Increased Expression of Androgen Receptor Coregulator MAGE-11 in Prostate Cancer by DNA Hypomethylation and Cyclic AMP

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Abstract
Melanoma antigen gene protein-A11 (MAGE-11) of the MAGE family of cancer germ-line antigens increases androgen receptor (AR) transcriptional activity through its interaction with the AR NH2-terminal FXXLF motif. The present study investigated the regulatory mechanisms that control MAGE-11 expression during androgen deprivation therapy and prostate cancer progression. Studies include the CWR22 xenograft model of human prostate cancer, clinical specimens of benign and malignant prostate, and prostate cancer cell lines. MAGE-11 mRNA levels increased 100- to 1,500-fold during androgen deprivation therapy and prostate cancer progression, with highest levels in the castration-recurrent CWR22 xenograft and clinical specimens of castration-recurrent prostate cancer.

Pyrosequencing of genomic DNA from prostate cancer specimens and cell lines indicated the increase in MAGE-11 resulted from DNA hypomethylation of a CpG island in the 5′ promoter of the MAGE-11 gene. Sodium bisulfite sequencing of genomic DNA from benign and malignant prostate tumors and prostate cancer cell lines revealed DNA hypomethylation at individual CpG sites at the transcription start site were most critical for MAGE-11 expression. Cyclic AMP (cAMP) also increased MAGE-11 expression and AR transcriptional activity in prostate cancer cell lines. However, cAMP did not alter DNA methylation of the promoter and its effects were inhibited by extensive DNA methylation in the MAGE-11 promoter region. Increased expression of the AR coregulator MAGE-11 through promoter DNA hypomethylation and cAMP provides a novel mechanism for increased AR signaling in castration-recurrent prostate cancer. (Mol Cancer Res 2009;7(4):523–35)

Introduction
The androgen receptor (AR) mediates androgen-stimulated growth of benign prostate and contributes to prostate cancer progression. Prostate cancers typically undergo remission during androgen deprivation therapy but recur with castration-recurrent growth and a poor prognosis despite low circulating levels of testosterone androgen. The CWR22 xenograft of human prostate cancer mimics clinical prostate cancer with regression after castration. Castration-recurrent growth of the CWR22 tumor begins ~120 days after castration to remove circulating testosterone androgens (1-3). AR functions in prostate cancer as a transcriptional regulator that drives androgen-stimulated and castration-recurrent growth. Inhibition of prostate cancer growth by reducing AR levels provides evidence that AR is a central mediator of prostate cancer progression during androgen deprivation therapy, although the underlying mechanisms remain to be established (4-8).

Prostate cancer cells use several mechanisms mediated through the AR to promote tumor growth. One explanation for persistent AR activity in castration-recurrent cancer is the local tissue production of active androgens, which provides a hormonal stimulus for AR function (9, 10). AR transcriptional activity is mediated through interactions with coregulator proteins (11), and increased levels of critical coregulators, such as the SRC/p160 family of coactivators, appear to contribute to prostate cancer growth and progression (12). In a few instances, and more often in castration-recurrent prostate cancer, naturally occurring AR somatic mutations promote androgen-stimulated prostate cancer growth. Gain-of-function AR mutations can increase AR transcriptional activity by stabilizing interactions with SRC/p160 coactivators (13, 14).

One recently identified AR coactivator is melanoma antigen gene protein-A11 (MAGE-11; ref. 15). MAGE-11 is a member of the MAGE-A subfamily of cancer-testis (or cancer germ-line) antigens (16) that is expressed in normal tissues of the human male and female reproductive tracts and in prostate cancer cells that express AR (15, 17). MAGE-11 is expressed only by human and nonhuman primates and is not present in the mouse or rat genomes, which suggests that the MAGE gene family is
undergoing rapid evolution (18, 19). MAGE-11 increases AR transcriptional activity by binding the AR NH2-terminal FXXLF motif through mechanisms that include epidermal growth factor-dependent phosphorylation and monoubiquitylation of MAGE-11 (20). The AR NH2-terminal FXXLF motif also mediates the androgen-dependent AR N/C interaction with activation function 2 in the AR ligand-binding domain (21). AR binding to MAGE-11 relieves competitive inhibition of AR transcriptional activity caused by the AR N/C interaction so that SRC/p160 coactivators are recruited more freely by activation function 2 (22, 23).

In the present study, MAGE-11 expression increased with time after castration, as AR becomes reactivated during prostate cancer progression in the CWR22 xenograft model of human prostate cancer and in clinical prostate cancer specimens. In three different experimental settings, which include prostate cell lines, CWR22 xenografts, and clinical specimens of benign and malignant prostate tissues, increased expression of MAGE-11 resulted from hypomethylation of CpG sites directly proximal to the MAGE-11 transcriptional start site. In studies using prostate cell lines, cyclic AMP (cAMP) signaling also increased MAGE-11 expression and AR transcriptional activity but did not alter DNA methylation of the MAGE-11 promoter. The data suggest a mechanism whereby increased expression of MAGE-11 facilitates prostate cancer progression by enhancing AR-dependent tumor growth.

**Results**

**Increased MAGE-11 Expression during CWR22 Tumor Progression after Castration**

Quantitative reverse transcription-PCR (RT-PCR) analysis of CWR22 tumor RNA obtained 2, 6, and 12 days after castration indicated increased levels of MAGE-11 mRNA in association with tumor progression (Fig. 1A). Highest levels of MAGE-11 mRNA occurred on day 120 or longer after castration when tumor growth recurred in the absence of circulating testicular androgens. MAGE-11 immunostaining was nuclear and most prominent on days 6 and 12 after castration (Fig. 1A). MAGE-11 expression increased with time after castration, as AR becomes reactivated during prostate cancer progression in the CWR22 xenograft model of human prostate cancer and in clinical prostate cancer specimens.

In three different experimental settings, which include prostate cell lines, CWR22 xenografts, and clinical specimens of benign and malignant prostate tissue, increased expression of MAGE-11 resulted from hypomethylation of CpG sites directly proximal to the MAGE-11 transcriptional start site. In studies using prostate cancer cell lines, cyclic AMP (cAMP) signaling also increased MAGE-11 expression and AR transcriptional activity but did not alter DNA methylation of the MAGE-11 promoter. The data suggest a mechanism whereby increased expression of MAGE-11 facilitates prostate cancer progression by enhancing AR-dependent tumor growth.

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CWR22 xenograft during progression to castration-recurrent growth.

In parallel studies, AR mRNA levels also increased in the CWR22 tumor on day 2 after castration and further at later times after castration in some samples (Fig. 1B). However, unlike the striking increase in MAGE-11 mRNA in the castration-recurrent CWR22 tumor, AR mRNA levels remained relatively constant after castration with few exceptions. On the other hand, AR immunostaining (Fig. 2A) and immunoblotting (Fig. 2B) showed increased AR protein in the castration-recurrent CWR22 tumor as reported previously (24). AR coactivators transcriptional intermediary factor 2 (TIF2) and p300 mRNA levels were variable and did not correlate with tumor progression (Fig. 1C and D). Immunostaining (data not shown) and immunoblots (Fig. 2B) indicated relatively constant levels of TIF2 and p300 during CWR22 tumor regression and regrowth, although p300 declined in the castration-recurrent CWR22 tumor.

The data indicated that both MAGE-11 and AR mRNA and protein levels increase during CWR22 tumor progression after castration. In the castration-recurrent CWR22 prostate cancer xenograft, MAGE-11 mRNA increased to a greater extent in association with prominent immunostaining of AR and MAGE-11.

**MAGE-11 Expression in Clinical Specimens of Benign and Malignant Prostate**

To investigate whether the increase in MAGE-11 during progression of the CWR22 xenograft is a characteristic feature of prostate cancer progression, tissue specimens of benign, androgen-stimulated, and castration-recurrent prostate cancer were analyzed. MAGE-11 mRNA levels were 10- to 1,500-fold greater in 4 of 11 clinical specimens of castration-recurrent prostate cancer than in androgen-stimulated benign prostate or prostate cancer (Fig. 3A). In one specimen, a Gleason score of 4 + 5 = 9 castration-recurrent prostate cancer from patient 5 (CR-CaP-5) obtained 49 months after the initiation of androgen deprivation therapy, MAGE-11 mRNA levels increased to a greater extent in association with prominent immunostaining of AR and MAGE-11.

**FIGURE 2.** Increase in MAGE-11 protein during CWR22 prostate cancer progression. A. MAGE-11 and AR immunostaining was analyzed in formalin-fixed, paraffin-embedded sections of the CWR22 xenograft excised from intact noncastrated mice and on days 2, 6, 12, and 120 after castration and longer for the castration-recurrent tumor, using MAGE-11 antibody MagAb94-108 (8 μg/mL) and AR PG21 (Upstate; 1:150 dilution). Brown reaction product indicates immunoreactivity against a toluidine blue counterstain. Original magnification, ×400.

B. Immunoblotting of MAGE-11, AR, TIF2, and p300 were assayed using CWR22 xenograft extracts prepared from intact-7 (day 0; lane 2), day 2 castrate-3 (lane 3), day 6 castrate-2 (lane 4), day 12 castrate-2 (lane 5), day 120 castrate-3 (lane 6), and castration-recurrent-4 (Rec; lane 7) according to the numbering of RNA analysis in Fig. 1. Protein extracts (100 μg/lane) were analyzed using antibodies as described in Materials and Methods. Combined extracts from COS cells transfected with pSG5-MAGE-11, pCMV-AR, pSG5-TIF2 and pSG5-HA-p300 served as protein controls (lanes 1 and 8), and endogenous β-actin served as a loading control.

Although AR mRNA was undetectable (Fig. 3B), AR mRNA levels also increased by 10- to 100-fold in 4 of 11 castration-recurrent prostate cancer specimens. However, castration-recurrent prostate cancer specimens with increased levels of AR mRNA had lower levels of MAGE-11 mRNA. In agreement with the results of CR-CaP-5, the data suggest an inverse relationship between MAGE-11 and AR mRNA in clinical specimens of castration-recurrent prostate cancer (Fig. 3C). MAGE-11 and AR protein levels both increased in specimens of castration-recurrent prostate cancer (Fig. 3D).

The results indicated that ∼30% of clinical specimens of castration-recurrent prostate cancer have elevated levels of MAGE-11 or AR mRNA, which suggests the presence of a compensatory relationship controlling AR transcriptional activity.

**MAGE-11 Promoter DNA Hypomethylation in the CWR22 Xenograft**

The molecular basis for increased expression of MAGE-11 during prostate cancer progression was investigated further based on evidence that DNA hypomethylation occurs for other cancer germ-line antigens (16, 25). The MAGE-11 gene is located at Xq28 on the human X chromosome (18) and contains a classic CpG island flanking the transcription start site (Fig. 4A). Pyrosequencing of 10 CpG sites ∼200 bp before the transcription start site indicated 5% DNA methylation of the MAGE-11 promoter in CWR22 xenografts excised from intact mice and on days 2, 6, and 12 after castration (Fig. 4B). In the CWR22 castration-recurrent tumor, DNA methylation decreased further to 2%.

Methylation of individual CpG sites was investigated further using sodium bisulfite sequencing of 20 CpG sites that included 10 additional downstream sites in closer proximity to the transcription start site. Unmethylated (open circles) and methylated (filled circles) CpG sites were determined for multiple alleles in CWR22 tumors on different days after castration (Fig. 4C). Hypermethylation at sites more distal to the transcription start site appeared to account for the overall low percentage of DNA methylation in the CWR22 tumors, whereas CpG sites near the transcription start site were methylated in intact CWR22.
tumors and on day 2 after castration (Fig. 4B and C). At later times after castration and in the castration-recurrent CWR22 tumor, DNA methylation sites near the transcription start site were hypomethylated.

The results suggested that hypomethylation at CpG sites in direct proximity to the transcription start site is associated with increased expression of MAGE-11 during CWR22 tumor progression.

MAGE-11 Promoter DNA Hypomethylation in Clinical Specimens of Prostate Cancer

To extend this analysis to clinical specimens, pyrosequencing of the MAGE-11 promoter was done using benign and malignant prostate tissues to investigate further the dependence of MAGE-11 expression on promoter DNA methylation during prostate cancer progression after castration. Pyrosequencing analysis revealed that MAGE-11 methylation levels decreased in castration-recurrent prostate cancer compared with androgen-stimulated benign prostate or androgen-stimulated prostate cancer (Fig. 5A). In particular, the MAGE-11 methylation level in CR-CaP-5, a castration-recurrent prostate cancer specimen that had 1,500-fold higher MAGE-11 mRNA levels than benign prostate (Fig. 3A), was reduced to 5% compared with ∼70% DNA methylation in androgen-stimulated benign prostate and androgen-stimulated prostate cancer specimens (Fig. 5A). DNA hypomethylation of CR-CaP-5 was evident at all CpG sites within the pyrosequenced region that was different than androgen-stimulated benign prostate (AS-BP-3) and androgen-stimulated prostate cancer (AS-CaP-3; Fig. 5B). The trend of relative uniformity in methylation levels at individual CpG sites across the pyrosequenced region was consistent across all samples analyzed (data not shown). Sodium bisulfite sequencing of CR-CaP-5 indicated almost complete loss of DNA methylation compared with dense DNA methylation of the MAGE-11 promoter in androgen-stimulated benign prostate (AS-BP-9; Fig. 5C). CR-CaP-4 MAGE-11 mRNA levels were 1,500-fold less than CR-CaP-5 (Fig. 3A) and dense methylation of CpG sites was retained in close proximity to the transcription start site, although more distal CpG sites were hypomethylated (Fig. 5C, middle).

The results from clinical specimens of benign prostate and prostate cancer provided further evidence that DNA hypomethylation of CpG sites proximal to the MAGE-11 transcription start...
site is correlated with increased expression of MAGE-11 in association with prostate cancer progression to castration-recurrent growth.

**MAGE-11 Expression in Prostate Cancer Cell Lines**

The direct relationship between increased expression of MAGE-11 and promoter DNA hypomethylation near the transcription start site prompted a study of cell lines that express AR and MAGE-11 at different levels. MAGE-11 mRNA levels were 10- to 100-fold higher in LAPC-4 prostate cancer cells than in LNCaP, LNCaP-C4-2, or CWR-R1 prostate cancer cells, each of which had high levels of AR mRNA (Fig. 6A). These results suggested a more direct relationship between AR and MAGE-11 mRNA than was seen in clinical specimens of prostate cancer. MAGE-11 mRNA levels were 10- to 1,000-fold higher in LAPC-4, LNCaP, LNCaP-C4-2, and CWR-R1 cells than in benign PWR-1E and RWPE-2 prostate cells, DU145 or PC-3 prostate cancer cells, or CV1 or COS-1 cells. In the majority of prostate cancer cell lines, AR mRNA levels were 100 to 1,000 times higher than MAGE-11.

MAGE-11 protein expression determined using immunoblot analysis correlated with MAGE-11 mRNA levels. Highest levels of MAGE-11 protein were in LAPC-4 cells, with lowest levels in RWPE-2 and PWR-1E benign prostate cells (Fig. 6B). AR protein in CWR-R1, CWR22-RV1, LNCaP, LNCaP-C4-2, and LAPC-4 prostate cancer cell extracts (Fig. 6B) correlated with AR mRNA levels (Fig. 6A). Under these assay conditions, AR protein was not detected in PC-3 or DU145 prostate cancer cells or in PWR-1E and RWPE-2 benign human prostate cells. Except for DU145 cells, in which AR mRNA was

![FIGURE 4. DNA hypomethylation of the MAGE-11 promoter during CWR22 prostate cancer xenograft progression. A. MAGE-A11 5′ promoter region and CpG island as predicted using http://www.uscnorris.com/cpgislands2/cpg.aspx in the range 148575477 to 148605507 on the reverse complement strand (accession no. NC-000023). CpG island characteristics are indicated. Black vertical lines, CpG sites in the 5′ CpG island of MAGE-11 (−367 to +133 relative to the transcription start site); red vertical lines, CpG sites analyzed using pyrosequencing. Regions analyzed using sodium bisulfite pyrosequencing and standard sodium bisulfite sequencing and the number of CpG sites analyzed using each method are indicated. B. Sodium bisulfite pyrosequencing of MAGE-11 promoter DNA methylation in CWR22 xenografts before and after castration. Shown is the percent DNA methylation averaged over 10 analyzed CpG sites in the MAGE-11 promoter region indicated in red in A. Human genomic DNA modified with bacterial CpG methylase SssI and DNMT-deficient HCT116 colorectal cancer cell DNA (DKO) served as positive and negative controls for MAGE-11 5′ region DNA methylation, respectively. Columns, average of four independent pyrosequencing analyses; bars, SE. C. Sodium bisulfite sequencing of the MAGE-11 promoter DNA methylation in CWR22 xenografts before and after castration. Tumors were analyzed from intact mice (intact-5) and on day 2 after castration (day 2 castrate-3), day 6 after castration (day 6 castrate-2), day 12 after castration (day 12 castrate-6), and during castration-recurrent tumor growth >120 d after castration (recurrent-1 and recurrent-5), with sample designations according to Fig. 1. Open circles, unmethylated CpG sites; filled circles, methylated CpG sites; rows, individually sequenced alleles; right arrow, transcription start site; brackets, region analyzed using pyrosequencing in B.](mcr.aacrjournals.org)
not detected, AR mRNA was expressed in benign prostate cells (Fig. 6A) but at levels too low to detect on immunoblots (Fig. 6B) and too low to activate androgen-responsive genes in transient reporter gene assays (data not shown).

As found in the CWR22 xenograft and tissue specimens of benign and malignant prostate, MAGE-11 mRNA levels correlated directly with DNA methylation status of the MAGE-11 promoter in the majority of cell lines. Pyrosequencing of 10 CpG sites preceding the MAGE-11 transcription start site showed 5% DNA methylation in LAPC-4 cells, which had the highest levels of MAGE-11 mRNA and protein (Figs. 6 and 7A). MAGE-11 CpG site methylation was 8% to 17% in LNCaP, LNCaP-C4-2, and CWR-R1 cells, 50% to 65% in DU145, CV1, and COS cells, 35% in PWR-1E and RWPE-2, and 12% in PC-3 cells (Fig. 7A). Representative examples of the extent of DNA methylation at the 10 individual CpG sites interrogated by pyrosequencing are shown for LAPC-4 and DU145 cells (Fig. 7B).

Sodium bisulfite sequencing of the MAGE-11 promoter in human specimens of androgen-stimulated benign prostate and castration-recurrent prostate cancer. Samples include AS-BP-9, CR-CaP-4, and CR-CaP-5 and are designated according to Fig. 2. Open and filled circles, unmethylated and methylated CpG sites, respectively; rows, individually sequenced alleles. Methylation status of CpG sites (hashed lines) could not be determined accurately. Right arrow, transcription start site; brackets, region analyzed using pyrosequencing in A and B.

FIGURE 5. DNA hypomethylation of the MAGE-11 promoter region in clinical specimens of benign and malignant prostate. A. Sodium bisulfite pyrosequencing analysis of MAGE-11 promoter DNA methylation in androgen-stimulated benign prostate, androgen-stimulated prostate cancer, and castration-recurrent prostate cancer specimens. Shown is the percent DNA methylation averaged over 10 CpG sites in the MAGE-11 promoter region indicated in red in Fig. 4A. Human genomic DNA modified with bacterial CpG methylase SsII and DNMT-deficient HCT116 colorectal cancer cells DNA (DKO) served as positive and negative controls for MAGE-11's region DNA methylation, respectively. Columns, average of four independent pyrosequencing analyses; bars, SE. B. DNA methylation at individual CpG sites in the MAGE-11 promoter region determined using pyrosequencing. Representative examples of AS-BP-3, AS-CaP-3, and CR-CaP-5. CpG sites are numbered 1 to 10 starting at the 5' site (nucleotide position -192 relative to the transcription start site). Columns, average of four independent pyrosequencing analyses; bars, SE. C. Sodium bisulfite sequencing of the MAGE-11 promoter in human specimens of androgen-stimulated benign prostate and castration-recurrent prostate cancer. Samples include AS-BP-9, CR-CaP-4, and CR-CaP-5 and are designated according to Fig. 2. Open and filled circles, unmethylated and methylated CpG sites, respectively; rows, individually sequenced alleles. Methylation status of CpG sites (hashed lines) could not be determined accurately. Right arrow, transcription start site; brackets, region analyzed using pyrosequencing in A and B.
Regulation of MAGE-11 Expression in Prostate Cancer Cell Lines by cAMP

Regulation of MAGE-11 expression was investigated further based on evidence that cAMP increases, and 17β-estradiol decreases, MAGE-11 expression in human endometrial cells (17). MAGE-11 mRNA levels increased up to 35-fold in a dose-dependent manner in response to dibutyryl-cAMP (db-cAMP) in LNCaP-C4-2, CWR22-RV1, LAPC-4, LNCaP, RWPE-2, and PWR-1E cells as shown for LNCaP-C4-2 and PWR-1E cells (Fig. 8A and B). The cAMP-dependent increase in MAGE-11 mRNA was evident within 12 h in LNCaP cells, indicative of a transcriptional effect, was maximal by 24 h, and was not influenced by 17β-estradiol (Fig. 8C).

In DU145 cells where the MAGE-11 promoter was densely methylated (Fig. 7C), MAGE-11 mRNA levels did not increase in response to cAMP relative to a 48 h control (Fig. 8D). The inability of cAMP to increase MAGE-11 expression in DU145 cells suggests that extensive promoter DNA methylation blocks cAMP signaling, and cAMP may not alter DNA methylation of the MAGE-11 promoter. DNA methylation-dependent inhibition of cAMP regulation was supported further by studies in CWR22-RV1 and LNCaP-C4-2 cells, where the extent of DNA methylation of the MAGE-11 promoter CpG sites determined by sodium bisulfite sequencing was unchanged after 48 h treatment with 8 mmol/L db-cAMP (data not shown). Furthermore, cAMP did not increase MAGE-11 mRNA levels in DU145 cells after treatment with the DNMT inhibitor, DAC, although a small potentiation effect was apparent in PC-3 cells (data not shown).

The results suggested that hypomethylation within the MAGE-11 promoter near the transcription start site is necessary but not sufficient for cAMP-dependent up-regulation of MAGE-11.

cAMP Regulation of AR Transcriptional Activity

To determine whether the cAMP-dependent increase in MAGE-11 is associated with an increase in AR transcriptional activity, AR and a prostate-specific antigen-luciferase reporter gene were coexpressed in LNCaP-C4-2 and DU145 prostate cancer cells, two cell lines that differ in the extent of MAGE-11 promoter DNA methylation, expression, and responsiveness to cAMP. AR transactivation increased up to 25-fold in response to db-cAMP in a dose-dependent manner in LNCaP-C4-2 cells in the absence and presence of 1 mmol/L dihydrotestosterone (Fig. 9A). In contrast, increased AR transactivation in DU145 cells required higher concentrations of cAMP and was evident only in the presence of dihydrotestosterone (Fig. 9B).

The results suggested that increased AR transcriptional activity in response to cAMP results at least in part from increased expression of MAGE-11. To test this further, MAGE-11 siRNA was used to reduce expression in CWR-R1 prostate cancer cells, where endogenous AR transcriptional activity can be measured using a MMTV-luciferase reporter vector. The cAMP-dependent increase in AR transcriptional activity was retained in CWR-R1 cells treated with MAGE-11 siRNA-3, which does not alter MAGE-11 expression, but was lost after treatment with MAGE-11 siRNA-2, which reduces MAGE-11 expression (ref. 20; Fig. 9C).

The results raised the possibility that the cAMP-dependent increase in AR transcriptional activity occurs through mechanisms that include increased expression of MAGE-11. On the other hand, the ability of higher concentrations cAMP to increase AR transcriptional activity in DU145 cells provided evidence that additional cAMP-dependent mechanisms increase AR transcriptional activity possibly independent of MAGE-11.

Discussion

MAGE-11 Expression during Prostate Cancer Progression

This report provides evidence that increased expression of the AR selective coregulator MAGE-11 represents a novel mechanism to promote AR-dependent growth of prostate cancer. In three experimental settings, MAGE-11 expression increased in prostate cancer as a result of MAGE-11 promoter DNA hypomethylation at the transcription start site or in response to cAMP. In clinical specimens of castration-recurrent prostate cancer, the MAGE-11 promoter region was hypomethylated and correlated
with increased expression of MAGE-11. MAGE-11 mRNA levels were highest in clinical specimens of castration-recurrent prostate cancer, where CpG sites were hypomethylated directly proximal to the transcription start site. In the CWR22 xenograft model of human prostate cancer, the MAGE-11 gene was hypomethylated in the androgen-stimulated tumor before castration, which suggests that the androgen-stimulated CWR22 tumor model represents an advanced stage of the disease. However,

**FIGURE 7.** MAGE-11 promoter DNA methylation status in benign and malignant cell lines. A. Sodium bisulfite pyrosequencing of MAGE-11 promoter DNA methylation in human and monkey benign and malignant cell lines. Shown is percent DNA methylation averaged over 10 CpG sites in the MAGE-11 promoter region indicated in red in Fig. 4A for human prostate cancer cell lines LNCaP, CWR-R1, LAPC-4, LNCaP-C4-2, PC-3, and DU145, benign human prostate cell lines PWR-1E and RWPE-2, human endometrial cancer Ishikawa, monkey kidney CV1 and COS-1, and human cervical cancer HeLa cells. Human genomic DNA modified with bacterial CpG methylase SssI and DNMT-deficient HCT116 colorectal cancer cells DNA (DKO) served as positive and negative controls for MAGE-11 5′ region DNA methylation, respectively. Columns, average of four independent pyrosequencing analyses; bars, SE. B. DNA methylation at individual CpG sites in the MAGE-11 promoter region in LAPC-4 and DU145 cells determined using pyrosequencing. Columns, average of four independent pyrosequencing analyses; bars, SE. C. Sodium bisulfite sequencing of the MAGE-11 promoter in LAPC-4, PC-3, and DU145 cells. Open and filled circles, unmethylated and methylated CpG sites, respectively; rows, sequenced alleles; right arrow, transcription start site; brackets, region analyzed using pyrosequencing in A and B. The percent methylation of three CpG sites proximal to the transcriptional start site is indicated and takes into account all sequenced alleles for each sample. D. Activation of MAGE-11 expression by DNA demethylation using 5-aza-2′-deoxycytidine (DAC). PC-3 and DU145 cells were treated at 0 and 48 h with 0, 0.1, 0.5, and 1 μmol/L DAC in PBS and RNA was harvested for analysis at 120 h (5 d post-treatment). MAGE-11 expression levels were determined using RT-PCR of total RNA extracted with Trizol. RT-PCR amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as control for cDNA input. Neg, a no template control reaction. E. Sodium bisulfite sequencing of the MAGE-11 promoter in PC-3 (left) and DU145 (right) cells following 0.5 μmol/L DAC 5 d post-treatment. Open and filled circles, unmethylated and methylated CpG sites, respectively; rows, sequenced alleles; right arrow, transcription start site. The percent methylation of three CpG sites proximal to the transcriptional start site is indicated and takes into account all sequenced alleles for each sample.
with progression to castration-recurrent growth, MAGE-11 expression increased ~100-fold in the CWR22 xenograft and correlated with hypo-methylation at CpG sites proximal to the transcription start site of the MAGE-11 promoter. MAGE-11 expression levels in cell lines similarly correlated with promoter DNA methylation proximal to the transcription start site, and hypo-methylation was associated with increased expression. The ability of DNA methylation to inhibit MAGE-11 expression was especially evident among the prostate cancer cell lines. Most striking was the DU145 cell line, where MAGE-11 expression was low and the promoter was densely methylated.

Although the predominant mechanism for increased expression of MAGE-11 during prostate cancer progression to castration-recurrent growth appears to be hypo-methylation at the transcription start site, MAGE-11 gene expression was also up-regulated by cAMP. cAMP increased MAGE-11 expression in prostate cancer cells that were hypo-methylated more extensively in the MAGE-11 promoter region that appeared to be independent of a change in DNA methylation. In contrast, the lack of cAMP stimulation of MAGE-11 expression in DU145 cells suggested that cAMP does not override the inhibitory effects of an extensively methylated MAGE-11 promoter.

The results suggest that basal expression of MAGE-11 is blocked by DNA methylation near the transcription start site, some extent of hypo-methylation in the MAGE-11 distal promoter is required for up-regulation by cAMP, and additional cell specific factors likely contribute MAGE-11 gene regulation by cAMP. MAGE-11 promoter DNA in clinical specimens of benign prostate tissue was methylated extensively but only methylated partially in the PWR-1E and RWPE-2 cell lines that derive from benign prostate. These findings make it difficult to predict whether MAGE-11 expression is sensitive to cAMP stimulation in benign prostate tissue under normal physiologic conditions.

The cAMP-dependent increase in AR transcriptional activity appears to result in part to an increase in the expression of MAGE-11. Depletion of MAGE-11 levels in CWR-R1 cells using siRNA that inhibits MAGE-11 expression inhibited the cAMP-dependent increase in AR transcriptional activity. However, cAMP also increased AR transcriptional activity when AR was expressed in DU145 cells, a cell line that lacks endogenous AR and MAGE-11, and where MAGE-11 mRNA levels were not increased by cAMP. AR transcriptional activity was increased by cAMP in LNCaP-C4-2 cells in the absence and presence of androgen, which may reflect the increased expression of MAGE-11. The data suggest that additional mechanisms, possibly involving other coregulatory proteins (26), contribute to the cAMP-dependent increase in AR transcriptional activity.

Relationship between AR and MAGE-11

AR transcriptional activity is increased through several mechanisms involving MAGE-11. Through its interaction with the AR NH2-terminal FXXLF motif, MAGE-11 increases AR recruitment of SRC/p160 coactivators by activation function 2 in the AR ligand-binding domain and inhibition of the AR N/C interaction (15). The effects of MAGE-11 on AR transcriptional activity are enhanced by epidermal growth factor signaling, and MAGE-11 stabilizes AR in the absence or at low levels of androgen (15, 17, 20). The stabilizing effect of MAGE-11 on AR under conditions of low androgen raises the possibility that increases in MAGE-11 in the CWR22 tumor on days 6 and 12 after castration and in the castration-recurrent tumor provides a mechanism to increase AR levels in the low androgen environment in the absence of the stabilizing effects of the androgen-dependent AR N/C interaction. AR stabilization by MAGE-11 in castration-recurrent prostate cancer may contribute to the apparent increase in AR protein when AR mRNA levels remained relatively constant after castration. Greater levels of AR could increase the sensitivity to low circulating androgen levels associated with androgen deprivation therapy in the treatment of prostate cancer. Increased levels of MAGE-11 may also increase ligand-independent AR transactivation as suggested by studies using the LNCaP-C4-2 prostate cancer cell line.

The apparent inverse relationship between AR and MAGE-11 mRNA levels identified in clinical specimens of castration-recurrent prostate cancer suggests a compensatory relationship between AR and MAGE-11 at the level of gene regulation. This relationship also was seen in some samples of the CWR22 xenograft but not in the prostate cancer cell lines. Present evidence indicates that MAGE-11 expression is not regulated by...
androgen (data not shown), and increased expression of MAGE-11 is associated with prostate cancer progression to recurrent growth. Hypomethylation and cAMP signaling may account for the increased expression of MAGE-11 during prostate cancer progression and contribute to AR-mediated castration-recurrent growth of prostate cancer. The molecular basis for the difference in relative levels of AR and MAGE-11 mRNA between castration-recurrent tumor specimens and prostate cancer cell lines remains to be established.

Epigenetic Regulation of MAGE-11

Increased DNA hypomethylation at CpG sites in the MAGE-11 promoter and the associated increase in MAGE-11 expression was greatest in castration-recurrent prostate cancer in the CWR22 xenograft model and clinical specimens of prostate cancer. Epigenetic abnormalities in gene silencing and activation observed for MAGE-11 are now well-established markers for cancer development (27). These results show that hypomethylation of specific CpG sites proximal to the transcription start site is most critical in regulating MAGE-11 expression, whereas more distal hypomethylation may influence regulation by cAMP. The lack of a cAMP-dependent increase in MAGE-11 associated with the densely methylated 5′ CpG island in DU145 cells suggests that MAGE-11 promoter DNA hypomethylation distal to the transcription start site is required for cAMP stimulation of MAGE-11 expression. On the other hand, recent genome-wide studies on DNA methylation, histone modification patterns and gene expression, support the importance of epigenetic marks proximal to the transcription start site in gene regulation (28, 29). Localized DNA hypomethylation may alter chromatin structure at the MAGE-11 transcription start site even when flanking DNA is hypermethylated.

Cancer cells exhibit global decreases in 5-methylcytosine and DNA hypomethylation to various extents depending on tumor stage and type, which may contribute to chromosomal instability and acquisition of mutations (30, 31). Growth-promoting genes can be activated by DNA hypomethylation in tumors (32). Although relatively little is known about the mechanisms of genome-wide or gene-specific DNA hypomethylation, recent evidence suggests that the expression of DNMT-3b isoforms or aberrant expression of the BORIS/CTCFL imprinting-related protein plays a role (33, 34). Germ-line-specific genes, such as MAGE-11 and other members of the MAGE family, become methylated during normal development (35) and some members of the family of cancer germ-line genes undergo DNA hypomethylation in cancer (16, 30). Germ-line antigen gene activation in cancer has led to the development of cancer vaccines targeting these antigens (16). DNA hypomethylation at CpG sites in the MAGE-11 promoter associated with the onset of castration-recurrent prostate cancer may have critical consequences to tumor growth based on the ability of MAGE-11 to increase AR transcriptional activity in the presence and especially in the relative absence of androgen. The AR coregulator MAGE-11 may be a viable therapeutic and/or vaccine target in some cases of castration-recurrent prostate cancer.

Materials and Methods

Clinical Prostate and CWR22 Xenograft Sample Preparation

Serially transplanted androgen-dependent and nonmetastatic CWR22 human prostate cancer xenografts derived from a primary human prostate cancer were propagated in athymic nu/nu mice (24, 36). Some animals were castrated and testosterone pellets removed after tumors reached ∼0.75 g. CWR22 tumors from intact mice and on days 2, 6, 12, and 120 or longer after castration when castration-recurrent tumors develop were formalin-fixed and paraffin-embedded for immunocytochemistry or frozen for DNA and RNA extraction. Patient specimens of androgen-stimulated benign prostate were obtained by radical prostatectomy from African and Caucasian Americans ages 47 to 72 years. Clinically localized androgen-stimulated prostate cancer was obtained from radical prostatectomy specimens from African and Caucasian Americans ages 44 to 62 years, and castration-recurrent prostate cancer (Gleason score 3 + 5 = 8 to 5 + 5 = 10) was obtained from transurethral resection specimens from men ages 61 to 86 years who suffered urinary
retention from local recurrence. Prostatectomy specimens were macrodissected (37) to enrich for benign or malignant epithelial cells when their content was <80%. This method resulted in an average of 92% malignant epithelial cells in androgen-stimulated prostate cancer specimens (9). Tissues were stored in liquid nitrogen or formalin-fixed and paraffin-embedded for immunocytochemistry. Animal procedures and patient sample collections were done in accordance with institutional guidelines.

**Cell Culture**

Cells were maintained in medium containing phenol red, penicillin (100 units/mL), streptomycin (100 μg/mL), and 2 mmol/L L-glutamine. LNCaP prostate cancer cells were maintained in RPMI 1640 (Cellgro) containing 1 mmol/L sodium pyruvate and 10% fetal bovine serum (FBS; Sigma). LNCaP-C4-2 prostate cancer cells were cultured in DMEM/F-12 containing 5% FBS, 5 μg/mL insulin, 13.65 μg/mL triiodothyronine, 5 μg/mL apotransferrin, 0.244 μg/mL α-biotin, and 25 μg/mL adenine hemisulfate (Sigma). CWR-R1 cells derived from the castration-recurrent CWR22 prostate cancer xenograft (12) were maintained in DMEM with high glucose, 5 μL of 90 mg/mL linoleic acid (Sigma), 0.6 g nicotinamide (Sigma), 0.5 mL insulin-transferrin-selenium mix (Roche), 10 mL FBS, and 10 ng/mL epidermal growth factor in 500 mL medium. Serum-free CWR-R1 cell medium was Improved Zinc Optimal with additives without serum, phenol red, and epidermal growth factor. CWR22-RR1 cells derived from the CWR22 human prostate xenograft (American Type Culture Collection) were maintained in RPMI 1640 (Cellgro) containing 10% FBS, 10% fetal bovine serum (FBS), and 25 mg bovine pituitary extract per 500 mL. Human prostate cancer (17). MAGE-11 antibody MagAb94-108 (8 μg/mL) recognizes amino acid residues 94 to 108 (17). Human p300 C-20 antibody was obtained from Santa Cruz Biotechnology (1:100 dilution). Tissue sections were treated with 0.01 μg/mL sodium citrate (pH 6.0) for 15 min in a microwave at high setting (38) to retrieve antigens for affinity-purified rabbit polyclonal PG21 AR antibody (Upstate; 1:150 dilution) and mouse monoclonal human TIF2 IgG1 antibody (BD Transduction Laboratories; 1:300 dilution). Sections were blocked with 2% goat serum, incubated overnight at 4°C with primary antibody, blocked, and incubated for 30 min with biotinylated secondary antibody (Vector Labs). For MAGE-11 immunostaining, CWR22 tumor sections were incubated for 30 min with biotinylated secondary antibody and 30 min with avidin DH-biotinylated horseradish peroxidase H complex Vectastain Elite ABC kit for MAGE-11 and AR Coregulator MAGE-11 Expression in Prostate Cancer

**Immunocytochemistry**

Immunocytochemistry was done on formalin-fixed, paraffin-embedded sections of CWR22 prostate cancer xenograft and clinical specimens of androgen-stimulated benign prostate, androgen-stimulated prostate cancer, and castration-recurrent prostate cancer (17). MAGE-11 antibody MagAb94-108 (8 μg/mL) recognizes amino acid residues 94 to 108 (17). Human p300 C-20 antibody was obtained from Santa Cruz Biotechnology (1:100 dilution). Tissue sections were treated with 0.01 μg/mL sodium citrate (pH 6.0) for 15 min in a microwave at high setting (38) to retrieve antigens for affinity-purified rabbit polyclonal PG21 AR antibody (Upstate; 1:150 dilution) and mouse monoclonal human TIF2 IgG1 antibody (BD Transduction Laboratories; 1:300 dilution). Sections were blocked with 2% goat serum, incubated overnight at 4°C with primary antibody, blocked, and incubated for 30 min with biotinylated secondary antibody (Vector Labs). For MAGE-11 immunostaining, CWR22 tumor sections were incubated for 30 min with biotinylated secondary antibody and 30 min with avidin DH-biotinylated horseradish peroxidase H complex Vectastain Elite ABC kit (Vector Labs). Benign prostate and prostate cancer slides were incubated using the Vectastain Standard ABC kit for MAGE-11 and AR. Slides were immersed in 3,3′-diaminobenzidine tetrahydrochloride (17), exposed to osmium vapors and counterstained with 0.05% toluidine blue in 30% ethanol, dehydrated, cleared in xylene, and mounted with Permount (Fisher).

For immunoblots, cells were solubilized in 0.3 to 0.4 mL of 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 0.5 mmol/L EDTA, 50 mmol/L NaF, 0.5 mmol/L Na2VO4 and 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL pepstatin A, and 5 μg/mL aprotinin and sedimented at 4°C for 30 min at 20,000 × g. Extracts from 0.1 to 0.2 g CWR22 tumor were lysed in 2 mL buffer with detergents added

**Real-time RT-PCR**

CWR22 tumors (50-150 mg) and cultured cells were extracted using Trizol reagent (Invitrogen). Tumors were disrupted using a Brinkmann polytron (Switzerland). RNA from patient samples was obtained by extracting 0.1 mL AllPrep DNA/RNA extraction kit lysate (Qiagen) with 3 mL Trizol. First-strand cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen). PCR primers and fluorogenic probes for peptidylprolyl isomerase A (cyclophilin A) and human AR and MAGE-11 were described (17); however, sequence homology for African green monkey COS and CV1 cells could not be confirmed. Primers and probes for TIF2 (Hs00197990-m1) from Assays-On-Demand (Applied Biosystems; 18 bp ampiclon) span the exons 14 to 15 junction at 3193 (NM006540.2). p300 primers and probes (Hs00914232-m1; Applied Biosystems; 138 bp ampiclon) span the exons 9 to 10 junction at assay location 2279 (NM001429.2). PCR (20 μL) contained cDNA from 0.4 μg total RNA for AR, TIF2, MAGE-11, and peptidylprolyl isomerase A or 0.04 μg total RNA for p300 and peptidylprolyl isomerase A. 4 μL Light Cycler TaqMan Master mix (Roche), and 0.5 μL of 20× TaqMan Mix (Applied Biosystems). Thermal cyclers were done at 95°C for 10 min and 55 cycles at 95°C for 15 s, 60°C for 25 s, and 72°C for 1 s. Standard curves were prepared as described (17).


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after homogenization using a Brinkmann polytron. Tissue extracts were sedimented at 4°C for 1 h at 27,000 rpm in a Ti60 Beckman ultracentrifuge rotor. Protein controls were from COS cells transfected with pSG5-MAGE-11 (15, 17), pcMV-hAR (39), pSG5-TIF2, and pSG5-hAR-p300. Protein concentration was determined using the Bio-Rad assay and bovine serum albumin as standard.

Protein extracts from cells (50 μg) and tissues (100 μg) were separated on 10% acrylamide gels containing SDS. Nitrocellulose transfer blots (Perkin-Elmer) were probed with MAGE-11 antibodies MagAb94-108, MagAb59-79, and MagAb13-26 (10 μg/mL each; ref. 17). AR32 rabbit polyclonal antipeptide antibody (2 μg/mL), mouse monoclonal TIF2 IgG1 antibody (BD Transduction Laboratories; 1:250 dilution), rabbit polyclonal p300 C-20 antibody (Santa Cruz Biotechnology; 1:800 dilution), and mouse monoclonal β-actin antibody (Abcam; 1:5,000 dilution). Blots were incubated with primary antibody overnight at 4°C and with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary IgG antibody (Amersham Biosciences; 1:10,000 dilution) for 1 h at room temperature, and except MAGE-11 antibody, the secondary antibody was incubated at 4°C for 1 h. Signals were detected using chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Pierce).

**DNA Methylation Analyses**

The 5′ end of MAGE-A11 transcript variant 1 (National Center for Biotechnology Information accession no. NM-005366) contains a CpG island (500 bp, GC = 59.4%, CpG observed/expected = 0.6) as predicted by www.uscnorris.com/cpgislands2/cpg.aspx. Sodium bisulfite sequencing was done as described (25) using genomic DNA from tissue samples (50-150 mg) extracted using the AllPrep DNA/RNA extraction kit (Qiagen). Frozen tissue was homogenized in RLT tissu buff er using a polytron. Extracts were centrifuged at 12,000 × g for 3 min and stored at -80°C. Genomic DNA was sodium bisulfite converted using the EZ DNA methylation kit (Zymo Research). Primers for amplification of the MAGE-11 CpG island were designed (New England Biolabs) served as positive control for DNA methylation: GGTATTTTTATGAT-3′ and +47 reverse 5′-CAGCTTCAAAAACACCTTCAAAA-3′. Sodium bisulfite pyrosequencing was done as described (40) using MAGE-A11 forward PCR primer -219 5′-GGAGGATGGTGA-GGTATTTTATG-3′ and MAGE-A11 reverse PCR primer -70 5′-biotin-AACTTCCCATAATTTCAAACAAA-3′, and MAGE-A11 sequencing primer -212 5′-TTGAGGTATTTT-TATGATT-3′. PCR cycling conditions were 95°C for 30 s, 59°C for 30 s, and 72°C for 1 min for 45 cycles. Pyrosequencing was done on duplicate samples repeated at least twice. Human genomic DNA modified with bacterial CpG methylase SssI (New England Biolabs) served as positive control for DNA methylation. Genomic DNA from DNMT1 and DNMT3b HCT116 colorectal cancer cells was used as a negative DNA methylation control (41).

**Cell Treatments with DAC**

PC-3 and DU145 cells were treated with 0, 0.1, 0.5, and 1.0 μmol/L DAC (Sigma) in PBS at 0 and 48 h. RNA was harvested 120 h (5 days) post-treatment and analyzed for MAGE-11 using RT-PCR (15).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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