation suggest that MAGE-11 may serve as a new biomarker for human endometrial staging.

Our studies in human endometrial cell lines demonstrate MAGE-11 expression is inhibited by estrogen. The importance of ERα-mediated down-regulation of genes in establishing the timing of receptivity to implantation (Lessey *et al*., 1988) is supported by studies on αvβ3, a proposed marker of receptivity whose expression is inhibited by $E_2$ (Somkuti *et al*., 1997). We observed a surprising time-dependent increase in MAGE-11 mRNA in endometrial cell cultures in phenol red-free, charcoal stripped serum, an increase that was blocked by $E_2$. The increase in MAGE-11 mRNA was more evident in ECC-1 than Ishikawa cells and appeared to reflect a time-dependent depletion of estrogenic activity from phenol red-free, charcoal stripped serum

containing medium. The concept that basal culture conditions exert an estrogenic effect on MAGE-11 expression was supported by the increase in MAGE-11 mRNA expression after treatment with the ERα antagonist, ICI-182780. Differences in sensitivity to $E_2$ inhibition of MAGE-11 expression between the endometrial cell lines correlated with ERα levels even though estrogen increased AR mRNA levels in both cell lines. In agreement with these findings, the concentration of $E_2$ required to inhibit MAGE-11 expression was ~100-fold less than the concentration required to increase AR expression. There may also be differences in autonomous second messenger signaling between the endometrial cell lines because cAMP reversed the inhibitory effect of $E_2$ to a greater extent in Ishikawa cells than in ECC-1 cells.

Timing of the post-ovulatory increase in MAGE-11 mRNA and protein in endometrial biopsies and the increase in MAGE-11 mRNA in response to dibutyryl-cAMP in endometrial cell lines raise the possibility that LH secreted by the pituitary acts on the endometrium to increase MAGE-11. Indeed, studies suggest direct effects of LH on the uterus, independent of the ovary, that help to prepare the endometrium for implantation (Tesarik *et al*., 2003). Progesterone also acts directly on endometrial epithelial cells to increase gene expression during the secretory phase and indirectly through stromal cells by increasing expression of paracrine factors (Lessey, 2003) such as calcitonin, a proposed marker of uterine receptivity that increases cAMP production in Ishikawa cells (Li *et al*., 2006). The effects of cAMP are enhanced by progesterone in stromal cells (Tang *et al*., 1993) and activin A is a component of the cAMP signaling pathway (Tierney and Giudice, 2004). Differentiation of human endometrial stromal cells is promoted by prostaglandin-E2, LH and relaxin (Telgmann *et al*., 1997), each of which increases adenylate 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 $E_2$-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, $E_2$ and progesterone and the mid-luteal decline in ERα. Changing levels of MAGE-11 provides 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; Horie *et al*., 1992; Mertens *et al*., 1996, 2001; Slayden *et al*.,

2001; Apparao *et al*., 2002; Burton *et al*., 2003; Narvekar *et al*., 2004; Villavicencio *et al*., 2006). The opposing actions of cAMP and $E_2$ 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 inter-relationship 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 androgen contingent on the AR N-terminal AF1 transcriptional activation domain (residues 142–337) that lies outside the $^{23}$FQNLF$^{27}$ AR F*XX*LF interaction site for MAGE-11. AR stabilization by MAGE-11 is also supported by the F*XX*LF 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-F*XX*AA mutant to completely eliminate the AR-dependent transcriptional effects of MAGE-11 provides further evidence that regions outside of the F*XX*LF 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 ubiquitinylation 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.

## Acknowledgements

We thank K. Michelle Cobb, Brian J. Kennerley, John T. Minges and Andrew T. Hnat for excellent technical assistance, and Peter Petrusz, Director of the Molecular Histology Core of the University of North Carolina at Chapel Hill Laboratories for Reproductive Biology, for advice on immunohistochemistry.

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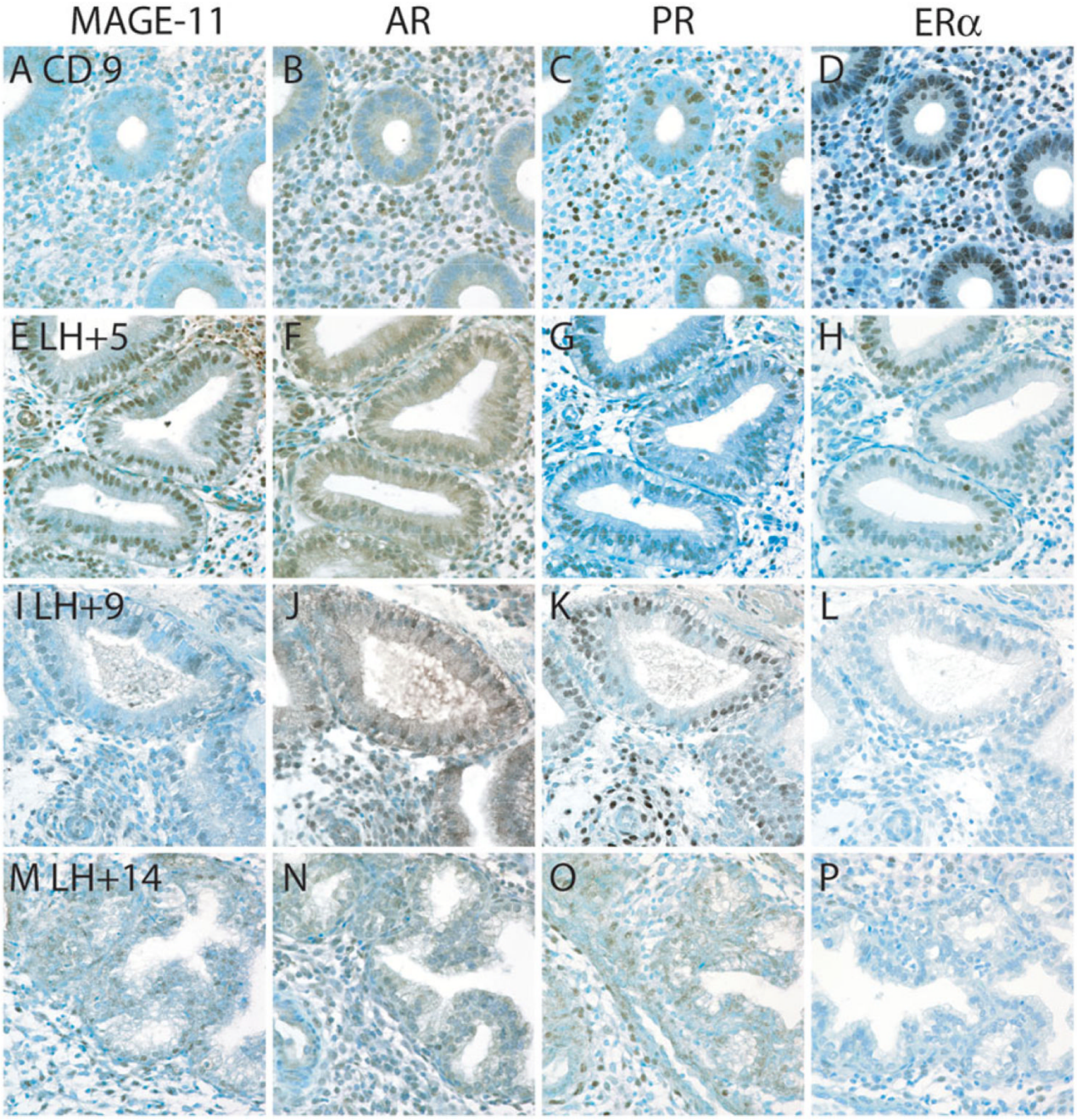


**Figure 1. Menstrual cycle stage dependence of MAGE-11, AR, PR and ERα immunostaining in human endometrium**

Paraffin-fixed sections of normal human endometrium were immunostained as described in the section Materials and Methods using antibodies against MAGE-11 (MagAb94–108, 9 μg/ml, **A**, **E**, **I** and**M**), AR (Abcam ab3510, 0.38 μg/ml, **B**, **F**, **J** and **N**), PR (Santa Cruz H-190, 2 μg/ml, **C**, **G**, **K** and **O**) and ERα (NovoCastra NCL-ER-6F11, 1:500 dilution, **D**, **H**, **L** and **P**). Representative sections are shown for proliferative (patient N003, cycle day 9 (CD 9), A–D), early secretory LH+5 (patient M135, CD 15, E–H), mid-secretory LH+9 (patient M122, CD 27, I–L) and late secretory LH+14 (patient M087, CD 24, M–P). Brown reaction product

represents positive immune reactivity against toluidine blue counterstain. Original magnification 60X

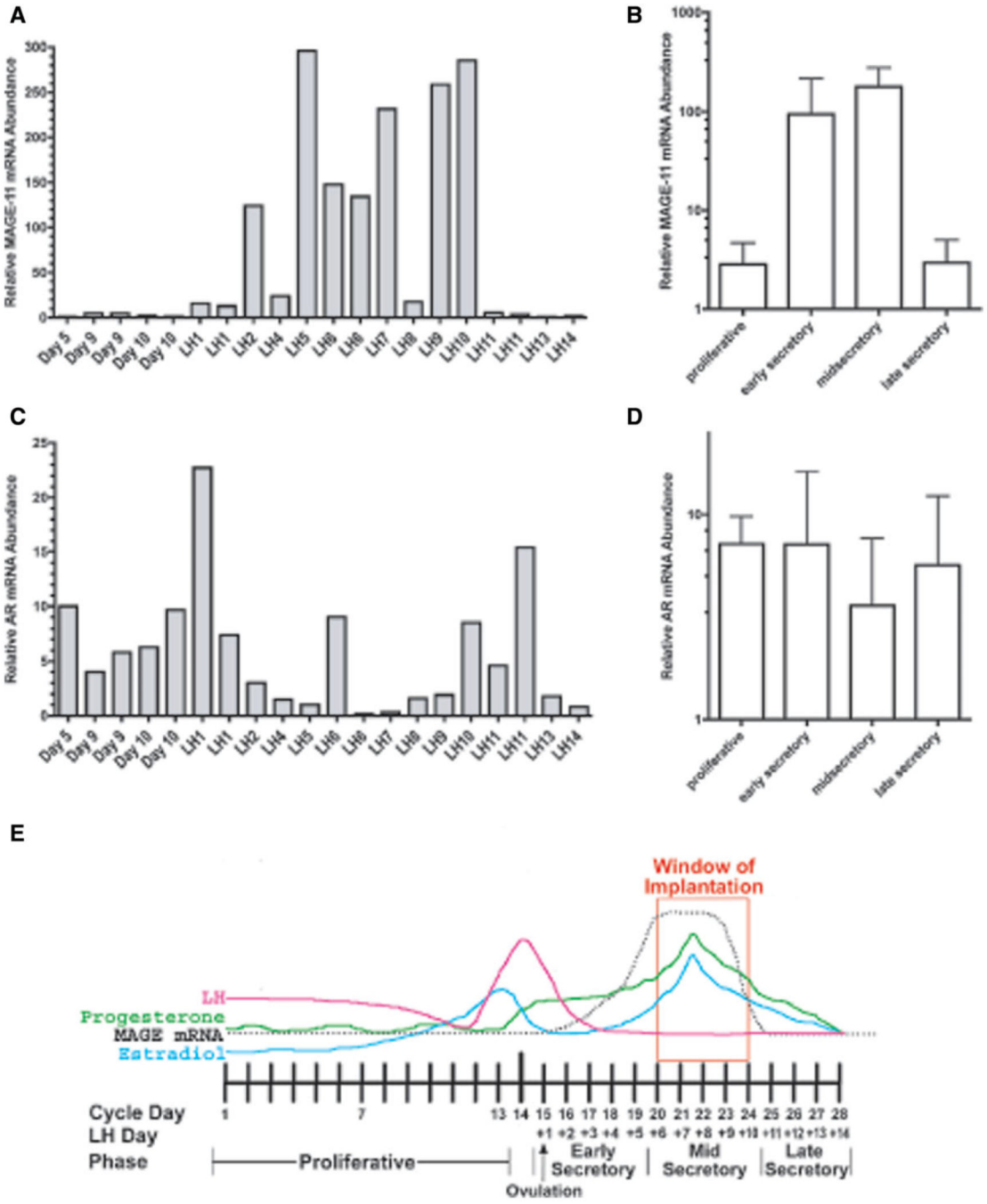


**Figure 2. MAGE-11 and AR mRNA levels in normal human endometrial biopsies through the menstrual cycle**

(**A**) Relative MAGE-11 mRNA levels in individual subjects by menstrual cycle day (CD) or day following the LH surge were assayed using total RNA from frozen human endometrium samples of normal cycling women. cDNA was prepared and amplified by real-time PCR as described in Materials and Methods. Normalized values are shown relative to LH+14. (**B**) Log scale plot of average relative MAGE-11 mRNA levels by menstrual cycle stage assayed as described in the section Materials and Methods and expressed as mean ± SD for proliferative phase (CD 1–14) and early (LH+1 to LH+5), mid (LH+6 to LH+10) and late secretory stage (LH+11 to LH+14). (**C**) Relative endometrial AR mRNA levels in individual subjects by

menstrual CD or days following the LH surge. Normalized values of AR mRNA are shown relative to LH+14. (**D**) Log scale plot of average relative AR mRNA levels by menstrual cycle stage assayed as described in the section Materials and Methods and (B) above. (**E**) Schematic of hormone profiles for LH (magenta), 17β-$E_2$ (cyan), progesterone (green), and MAGE-11 mRNA levels (black dotted line) based on the 28 day human menstrual cycle relative to the window of endometrial receptivity to embryo implantation at LH+6 through LH+10 (orange box), CD and day following the LH surge. Proliferative stage is CD 1–13, LH surge at CD 14, ovulation at LH+1, early secretory LH+1 to LH+5 (CD 15–19), mid-secretory LH+6 to LH +10 (CD 20–24) and late secretory LH+11 to LH+14 (CD 25–28)

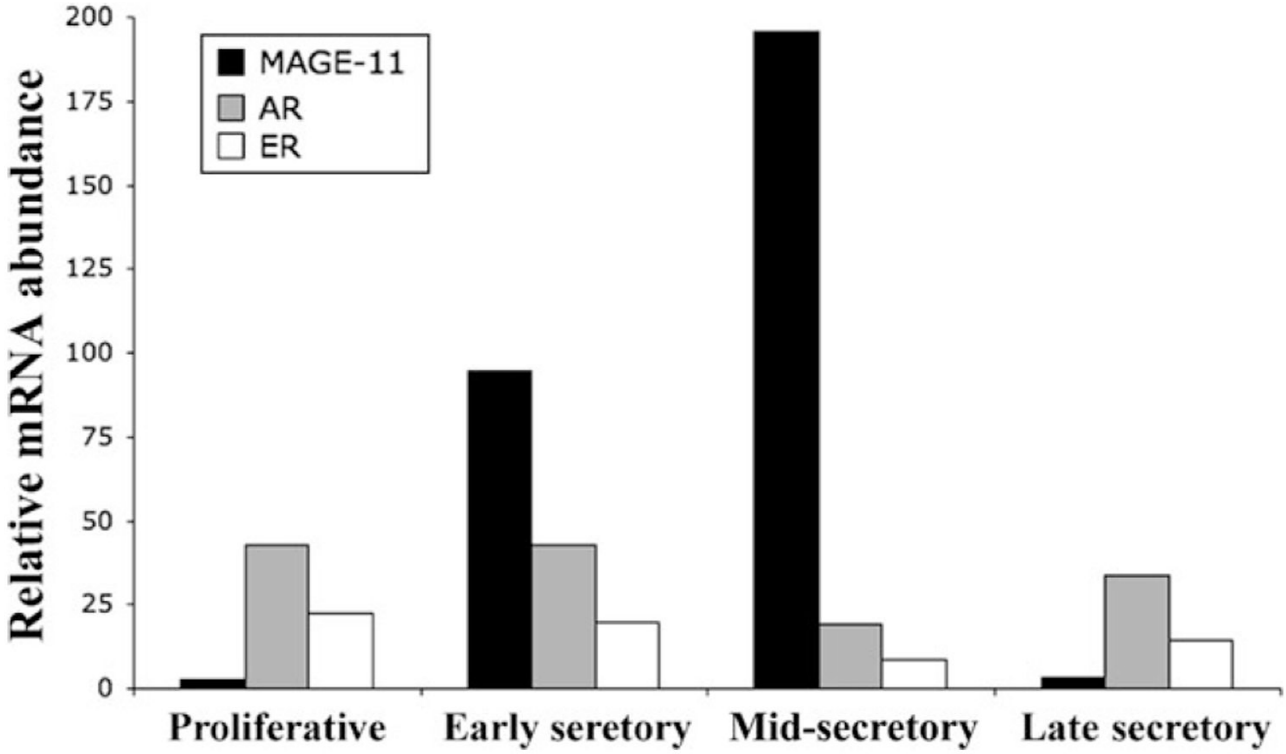


**Figure 3. Linear plot of stage-dependent changes in MAGE-11, AR and ERα mRNA in normal human endometrium through the menstrual cycle**

MAGE-11 (black), AR (gray) and ERα mRNA (white) were assayed as described in the section Materials and Methods. Relative mean mRNA levels in different stages of the cycle were not indicative of absolute levels but illustrate greater AR than MAGE-11 mRNA levels in the proliferative and late secretory phase and greater MAGE-11 than AR mRNA in the early and mid-secretory stages

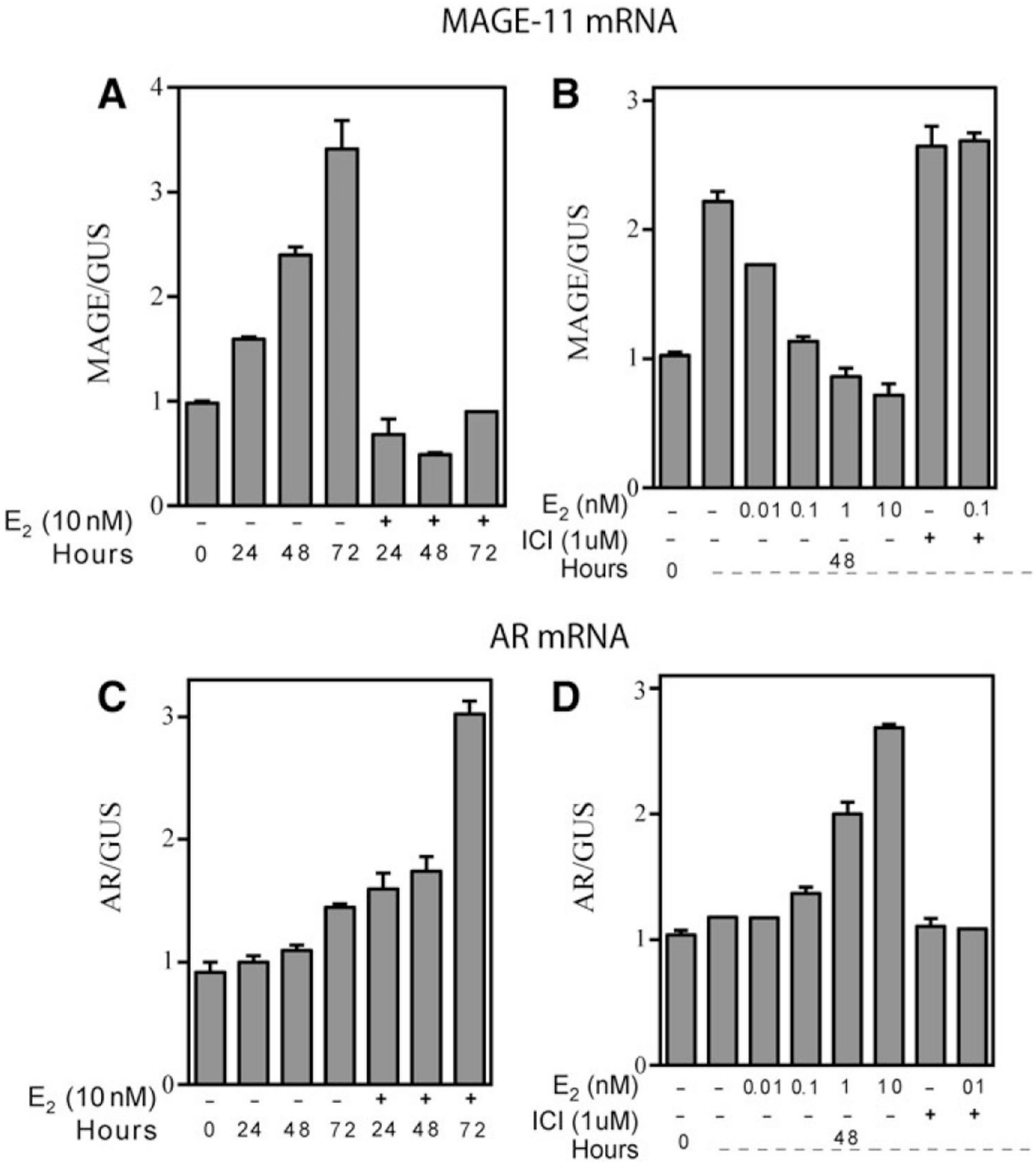


**Figure 4. Estrogen regulation of MAGE-11 and AR mRNA in ECC-1 cells**

ECC-1 cells were cultured and plated in phenol red free, 5% charcoal stripped serum medium and the next day harvested (0 time) or the medium replaced with or without 10 nM $E_2$ and harvested 24, 48 and 72 h later (**A** and **C**). Dose response studies were performed at increasing $E_2$ concentrations or 1 μM ICI-182780 with and without 0.1 nM $E_2$ as indicated and harvested 48 h later (**B** and **D**). Shown are relative levels of MAGE-11 (A and B) and AR (C and D) mRNA determined from total RNA by real-time PCR as described in the section Materials and Methods from duplicates of two 10 cm dish cultures extrapolated from standard curves and threshold cycle Ct values and expressed as ratios of target gene to control gene β-glucuronidase (GusB) ± SD

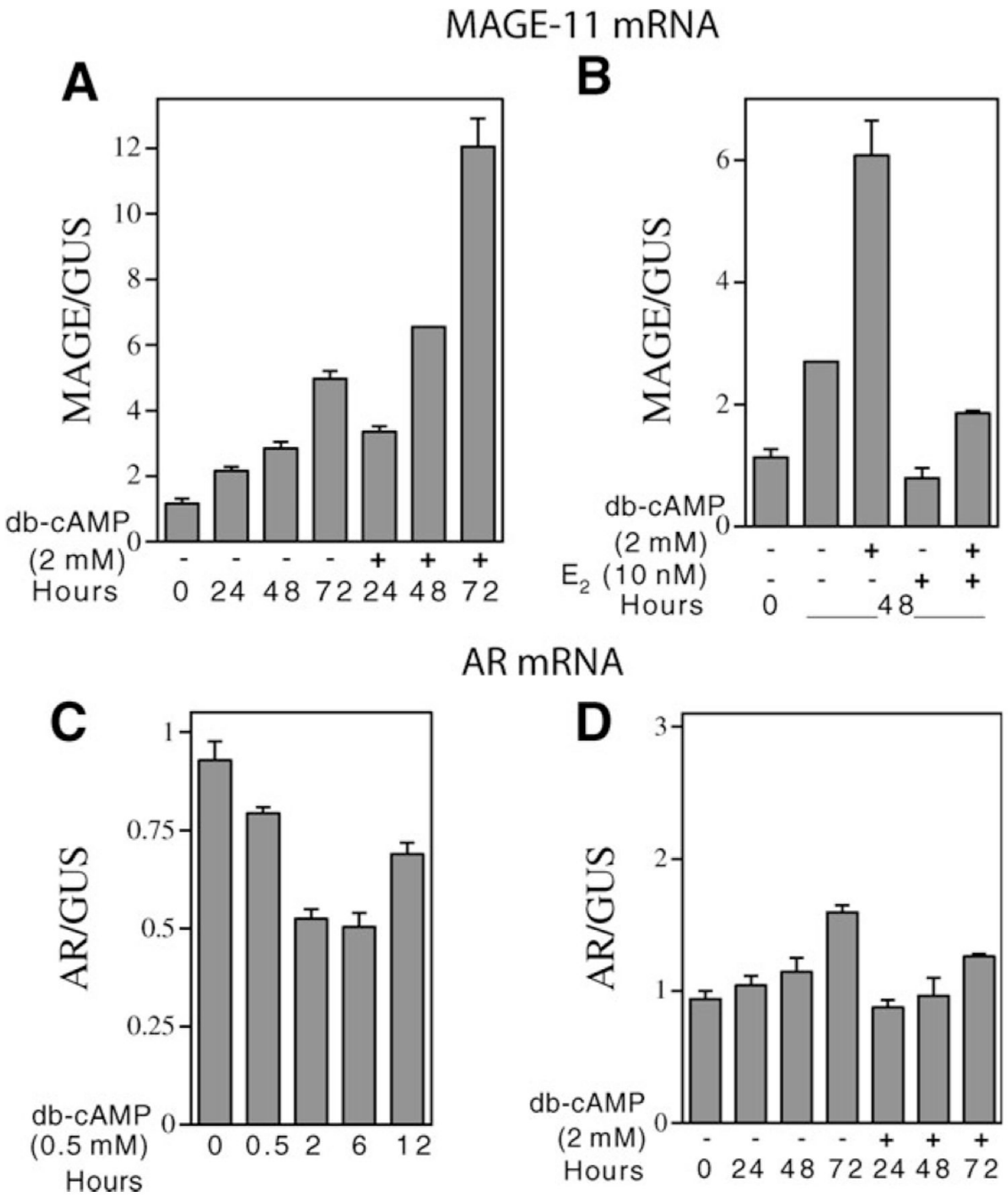


**Figure 5. cAMP regulation of MAGE-11 and AR mRNA in ECC1 cells**

ECC-1 cells were cultured, plated and treated in phenol red free, 5% charcoal stripped serum medium with and without 2 mM dibutyryl-cAMP (**A** and **D**), 2 mM dibutyryl-cAMP with and without 10 nM $E_2$ (**B**) and 0.5 mM dibutyryl-cAMP (**C**) for the times indicated. Extracted RNA was analyzed as described in the section Materials and Methods. Shown are relative levels of MAGE-11 (**A** and **B**) and AR (**C** and **D**) mRNA from duplicates of two 10 cm dish cultures extrapolated from standard curves and threshold cycle Ct values expressed as ratios of target gene to control gene β-glucuronidase (GusB) ± SD

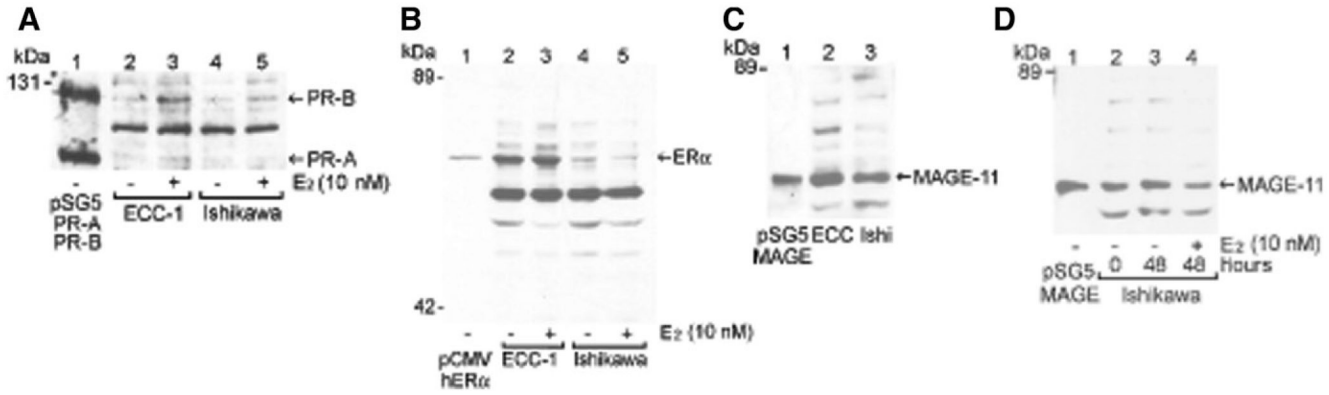


**Figure 6. Immunoblots of PR, ERα and MAGE-11 in ECC-1 and Ishikawa cells**

(**A**) For PR, ECC-1 (lanes 2 and 3) and Ishikawa cells (lanes 4 and 5) were treated with and without 10 nM $E_2$ for 48 h as indicated and protein (50 μg/lane) probed using PR H-190 antibody (Santa Cruz Biotechnology, 1:500 dilution). Human PR-B and PR-A were expressed together in COS cells from pSG5hPR-B and pSG5hPR-A and analyzed by immunoblot as controls (lane 1, 2 μg total protein). Protein extracts were separated on 10% acrylamide gels containing SDS calibrated using Kaleidoscope prestained molecular weight markers (BioRad) indicated on the left. (**B**) For ERα, ECC-1 (lanes 2 and 3) and Ishikawa cells (lanes 4 and 5) were treated with and without 10 nM $E_2$ for 24 h as indicated and protein (80 μg/lane) probed using a mouse monoclonal human ERα antibody (NovoCastra NCL-ER-6F11, 1:150 dilution). Human ERα was expressed in COS cells from pCMVhERα and included on the immunoblot (lane 1, 1 μg total protein) as a control. (**C**) ECC-1 (lane 2) and Ishikawa cells (lane 3) were untreated and protein extracts (50 μg/lane) were probed on immunoblots using MagAb94–108 immunoglobulin G (8 μg/ml). Human MAGE-11 was expressed in COS cells from pSG5-MAGE-11 and included on the immunoblot as a control (lane 1, 0.5 μg total protein). (**D**) Ishikawa cells were treated with and without 10 nM $E_2$ for 48 h as indicated and protein (50 μg/lane) probed with MagAb94–108 immunoglobulin G (8 μg/ml). Expression of pSG5-MAGE-11 was included as a positive control (lane 1)

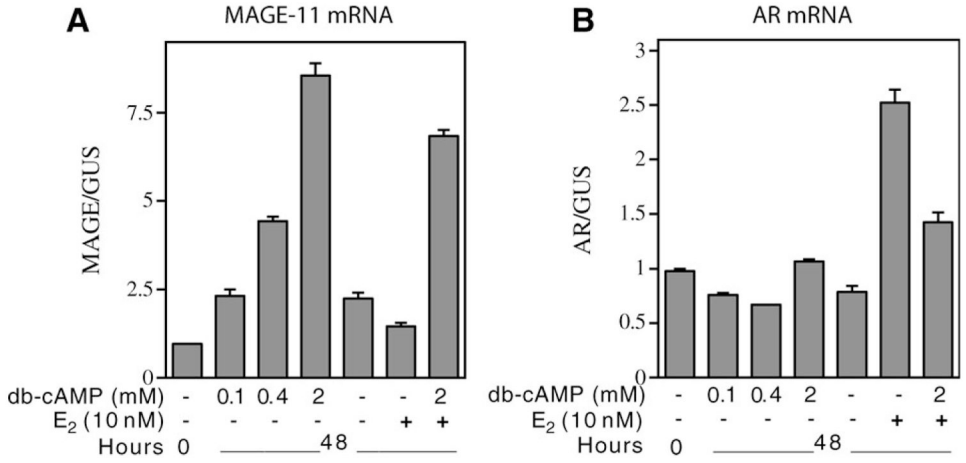


**Figure 7. Estrogen and cAMP regulation of MAGE-11 and AR mRNA in Ishikawa cells**

Ishikawa cells were cultured, plated and treated in phenol red free, 5% charcoal stripped serum medium with and without 0.1, 0.4 and 2 mM dibutyryl-cAMP and/or 10 nM $E_2$ for 48 h. RNA was analyzed as described in the section Materials and Methods. Shown are relative MAGE-11 (**A**) and AR (**B**) mRNA levels from duplicates of two 10 cm dish cultures extrapolated from standard curves and threshold cycle Ct values expressed as ratios of target gene to control gene β-glucuronidase (GusB) ± SD

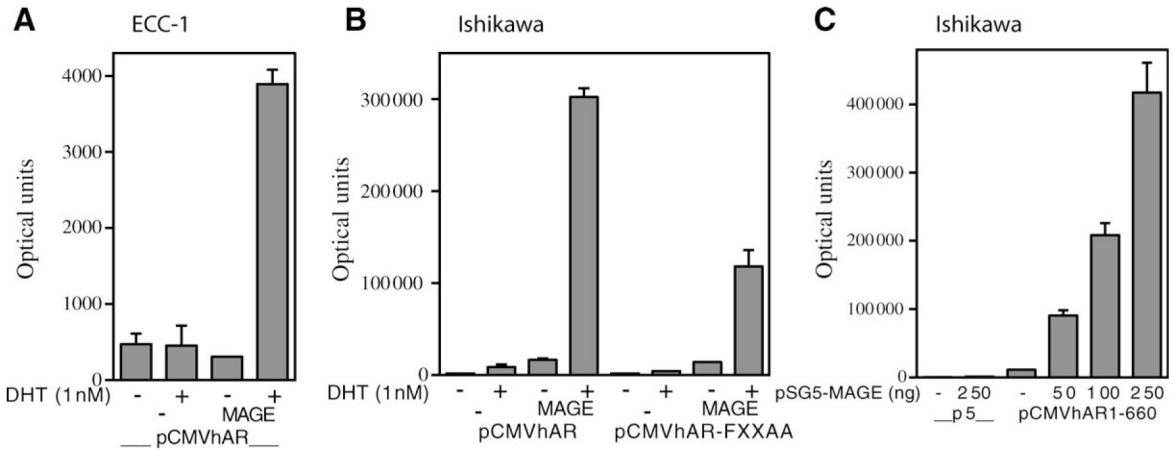


**Figure 8. Increase in AR transcriptional activity by MAGE-11 in endometrial cells**

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-F*XX*AA ($^{23}$FQNAA$^{27}$) 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

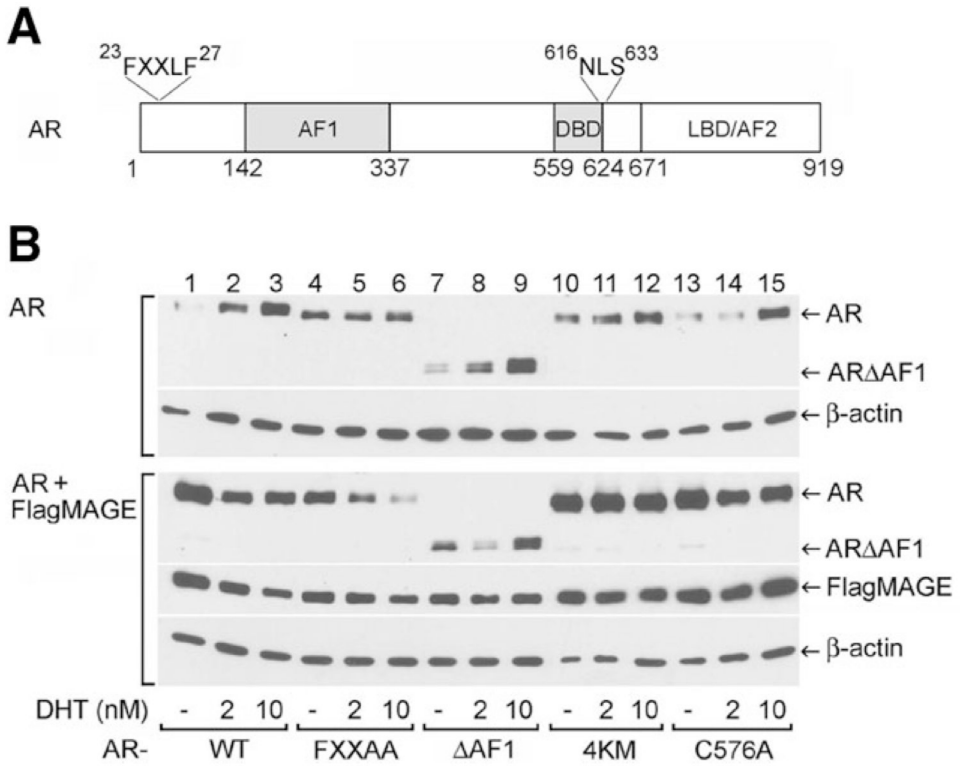


**Figure 9. Modulation of AR and MAGE-11 protein levels**

(**A**) Schematic of full-length human AR amino acid residues 1–919 including AR F*XX*LF motif $^{23}$FQNLF$^{27}$, 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-F*XX*AA with $^{23}$FQNLF$^{27}$ 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% charcoalstripped 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 $M_2$ 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