



Published in final edited form as:

*Prostate*. 2012 November ; 72(15): 1659–1668. doi:10.1002/pros.22519.

## MUC1-C ONCOPROTEIN CONFERS ANDROGEN-INDEPENDENT GROWTH OF HUMAN PROSTATE CANCER CELLS

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### Abstract

**Background**—The mucin 1 (MUC1) heterodimeric oncoprotein is overexpressed in human prostate cancers with aggressive pathologic and clinical features. However, few insights are available regarding the functional role of MUC1 in prostate cancer.

**Methods**—Effects of MUC1-C on AR expression were determined by RT-PCR, immunoblotting and AR promoter activation. Coimmunoprecipitations, direct binding assays and chromatin immunoprecipitation (ChIP) studies were performed to assess the interaction between MUC1-C and AR. Cells were analyzed for invasion, growth in androgen-depleted medium and sensitivity to MUC1-C inhibitors.

**Results**—The present studies in androgen-dependent LNCaP and LAPC4 prostate cancer cells demonstrate that the oncogenic MUC1-C subunit suppresses AR expression. The results show that MUC1-C activates a posttranscriptional mechanism involving miR-135b-mediated downregulation of AR mRNA levels. The results further demonstrate that MUC1-C forms a complex with AR through a direct interaction between the MUC1-C cytoplasmic domain and the AR DNA-binding domain. In addition, MUC1-C associates with AR in a complex that occupies the PSA promoter. The interaction between MUC1-C and AR is associated with induction of the epithelial-mesenchymal transition (EMT) and increased invasion. MUC1-C also conferred growth in androgen-depleted medium and resistance to bicalutamide treatment. Moreover, expression of MUC1-C resulted in sensitivity to the MUC1-C inhibitor GO-203 with inhibition of growth in vitro. GO-203 treatment also inhibited growth of established tumor xenografts in nude mice.

**Conclusions**—These findings indicate that MUC1-C suppresses AR expression in prostate cancer cells and confers a more aggressive androgen-independent phenotype that is sensitive to MUC1-C inhibition.

### Keywords

MUC1-C; androgen receptor; prostate cancer; androgen-independent growth; targeted therapy

### Introduction

Mucin 1 (MUC1) is a heterodimeric complex that is aberrantly overexpressed in human prostate and other carcinomas [1]. As such, MUC1 has been considered an attractive target for the development anti-cancer agents. However, early attempts at targeting MUC1, particularly with antibodies, were largely unsuccessful [2]. In this regard, MUC1 consists of

two subunits that result from autocleavage of a single polypeptide product from the *MUC1* gene [1]. The MUC1 N-terminal cleavage fragment (MUC1-N) contains the characteristic glycosylated tandem repeat structure of mucin family members [1]. MUC1-N forms a cell surface complex with the MUC1 C-terminal fragment that spans the cell membrane and includes a transforming cytoplasmic domain [1,3,4]. The demonstration that MUC1-C is oncogenic provided the basis for the design of inhibitors that block its function [5]. In this capacity for inducing transformation, MUC1-C interacts with the epidermal growth factor receptor (EGFR) and other receptor tyrosine kinases (RTKs) at the cell membrane [1,6]. In addition, with overexpression as found in carcinoma cells, MUC1-C accumulates in the cytoplasm and localizes to the nucleus, where it contributes to the regulation of gene expression [1,7]. In the nucleus, MUC1-C interacts with certain transcription factors, such as NF- $\kappa$ B p65 and STAT3, and promotes activation of their target genes [8,9]. The *MUC1* gene itself is activated by NF- $\kappa$ B p65 and STAT3, thus forming an autoinductive loop in which MUC1-C contributes to the overexpression of MUC1 in carcinoma cells [8,9]. As an integral part of this loop, the MUC1-C cytoplasmic domain contains a CQC motif that is necessary for the formation of MUC1-C dimers and the import of MUC1-C to the nucleus [10]. Blocking the MUC1-C CQC motif with cell-penetrating peptides thus inhibits nuclear MUC1-C localization and its transforming function [11].

MUC1 is overexpressed in prostate cancers that are associated with more aggressive disease [12–17]. In this context, MUC1 expression was detected in ~90% of primary prostate cancers that were Gleason grade 7 or were metastatic to lymph nodes [12,13]. Moreover, gene expression profiling of prostate cancers has shown that MUC1 is highly expressed in those with aggressive clinicopathologic features and an elevated risk of recurrence [18]. In human prostate cancer cell lines, MUC1 is expressed at high levels in the androgen-independent DU145 and PC3 models, which have low to undetectable androgen receptor (AR) abundance [8,19]. By contrast, the androgen-dependent LNCaP, androgen-responsive CWR22Rv1, and androgen-sensitive MDA PCa 2b prostate cancer cells express AR and little if any MUC1, supporting a potential inverse relationship between these two proteins [8,19]. Indeed, stable introduction of AR in PC3 cells was associated with downregulation of MUC1 expression [19]. The basis for this effect was attributed in part to AR occupancy of the *MUC1* promoter and suppression of *MUC1* gene transcription [19]. In addition, AR-mediated upregulation of miR-125b [20] was shown to contribute to suppression of MUC1 translation [19,21]. Thus, AR signaling suppresses MUC1 expression by transcriptional and posttranscriptional mechanisms. In concert with these observations, treatment of LNCaP, CWR22Rv1 and MDA PCa 2b cells with a MUC1-C inhibitor had no apparent effect on growth or survival [22]. However, the MUC1-C-positive DU145 and PC3 cells responded to MUC1-C inhibition with induction of cell death in vitro [22]. Established DU145 and PC3 tumor xenografts in nude mice were also sensitive to MUC1-C inhibitor treatment as evidenced by prolonged regressions [22]. These findings indicate that AR signaling downregulates MUC1 abundance and that MUC1 is overexpressed in certain prostate cancer cells, which in turn are sensitive to MUC1-C inhibitors.

The present studies demonstrate that MUC1-C suppresses AR expression in prostate cancer cells by a posttranscriptional miR-135b-mediated mechanism. The results also show that MUC1-C interacts directly with AR and forms complexes with AR on the promoter of the *PSA* gene. The interaction between MUC1-C and AR is associated with induction of (i) EMT, (ii) invasion, (iii) androgen-independent growth, and (iv) sensitivity to MUC1-C inhibition.

## Materials and Methods

### Cell culture

Human LNCaP prostate cancer cells (ATCC) were cultured in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. LAPC4 cells were grown in Iscove's Modified Dulbecco's medium (IMDM) with 5% HI-FBS, antibiotics and L-glutamine. LNCaP and LAPC4 cells were infected with lentiviruses expressing GFP or MUC1-C and selected in hygromycin. In certain experiments, the cells were grown in phenol red-free medium containing charcoal-stripped serum (CSS) (Cellgro). Cells were treated with bicalutamide (Sigma). Cells were also treated with the MUC1-C inhibitor GO-203 or the control peptide CP-2 as described [23].

### Immunoprecipitation and immunoblotting

Lysates from subconfluent cells were prepared as described [8]. Soluble proteins were precipitated with anti-AR (H-280; Santa Cruz Biotechnology) or anti-MUC1-C [24]. Immune precipitates and lysates not subjected to precipitation were immunoblotted with anti-AR, anti-MUC1-C, anti-PSA (Santa Cruz Biotechnology), anti-β-actin (Sigma), anti-E-cadherin (Cell Signaling Technology) and anti-vimentin (Santa Cruz Biotechnology). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare Biosciences).

### RT-PCR analysis of mRNA expression

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). RNAs were analyzed using the One-Step RT-PCR Kit with Platinum Taq (Invitrogen) and AR-specific primers or GAPDH-specific primers (Supplemental Table 1). Amplified fragments were analyzed by electrophoresis in 2% agarose gels.

### AR promoter-luciferase assays

Cells were transfected with pGL3 or pGL3-pAR-Luc and, as an internal control, SV-40-*Renilla*-Luc (Promega) in the presence of Lipofectamine 2000. After 48 h, the cells were lysed in passive lysis buffer. The lysates were analyzed for firefly and *Renilla* luciferase activities using the dual luciferase assay kit (Promega).

### Analysis of miRNA expression

Total RNA was assessed for miR-135b and miR-147 expression using a small RNA specific RT-PCR kit (Systems Biosciences) with a universal reverse primer and specific forward primers (Supplemental Table 2). Human U6 small RNA was used as a control.

### In vitro binding assays

GST, GST-MUC1-CD, GST-MUC1-CD(1–46), and GST-MUC1-CD(46–72) were prepared as described [8]. GST-AR, GST-AR(3–557), GST-AR(528–627) and GST-AR(625–910) were generated as described [25]. Purified GST-MUC1-CD and GST-AR(528–627) were cleaved with thrombin to remove the GST moiety. Adsorbates to glutathione-conjugated beads were analyzed by immunoblotting.

### Chromatin immunoprecipitation (ChIP) assays

Soluble chromatin was prepared as described [26] and precipitated with anti-AR, anti-MUC1-C or a control nonimmune IgG. For re-ChIP assays, AR complexes from the initial ChIP were eluted and reimmunoprecipitated with anti-MUC1-C as described [19]. For PCR,

2  $\mu$ l for a 50  $\mu$ l DNA extraction were used with 25 to 35 cycles of amplification. The primers used for PCR and qPCR are listed in Supplemental Tables 3 and 4, respectively.

### Cell invasion assays

Cell invasion assays were performed using 8 mm pores size transwell chambers (Costar) coated with 1:1000 Matrigel (BDBioscience) as described [27].

### Tumor xenograft studies

Four- to 6-week-old castrated BALB/c nu/nu male mice were injected subcutaneously with  $1 \times 10^7$  LNCaP/MUC1-C cells in the flank. When tumors were detectable, the mice were pair-matched into control and treatment groups of 10 mice each. Mice were excluded if the tumors were not within 15% of the mean tumor volume. PBS (control vehicle) or 7.5 mg/kg body weight GO-203 (dissolved in PBS) were administered daily by intravenous injection for 21 days. Tumor volume was calculated as described [23].

## Results

### MUC1-C downregulates AR expression

Previous studies showed that AR regulates MUC1-C expression in prostate cancer cells [19]. The androgen-dependent LNCaP prostate cancer cells express AR and low to undetectable levels of MUC1-C (Fig. 1A). By contrast, the androgen-independent DU-145 prostate cancer cells have the opposite pattern, that is, undetectable levels of AR and upregulation of MUC1-C (Fig. 1A). To assess the effects of MUC1-C on AR levels, we stably expressed MUC1-C or, as a control, GFP in LNCaP cells (Fig. 1B). Notably, MUC1-C expression was associated with marked downregulation of AR protein (Fig. 1B). MUC1-C expression was also associated with decreases in PSA levels, consistent with AR-dependent regulation of the *PSA* gene (Fig. 1B). To extend these observations, androgen-dependent LAPC4 prostate cancer cells were stably transfected to express GFP or MUC1-C. Like LNCaP cells, LAPC4 cells express AR, but not MUC1-C (Fig. 1C). Moreover, expression of MUC1-C in LAPC4 cells resulted in downregulation of AR and PSA protein (Fig. 1C). RT-PCR further demonstrated that MUC1-C decreases AR expression at the mRNA level in LNCaP (Fig. 1D) and LAPC4 (Fig. 1E) cells. These findings indicate that MUC1-C suppresses AR expression.

### MUC1-C decreases AR mRNA levels by a miRNA-dependent mechanism

To assess the effects of MUC1-C on *AR* gene transcription, we transfected the LNCaP/GFP and LNCaP/MUC1-C cells with a vector containing the *AR* promoter upstream to the *luciferase* gene (pAR-Luc). Analysis of luciferase activity demonstrated that MUC1-C has little if any effect on *AR* promoter activation (Fig. 2A). Similar results were obtained in LAPC4 cells (data not shown), indicating that MUC1-C downregulates AR expression at the post-transcriptional level. Previous work has identified certain miRNAs downregulate AR expression in prostate cancer cells [28,29]. Among these, we found that expression of MUC1-C in LNCaP cells is associated with upregulation of miR-135b and miR-147 (Fig. 2B). To confirm that miR-135b downregulates AR mRNA levels, we overexpressed miR-135b in LNCaP cells. Analysis of AR mRNA levels at 48 and 96 h after transfection demonstrated decreases in AR transcripts (Fig. 2C). Overexpression of miR-135b was also associated with decreases in AR protein (Fig. 2D). These findings indicate that MUC1-C decreases AR expression at least in part by upregulation of miR-135b.

### MUC1-C cytoplasmic domain binds directly to AR

Previous work demonstrated that MUC1-C associates with ER $\alpha$  and that the MUC1-C cytoplasmic domain binds directly to the ER $\alpha$  DNA binding domain (DBD) [26]. To determine whether MUC1-C associates with AR, coimmunoprecipitation studies were performed on lysates from LNCaP/MUC1-C cells. The detection of AR-MUC1-C complexes provided support for the contention that MUC1-C interacts with AR (Fig. 3A). To define in part the basis for the AR-MUC1-C interaction, LNCaP cell lysates were incubated with GST or GST-MUC1-CD. The results show that AR binds to GST-MUC1-CD and not GST, indicating that AR associates with the MUC1-C cytoplasmic domain (Fig. 3B). AR consists of an AF1 domain, DNA-binding domain (DBD), hinge region (H) and the AF2/ligand binding domain (LBD) (Fig. 3C). Incubation of MUC1-CD with GST-AR(3–557), GST-AR(528–627) or GST-AR(625–910) demonstrated that MUC1-CD binds to the AR DBD region (Fig. 3D). To further assess the interaction between MUC1-CD and the AR DNA binding domain, we incubated AR(528–627) with GST or GST-MUC1-CD. Analysis of the adsorbates with an anti-AR-DBD antibody confirmed direct binding of MUC1-CD to the AR-DBD (Fig. 3D). The MUC1-C cytoplasmic domain consists of 72 amino acids. The results also demonstrate that MUC1-CD(46–72), and not MUC1-CD(1–46), confers the interaction (Fig. 3E). These findings indicate that the MUC1-C cytoplasmic domain binds directly to AR at the DBD.

### MUC1-C associates with AR on the PSA promoter

To determine whether AR associates with MUC1-C in the nucleus, we performed chromatin immunoprecipitation (ChIP) studies on the *PSA* gene promoter, which contains an androgen response element at positions –170 to –165 (AGAACA) (Fig. 4A). AR occupancy was detectable on the *PSA* promoter in LNCaP/GFP and LNCaP/MUC1-C cells (Fig. 4B). ChIP analysis further demonstrated that MUC1-C also occupies the *PSA* promoter in LNCaP/MUC1-C, but not LNCaP/GFP cells (Fig. 4B). ChIP qPCR analysis confirmed that both AR and MUC1-C occupy the *PSA* promoter in the LNCaP/MUC1-C cells (Fig. 4C). Re-ChIP studies were then performed to determine whether AR and MUC1-C are present as a complex. In contrast to the control LNCaP/GFP cells, re-ChIP analysis of the LNCaP cells demonstrated that AR associates with MUC1-C on the *PSA* promoter (Fig. 4D). Re-ChIP qPCR analysis confirmed that AR and MUC1-C form a complex on the *PSA* promoter in the LNCaP/MUC1-C cells (Fig. 4E). These findings indicate that MUC1-C interacts with AR on the *PSA* promoter.

### MUC1-C induces EMT and invasion

Overexpression of MUC1 in breast and pancreatic cancer cells has been associated with induction of EMT [30,31]. Moreover, downregulation of AR abundance has been linked to EMT and invasion of prostate cancer cells [32]. Accordingly, we asked if MUC1-C-induced suppression of AR expression is associated with markers of EMT. Indeed, the LNCaP/MUC1-C cells exhibited a decrease in E-cadherin levels as compared to that in LNCaP/GFP cells (Fig. 5A). In addition, MUC1-C expression was associated with upregulation of vimentin, consistent with the induction of EMT (Fig. 5A). Consistent with these results, MUC1-C decreased E-cadherin expression and upregulated vimentin levels in LAPC4 cells (Fig. 5B). To assess the effects of MUC1-C on invasion, studies were performed by seeding LNCaP cells in transwell plates. Invasion of the LNCaP/MUC1-C cells was increased visually compared to that found for the LNCaP/GFP cells (Fig. 5C). Quantification of the results confirmed that MUC1-C significantly increases LNCaP cell invasion (Fig. 5D).

## MUC1-C confers androgen-independent growth

In association with the induction of EMT and invasion, LNCaP cell proliferation in complete medium was increased by MUC1-C expression (Fig. 6A). Moreover, as expected, proliferation of LNCaP and LNCaP/GFP cells was suppressed in charcoal-stripped medium (Fig. 6B). Importantly and in contrast, LNCaP/MUC1-C cells readily proliferated in this setting of androgen depletion (Fig. 6B). We also found that the LNCaP/MUC1-C cells grow in the presence of the anti-androgen, bicalutamide (Fig. 6C). Consistent with these results, LAPC4/MUC1-C, but not LAPC4/GFP, cells proliferated in androgen-depleted medium (Fig. 6D), indicating that MUC1-C confers androgen-independent growth.

## Inhibition of MUC1-C blocks binding to AR and induces death of LNCaP/MUC1-C cells

The MUC1-C cytoplasmic domain contains a CQC motif that is necessary for MUC1-C dimerization and interaction with other proteins (Fig. 7A) [10]. To inhibit MUC1-C dimerization, the cell-penetrating peptide GO-203 was synthesized that binds directly to the MUC1-C CQC motif in the cytoplasmic domain and blocks reactivity of this site (Fig. 7B) [11,23]. As a control, the CP-2 peptide, in which the Cys residues have been altered to Ala, is inactive in binding to the MUC1-C CQC motif (Fig. 7B). Previous studies have shown that inhibition of MUC1-C has no effect on growth of MUC1-negative LNCaP cells [22]. Notably, however, GO-203 was highly effective in inhibiting growth of LNCaP/MUC1-C cells (Fig. 7B). By contrast, the control CP-2 had no apparent effect on LNCaP/MUC1-C cell growth (Fig. 7B), indicating that expression of MUC1-C in LNCaP cells confers sensitivity to MUC1-C inhibition. To extend this analysis, we injected LNCaP/MUC1-C cells into the flanks of castrated nude mice. In contrast to LNCaP cells which do not grow in castrated mice [33], LNCaP/MUC1-C cells formed aggressive tumors that reached 3000 mm<sup>3</sup> by day 14 when they were sacrificed (Fig. 7C). In addition, treatment with 7.5 mg/kg/d GO-203 intravenously for 21 d was associated with a significant delay in LNCaP/MUC1-C tumor growth (Fig. 7C).

## Discussion and Conclusions

### MUC1-C oncoprotein suppresses AR expression

Previous work in human prostate cancer cell lines demonstrated a reciprocal relationship between MUC1 and AR expression [19,22]. AR signaling was thus shown to suppress MUC1 abundance by direct downregulation of the MUC1 promoter and by a posttranscriptional miR-125b-mediated mechanism [19]. These findings, however, were not sufficient to explain why overexpression of MUC1 is associated with low AR levels in androgen-independent prostate cancer cell lines. In the present studies, androgen-dependent LNCaP and LAPC4 cells, which express AR, but not MUC1, were used as a model to assess the effects of the oncogenic MUC1-C subunit. Interestingly, we found that MUC1-C downregulates AR levels and that this response is conferred by a posttranscriptional mechanism (Fig. 7D). Other work had shown that AR expression is suppressed by certain miRNAs [28,29]; consequently, we searched for the induction of those miRNAs by MUC1-C and identified miR-135b. We also found that MUC1-C increases miR-147 expression, which has similarly been shown to downregulate AR expression [29]. Thus, MUC1-C increases levels of at least two miRNAs that decrease AR abundance (Fig. 7D), and the present results do not exclude the possibility that other miRNAs may be involved in MUC1-C-mediated AR suppression. Decreases in AR expression have been associated with induction of EMT and the invasion potential of prostate cancer cells [32]. Moreover, MUC1 has been shown to confer the induction of EMT in other types of carcinoma cells [30,31]. In concert with these findings, MUC1-C-mediated AR suppression was associated with induction of EMT as detected by loss of the epithelial marker E-cadherin and gain of the mesenchymal marker vimentin. EMT has been linked to invasion and metastases [34].

Accordingly, we also found that MUC1-C expression in LNCaP cells is associated with significant increases in invasion. Overexpression of MUC1 in primary prostate cancer cells has been linked to more aggressive disease, recurrence and metastases [12,13,18]. The present findings that MUC1-C induces EMT may thus explain, at least in part, how MUC1-C contributes to the aggressiveness of prostate cancers.

### **MUC1-C interacts directly with AR**

MUC1-C localizes to the nucleus of carcinoma cells and contributes to the regulation of gene expression [1]. In breast cancer cells, MUC1-C binds directly to ER $\alpha$  at its DBD and stimulates ER $\alpha$ -mediated transcription [26]. The present studies demonstrate that the MUC1-C cytoplasmic domain binds to the AR DBD (Fig. 7D). Assessment of the binding with fragments of the 72 aa MUC1-C cytoplasmic domain showed that the region from aa 46 to 72 is responsible for the interaction. This region of the MUC1-C cytoplasmic domain is phosphorylated by EGFR and SRC [35,36], and contains a SAGNGGSSLS motif that confers direct binding to the Wnt effector  $\beta$ -catenin [4,37]. Protein inhibitor of activated STAT  $\gamma$  (PIAS $\gamma$ ) binds to the AR DBD and suppresses AR activity without affecting its binding to DNA [38]. The MST1 serine-threonine kinase also interacts with the AR DBD and inhibits AR activity, but by a mechanism that involves decreases in DNA binding [39]. In addition, the FOS [40] and FKHR [25] transcription factors have been shown to interact with the AR DBD and affect its activity. To assess in part the functional significance of the MUC1-C-AR interaction, ChIP studies showed that MUC1-C forms a complex with AR on the ARE of the *PSA* promoter (Fig. 7D). How MUC1-C affects AR-mediated transcription will require further study. However, the Wnt/ $\beta$ -catenin and PI3K/AKT pathways, which are both activated by MUC1-C [4,41,42], have been shown to stimulate AR function in positive feed-forward loops that lead to androgen-independent AR activation [43–45]. By extension, MUC1-C binds directly to  $\beta$ -catenin [4,37] and could contribute to the formation of AR- $\beta$ -catenin complexes, which enhance AR-mediated transcriptional activity [46].

### **Does MUC1-C contribute to the survival of prostate cancer cells?**

The development of cell-penetrating peptide [11] and small molecule [47] inhibitors of MUC1-C has permitted the assessment of malignant cell dependence on this oncoprotein for growth and survival. Studies in androgen-independent DU145 and PC3 prostate cancer cells demonstrated that MUC1-C inhibition is associated with slowing of growth and induction of death in a setting of MUC1-C overexpression and low AR abundance [22]. By contrast, inhibition of MUC1-C in androgen-dependent LNCaP cells had no effect on proliferation or viability [22], indicating that dependence on MUC1-C may be associated with androgen-independence. The present studies in LNCaP and LAPC4 cells expressing MUC1-C lend support to that contention. MUC1-C conferred cell growth in the presence of androgen depletion and bicalutamide, promoting the conversion from androgen-dependence to androgen-independence. Importantly, the LNCaP/MUC1-C cells also developed sensitivity to MUC1-C inhibition as indicated by loss of survival in vitro. MUC1-C inhibitor treatment of mice bearing established LNCaP/MUC1-C tumors further demonstrated growth delay. These results and those obtained with DU145 and PC3 tumors indicate that certain prostate cancer cells [22], particularly those with MUC1-C overexpression and androgen-independence, are sensitive to MUC1-C inhibitors. The development of castrate-resistant prostate cancer occurs in the presence of continued AR activation by multiple mechanisms that include AR gene amplification and mutations, intracellular production of AR ligands and interactions with other signaling pathways and effectors, such as  $\beta$ -catenin [48–51]. Thus, the present results and the demonstration that MUC1 is overexpressed in certain subsets of primary prostate cancers invokes the possibility that MUC1-C inhibitors may be effective in the treatment of this disease. In this regard, the first-in-man MUC1-C inhibitor, GO-203-2C, has entered Phase I evaluation in patients with refractory solid tumors. Based

on the present results, it is anticipated that this agent will be studied for effectiveness against MUC1-C expressing castrate-resistant prostate cancers that are metastatic and unresponsive to available therapies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by Department of Defense Prostate Cancer Idea Award W81XWH-08-1-0093 and National Cancer Institute Grants CA97098 and CA42802.

D. Kufe holds equity in Genus Oncology and is a consultant to the company.

The authors thank Dr. Wenlong Bai for providing the GST-AR constructs.

## Abbreviations

<b>MUC1</b>	mucin 1
<b>MUC1-C</b>	MUC1 C-terminal subunit
<b>MUC1-CD</b>	MUC1 cytoplasmic domain
<b>AR</b>	androgen receptor
<b>EMT</b>	epithelial-mesenchymal transition
<b>PSA</b>	prostate specific antigen

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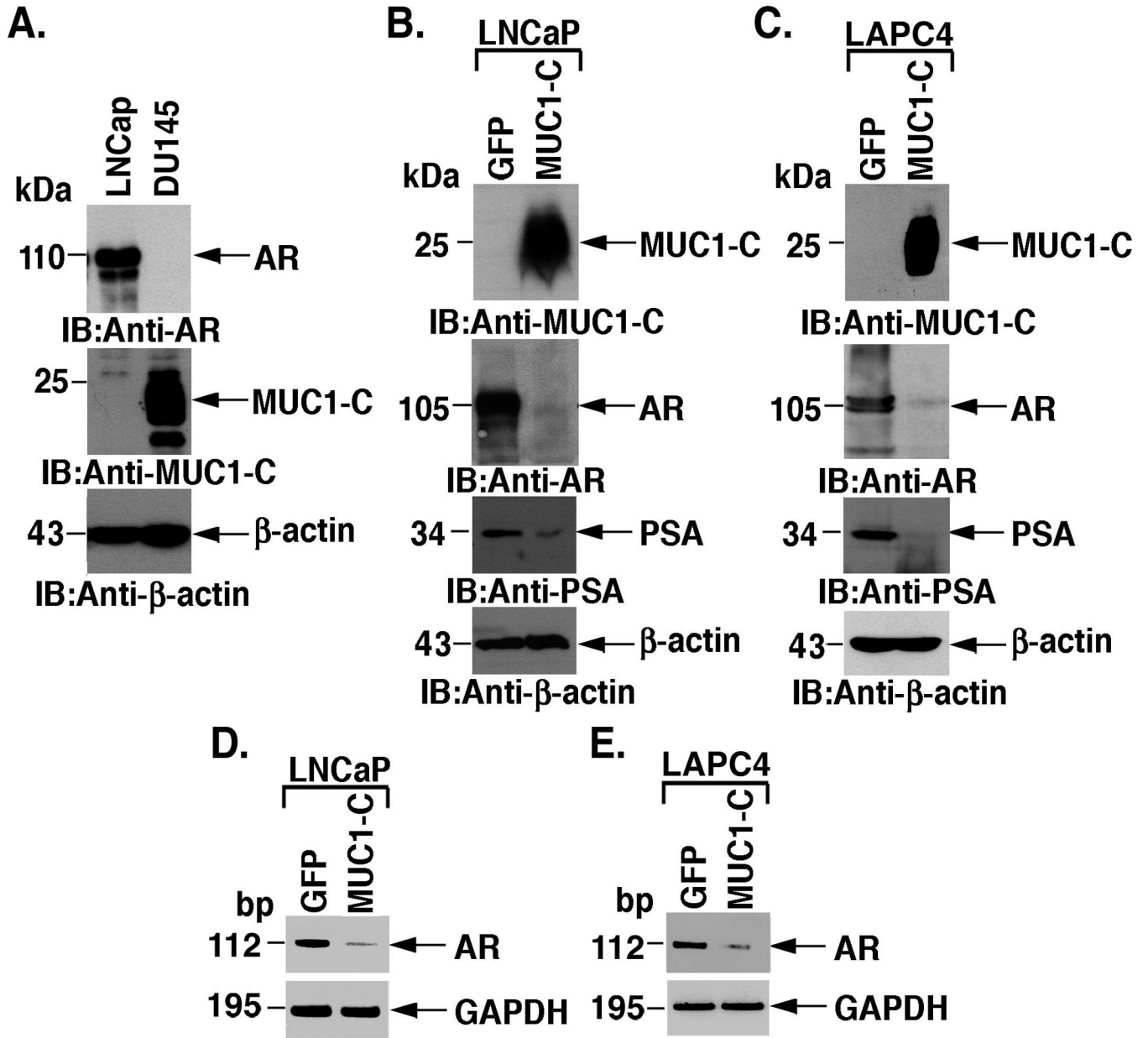
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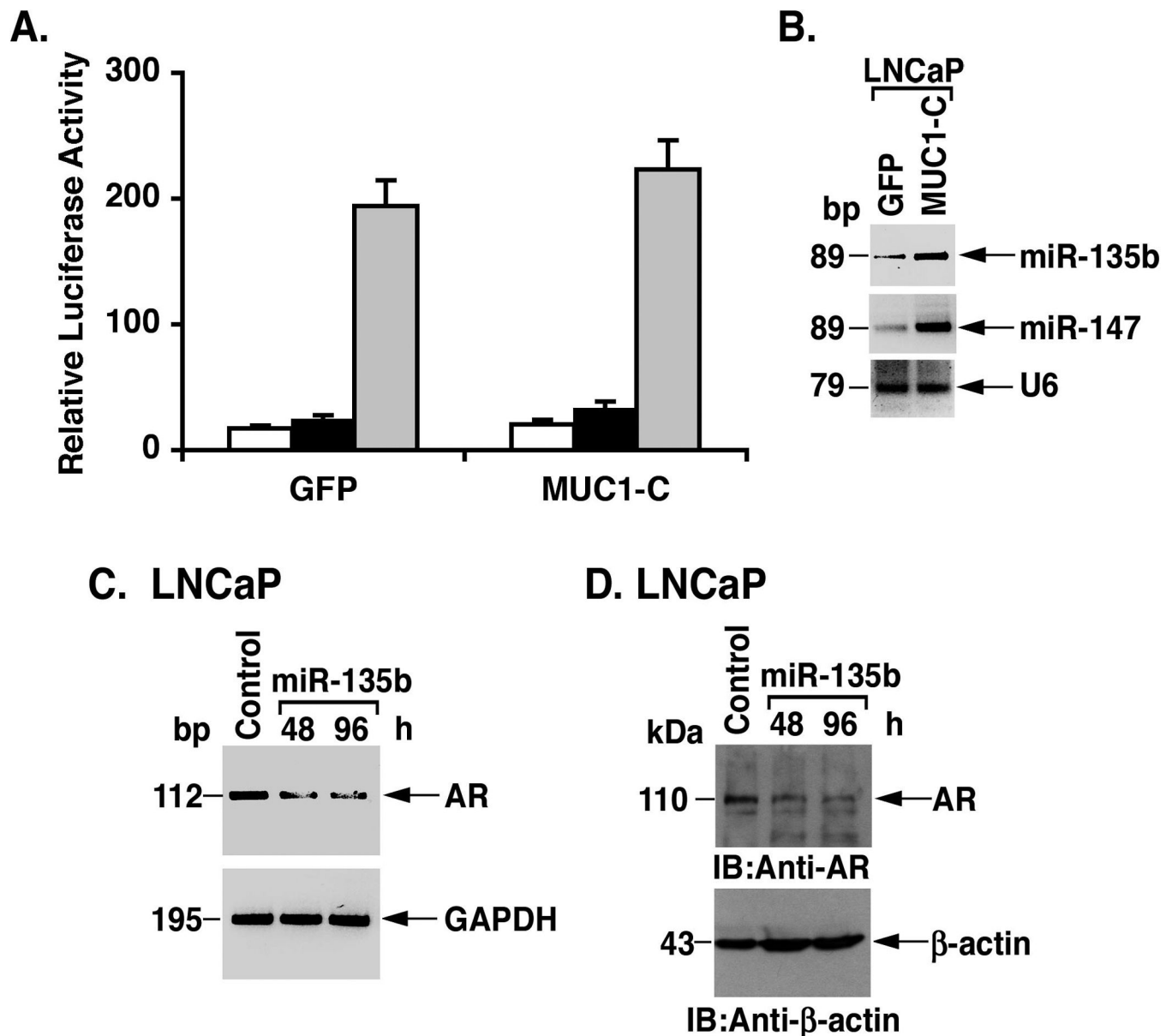
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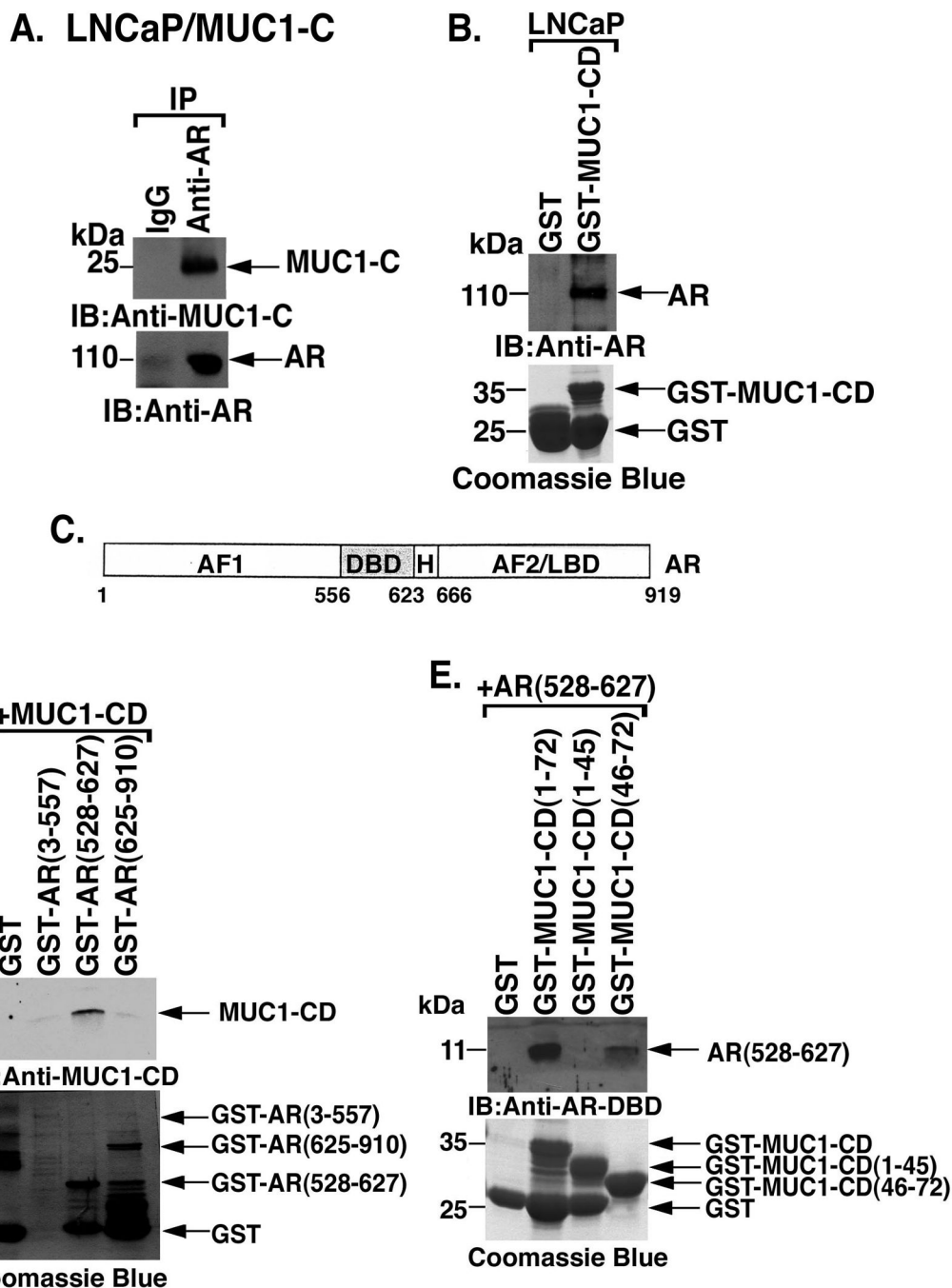


**Figure 1. MUC1-C suppresses AR expression**

A. Lysates from LNCaP and DU145 cells were immunoblotted with the indicated antibodies. B and C. LNCaP (B) and LAPC4 (C) cells were stably transfected to express GFP or MUC1-C. Lysates were immunoblotted with the indicated antibodies. D and E. Total RNA from the indicated LNCaP (D) and LAPC4 (E) cells was analyzed by RT-PCR for AR and, as a control, GAPDH mRNA levels.



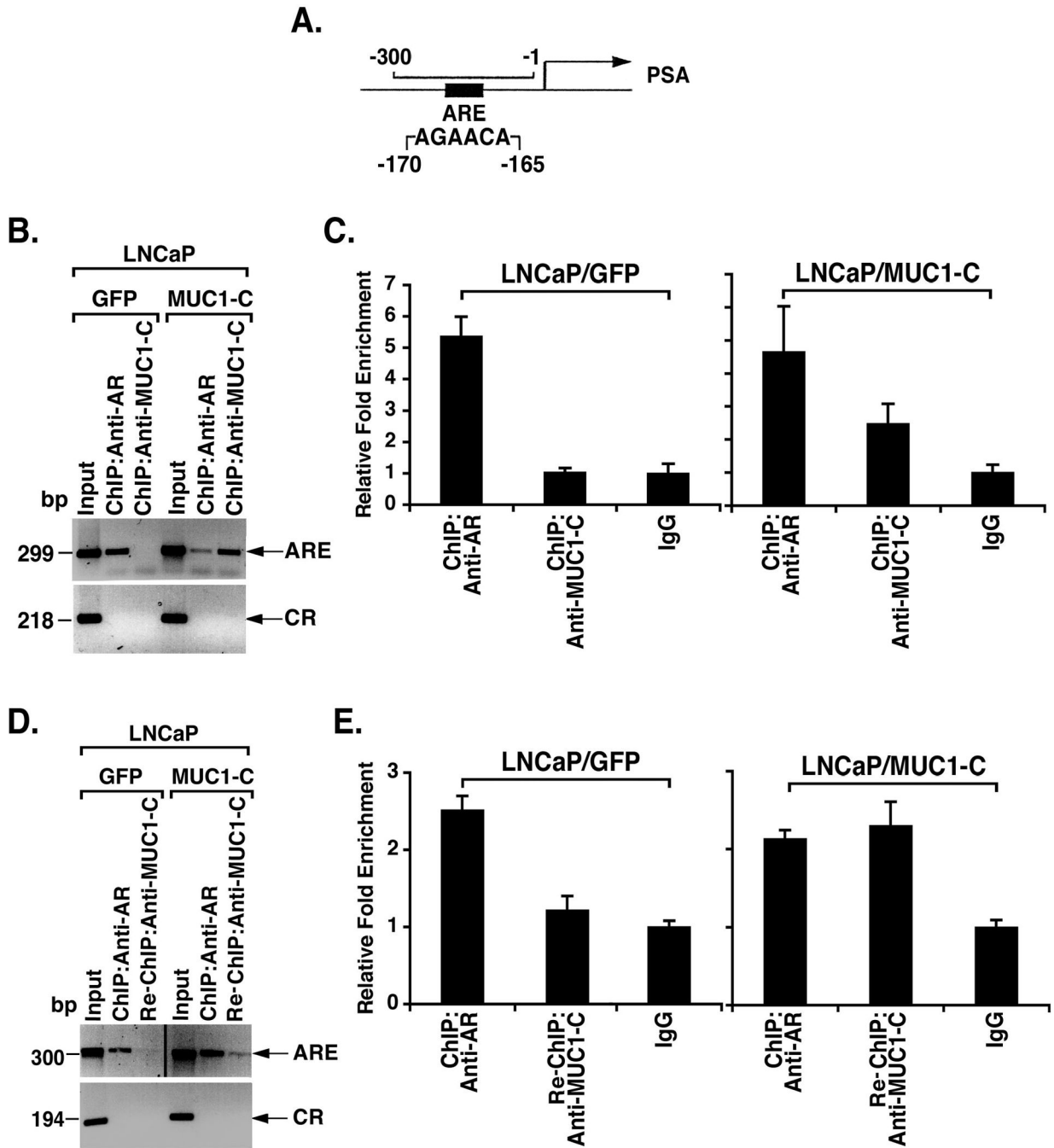
**Figure 2. MUC1-C suppresses AR expression by a miR-135b-mediated mechanism**  
 A. LNCaP/GFP and LNCaP/MUC1-C cells were transfected with the empty pGL3 vector or 0.1 µg (open bars), 0.2 µg (shaded bars) and 0.5 µg (solid bars) pAR-Luc. As an internal control, the cells were also transfected with the SV-40-Renilla-Luc plasmid. Luciferase activity was measured at 48 h after transfection. The results are expressed as relative luciferase activity (mean±SD from three separate experiments) compared to that obtained from cells transfected with the empty pGL3 vector (assigned a value of 1). B. Total RNA from the indicated LNCaP cells was analyzed for miR-135b and miR-147 levels. U6 expression was included as a control. C and D. LNCaP cells were transfected with a control scrambled siRNA for 48 h or miR-135b for the indicated times. Total RNA was analyzed for AR and GAPDH mRNA levels by RT-PCR (C). Lysates were immunoblotted with the indicated antibodies (D).



**Figure 3. MUC1-C cytoplasmic domain binds directly to the AR DNA-binding domain**

A. Lysates from LNCaP/MUC1-C cells were precipitated with anti-MUC1-C or a control IgG. The precipitates were immunoblotted with the indicated antibodies. B. Lysates from LNCaP cells were incubated with GST or GST-MUC1-CD. The adsorbates were immunoblotted with anti-AR. Input of the GST proteins was assessed by Coomassie blue staining. C. Schematic representation of the AR protein with the AF1 domain, the DNA-binding domain (DBD), hinge region (H) and the AF2/ligand binding domain (LBD). D. MUC1-CD was incubated with GST, GST-AR(3-557), GST-AR(528-627) or GST-AR(625-910). The adsorbates were immunoblotted with anti-MUC1-CD. Input of the GST proteins was assessed by Coomassie blue staining. E. AR(528-627) was incubated with

GST, GST-MUC1-CD, GST-MUC1-CD(1–45) or GST-MUC1-CD(46–72). The adsorbates were immunoblotted with anti-AR-DBD. Input of the GST proteins was assessed by Coomassie blue staining.

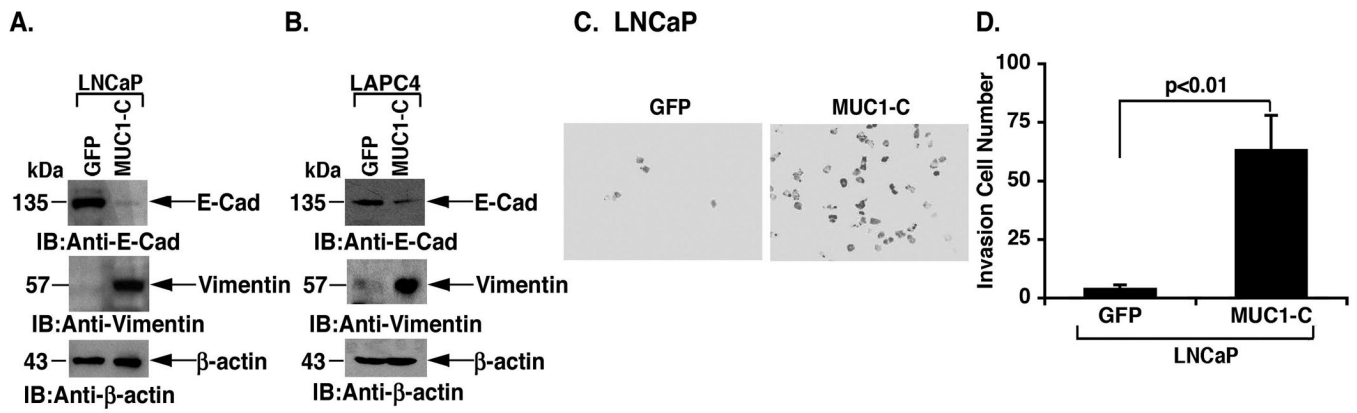


**Figure 4. MUC1-C associates with AR on the PSA promoter**

A. Schematic representation of the *PSA* gene promoter with positioning of the androgen response element (ARE). B and C. Soluble chromatin from LNCaP/GFP and LNCaP/MUC1-C cells was precipitated with anti-AR or anti-MUC1-C and then analyzed for *PSA* promoter sequences. The final DNA samples were amplified by PCR (B) and qPCR (C) with pairs of primers for the AR binding region (−300 to −1) or a control region (CR; −4893 to −4675). The results (mean±SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with the IgG control (C). D and E. Soluble chromatin from LNCaP/GFP and LNCaP/MUC1-C cells was precipitated with anti-AR. The anti-AR precipitates were released, reimmunoprecipitated with anti-MUC1-C, and then analyzed for

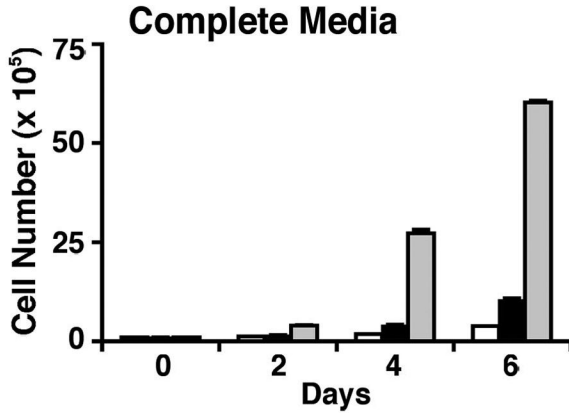
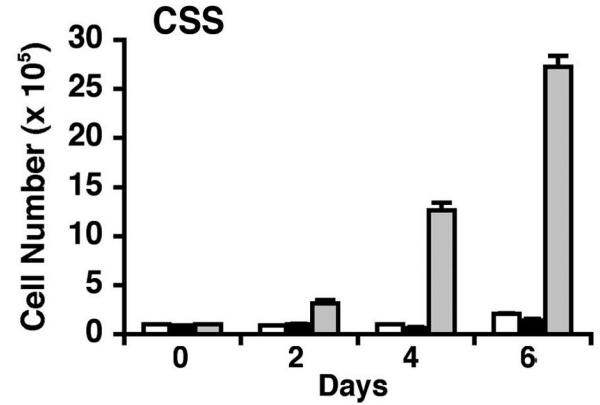
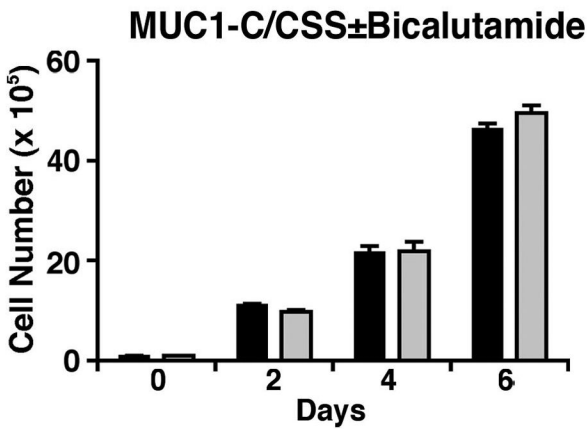
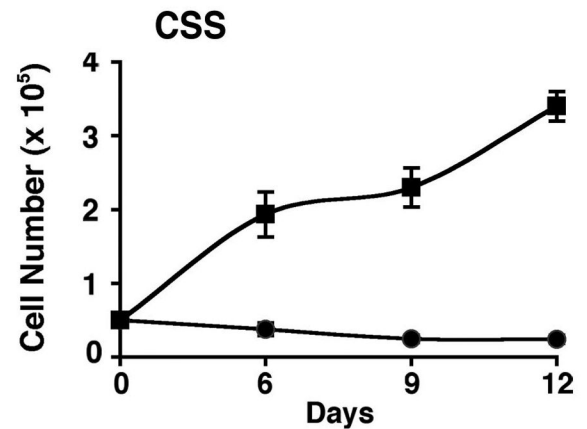


*PSA* promoter sequences. The final DNA samples were amplified by PCR (D) and qPCR (E). The results (mean $\pm$ SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with the IgG control (right).

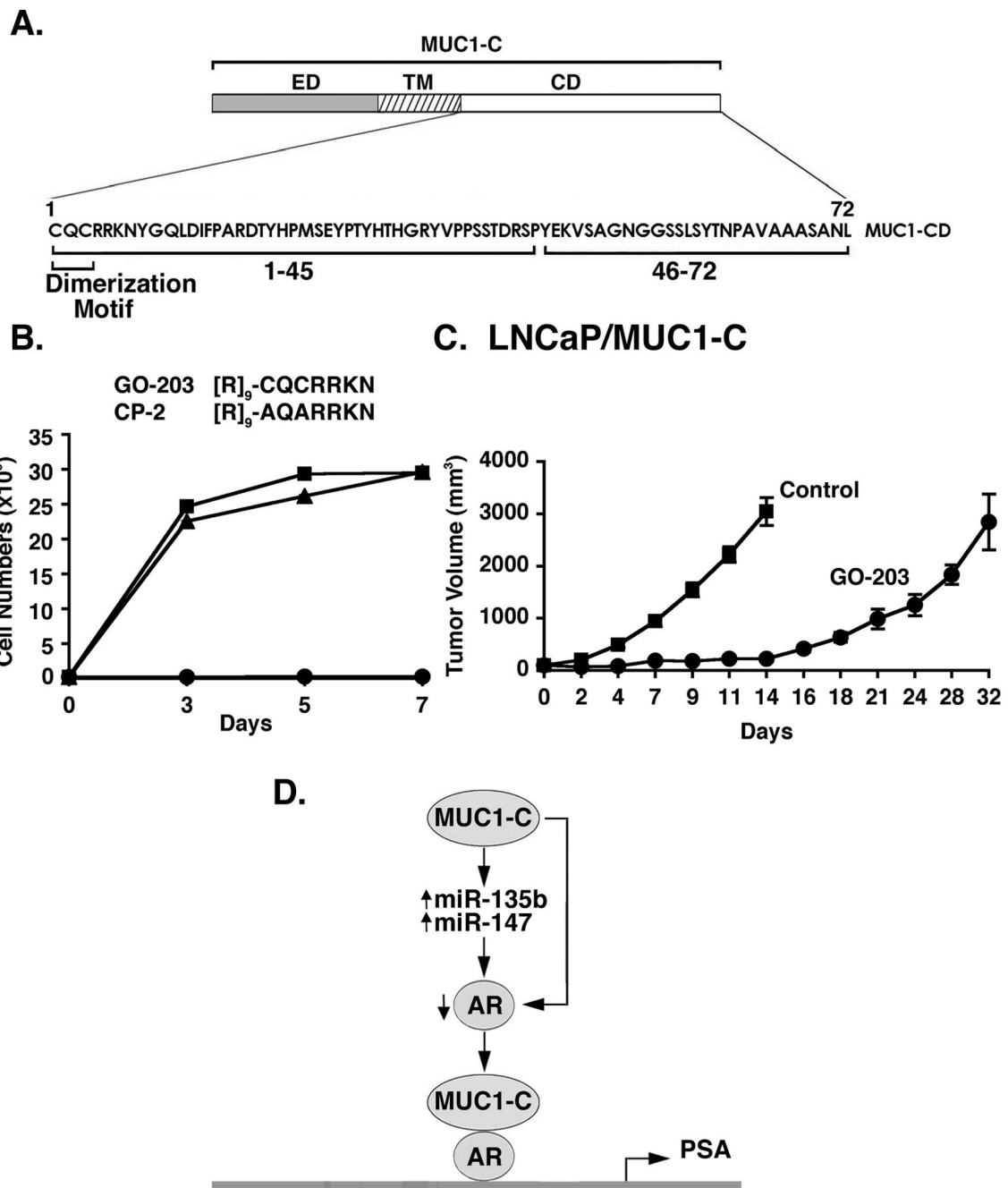


**Figure 5. Effects of MUC1-C on EMT and invasion**

A and B. Lysates from the stably transfected LNCaP (A) and LAPC4 (B) cells were immunoblotted with the indicated antibodies. C and D. LNCaP/GFP and LNCaP/MUC1-C cells ( $1 \times 10^5$ ) were seeded in 8  $\mu$ m pore transwell chambers coated with 1:1000 matrigel and incubated for 24 h (C). The results are expressed as the invasion cell number (mean  $\pm$  SD of 5 fields) at 24 h (D).

**A. LNCaP****B. LNCaP****C. LNCaP/MUC1-C****D. LAPC4**

**Figure 6. MUC1-C confers androgen independent growth of LNCaP and LAPC4 cells**  
 A–B. LNCaP (open bars), LNCaP/GFP (solid bars) and LNCaP/MUC1-C (shaded bars) cells were seeded at  $1 \times 10^5$  cells/ml in complete medium (A) or phenol red-free medium containing charcoal-stripped serum (CSS) (B). C. LNCaP/MUC1 cells were seeded at  $1 \times 10^5$  cells/ml in CSS (solid bars) or CSS containing 100  $\mu$ g/ml bicalutamide (shaded bars). D. LAPC4/GFP (circles) and LAPC4/MUC1-C (squares) cells were seeded at  $0.5 \times 10^5$  cells/ml in CSS. Viable cell number was determined by trypan blue exclusion. The results are expressed as the cell number (mean $\pm$ SD of three determinations).



**Figure 7. Targeting of MUC1-C inhibits growth of LNCaP/MUC1-C cells**

A. Schematic of the MUC1-C subunit with the amino acid sequences of the MUC1-C cytoplasmic domain highlighting the CQC dimerization motif. B. D-amino acid sequences of the GO-203 and control CP-2 peptides. LNCaP/MUC1-C cells were left untreated (Control; squares) and treated with 5  $\mu$ M GO-203 (circles) or CP-2 (triangles) each day for the indicated days. Cell numbers are expressed as the mean $\pm$ SD of three determinations. C. Castrated male BALB/c nu/nu male mice were injected subcutaneously in the flank with  $1 \times 10^7$  LNCaP/MUC1-C cells. The mice were pair-matched when the tumors were  $\sim 100 \pm 10$  (mean $\pm$ SE) mm<sup>3</sup>. Treatment groups consisted of 8 mice injected intravenously with PBS (vehicle control; squares) or 7.5 mg/kg GO-203 (circles) each day for 21 days. Tumor

measurements were performed on the indicated days. Mice were weighed twice weekly. There was no weight loss in either group. D. Proposed model for the interaction between MUC1-C and AR.