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TITLE: A Novel Anti-Beta2-Microglobulin Antibody Inhibition of Androgen Receptor Expression, Survival, and Progression in Prostate Cancer Cells

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β2-microglobulin (β2M) is a signaling and growth-promoting factor stimulating prostate cancer cell proliferation and progression. Blockade of the β2M signaling axis resulted in the inhibition of androgen receptor (AR) and its target gene, prostate-specific antigen (PSA), and the induction of programmed death of prostate cancer cells through activation of a caspase-dependent pathway in vitro and in vivo. In this annual summary report, we identified a cis-acting element, sterol regulatory element-binding protein-1 (SREBP-1) binding site, within the 5’-flanking human AR promoter region and its binding transcription factor, SREBP-1, regulating AR transcription by a new agent, anti-β2M monoclonal antibody (β2M mAb), in prostate cancer cells using the promoter deletion assay, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). The functional study of SREBP-1 revealed that knocked-down or overexpressed SREBP-1 utilizing a sequence-specific siRNA or an expression vector showed to decrease or increase total and nuclear AR proteins and cell viability in prostate cancer cells. These results provided a new concept to reveal the role of β2M and its related signaling pathways in regulation of AR expression, cell proliferation, survival and progression of human prostate cancers.
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INTRODUCTION:

Prostate cancer progression from an androgen-dependent (AD) to an androgen-independent (AI) state is well recognized clinically as a fatal event. Androgen signaling mediated by the androgen receptor (AR), a ligand-activated transcription and survival factor, is known to play a key role regulating this lethal progression (1, 2). The central molecule of this project is β2-microglobulin (β2M). β2M is a non-glycosylated protein composed of 119 amino acid residues, and the mature (secreted) form contains 99 amino acid residues with a molecular mass of 11,800 Da (3, 4). β2M associates with the heavy chain of major histocompatibility complex class I (MHC I) on cell surfaces (5). This complex is essential for the presentation of protein antigens recognized by cytotoxic T lymphocytes (6) and serves as a major component of body’s immune surveillance mechanism (7). We previously showed that β2M plays an unexpected role mediating prostate cancer osteomimicry, cell growth, survival and progression (8, 9), and AR expression. In this project, we evaluate the molecular mechanism of AR gene expression at the transcriptional level regulated by β2M during prostate cancer progression. We also focus on the β2M-mediated signaling and AR as a therapeutic target using a novel anti-β2M monoclonal antibody (β2M mAb) for the treatment of lethal prostate cancer malignancy. There are two specific aims proposed in this project: **Specific Aim 1:** To determine the molecular mechanism by which the β2M-mediated signaling regulates AR expression in prostate cancer cells. **Specific Aim 2:** To determine the anti-tumor efficacy of β2M Ab on prostate cancer cells *in vitro* and pre-established prostate tumors in mice *in vivo*.

BODY:

1) Blockade of β2M downregulated AR and PSA expression in human prostate cancer cells—We previously reported that β2M is a growth and signaling-promoting factor for human prostate cancer cells (8). Target β2M using a sequence specific β2M siRNA (8) or β2M Ab (10) greatly inhibited prostate tumor growth and induced cell programmed death via a caspase-9 cascade pathway *in vitro* and *in vivo*. To further investigate the molecular mechanism of the downstream target of β2M and its related signaling interruption in cell cultures and an animal model, we established permanent β2M knocking-down prostate cancer cells, C4-2B, by β2M...
siRNA (8). We observed that the steady-state mRNA levels of \( \beta 2M \), AR and PSA were dramatically decreased in \( \beta 2M \) knocking-down (si\( \beta 2M \)) C4-2B cells compared with parental (P) and control scramble siRNA (Scramble) C4-2B cells (Fig. 1A). Not only mRNA levels of \( \beta 2M \), AR and PSA were decreased, the endogenous proteins of secreted (from conditioned media, CM) and soluble (from cell lysates) \( \beta 2M \), AR and PSA were also greatly reduced in si\( \beta 2M \) C4-2B cells compared with P and Scramble C4-2B cells (Fig. 1B). Furthermore, we utilized a subcutaneous xenograft mouse model of prostate tumor C4-2B (8) to evaluate the in vivo effect of \( \beta 2M \) siRNA on AR and PSA expression. Likewise, the immunohistochemical staining results showed that AR expression was greatly decreased in si\( \beta 2M \)-treated C4-2B tumors compared with P and Scramble siRNA-treated C4-2B tumors (Fig. 1C). Mouse serum PSA levels were also markedly decreased in si\( \beta 2M \)-treated C4-2B tumors (1.03±0.52 ng, \( N=5 \), after 28-day treatment) compared with Scramble siRNA-treated C4-2B tumors (19.70±9.04 ng, \( N=5 \)). These in vitro and in vivo data suggested that blockade of intracellular \( \beta 2M \) by \( \beta 2M \) siRNA greatly inhibited mRNA and protein expression of AR and PSA in prostate cancer cells.

To test if interrupting \( \beta 2M \) from extracellular sources may also affect AR and PSA expression as well as cell growth of prostate cancer cells, we employed a new agent, anti-\( \beta 2M \) monoclonal antibody (\( \beta 2M \) mAb), to neutralize extracellular \( \beta 2M \) and interrupt its downstream signaling. As shown in Fig. 2A and 2B, \( \beta 2M \) mAb (0

![Fig. 2. \( \beta 2M \) mAb decreased AR and PSA expression in prostate cancer cells. A, \( \beta 2M \) mAb decreased AR and PSA mRNA expression in a dose-dependent manner (0 to 10 µg/mL) in both LNCaP (AD) and C4-2B (AI) prostate cancer cells determined by semi-quantitative RT-PCR. The inhibitory effect was restored by pre-incubation of \( \beta 2M \) mAb with the same amounts of purified \( \beta 2M \) protein. Isotype control IgG (10 µg/mL) did not significantly affect AR and PSA mRNA expression. The relative mRNA levels of AR and PSA, normalized by GAPDH mRNA, were measured by Gel Doc gel documentation software (Bio-Rad). The relative mRNA levels (%) were assigned as 100% in the absence of \( \beta 2M \) mAb treatment. *, \( P < 0.05 \) and **, \( P < 0.005 \), significant differences from the \( \beta 2M \) mAb-untreated group. Data represent the mean ± SD of independent triplicate experiments. B, \( \beta 2M \) mAb also inhibited total AR, nuclear AR (NE) and PSA protein expression in a dose-dependent pattern (0 to 10 µg/mL) in LNCaP and C4-2B cells assayed by Western blot. The inhibitory effect was abrogated by pre-incubation of \( \beta 2M \) mAb with \( \beta 2M \) protein. Control IgG (10 µg/mL) did not change AR and PSA protein expression. \( \beta \)-actin was used as an internal loading control.
to 10 µg/mL) significantly decreased both mRNA and protein levels of AR and PSA in LNCaP (AD) and C4-2B (AI) cells in a dose-dependent pattern determined by semi-quantitative RT-PCR and Western blot. Considered the specificity of β2M mAb inhibitory effect, purified β2M protein could rescue AR and PSA inhibition by β2M mAb in prostate cancer cells. Control IgG did not affect AR and PSA expression. Not only decreased endogenous total AR protein, β2M mAb also showed to inhibit nuclear AR protein expression in LNCaP and C4-2B cells (Fig. 2B). These data demonstrated that antagonizing extracellular β2M by β2M mAb also reduced AR and PSA expression at transcriptional and translational levels in prostate cancer cells.

2) β2M mAb induced apoptotic death in prostate cancer cells—To determine the molecular mechanism by which β2M mAb inhibited the growth of prostate cancer cells, we first examined apoptotic death in LNCaP and C4-2B cells, including sub-G₁ DNA content analysis and activation of caspase (19) and PARP expression. The results of flow cytometric analysis revealed that β2M mAb greatly increased sub-G₁ DNA contents in LNCaP (%sub G₁ = 82.49) and C4-2B (%sub G₁ = 79.45) cells compared to control IgG-treated LNCaP (%sub G₁ = 0.86) and C4-2B (%sub G₁ = 0.54) cells (Fig. 3A). Western blot analysis of caspases showed that cleaved caspase-9, caspase-3, and PARP, a downstream factor of caspases, were increased by exposing LNCaP and C4-2B cells to β2M mAb but not control IgG for a 48-h incubation (Fig. 3B). The induction of cleaved caspases and PARP was attenuated by pre-incubation of β2M mAb with purified β2M protein. In addition, cell death induced by β2M mAb was also confirmed at the level of light microscopy in LNCaP and C4-2B cells (Fig. 3C).

Fig. 3. β2M mAb induced cell death of prostate cancer cells through an apoptotic cascade pathway. A, LNCaP and C4-2B cells were exposed to either β2M mAb or isotype control IgG (10 µg/mL) for 48 h incubation and subjected to cell cycle analysis determined by flow cytometry. Both LNCaP and C4-2B cells treated with β2M mAb showed a marked increase in the sub-G₁ DNA contents compared with IgG-treated cells. B, β2M mAb (0 to 10 µg/mL, 48 h treatment) activated the expression of cleaved caspase-9, caspase-3 and PARP proteins in a dose-dependent pattern in LNCaP and C4-2B cells as assayed by Western blot. β2M protein rescued the apoptotic effect of β2M mAb. Control IgG (10 µg/mL) did not activate cleaved caspase and PARP expression. C, LNCaP and C4-2B cells were treated with β2M mAb, β2M mAb pre-incubated with β2M protein or control IgG (10 µg/mL) for 48 h and examined by light microscopy. Scale bars = 250 µm.
3) Sterol regulatory element-binding protein-1 binding site within the 5'-flanking promoter region of human AR gene is responsible for AR transcriptional activity regulated by β2M mAb—Subsequently, we sought to characterize the transcriptional mechanism of AR expression regulated by β2M mAb in prostate cancer cells. A luciferase reporter construct that contained the 5'-flanking human AR (hAR) promoter fragment (-5400 to +580) was transiently transfected into LNCaP and C4-2B cells. Consistent with previous RT-PCR and Western blot results (Fig. 2A and 2B), β2M mAb (0 to 10 µg/mL) significantly decreased hAR promoter luciferase activity in a concentration-dependent pattern (Fig. 4A). Purified β2M protein could restore the inhibition of hAR promoter reporter activity by β2M mAb as well. Isotype control IgG did not decrease hAR promoter luciferase activity in LNCaP and C4-2B cells. To further identify the responsible cis-acting element in the hAR promoter region, we conducted hAR promoter deletion study. Three deletion constructs of hAR promoter fragment (ΔA, ΔB and ΔC, Fig. 4B) were generated and confirmed the DNA sequence. After

![Graph A: Luciferase Activity vs. β2M mAb Concentration](Fig. 4A)

![Graph B: Luciferase Activity vs. Promoter Deletions](Fig. 4B)

**Fig. 4.** Sterol regulatory element-binding protein-1 (SREBP-1) binding site within the 5'-flanking promoter region of human AR (hAR) gene is responsible for AR transcriptional activity mediated by β2M mAb. **A**, β2M mAb decreased hAR promoter (-5400 to +580) luciferase activity with a concentration-dependent pattern (0 to 10 µg/mL) in LNCaP and C4-2B cells. Purified β2M protein could restore the inhibitory effect of hAR promoter activity regulated by β2M mAb. Control IgG did not suppress hAR promoter reporter activity. **B**, Region A (-600 to -40) is responsible for hAR promoter luciferase activity mediated by β2M mAb in LNCaP and C4-2B cells. β2M mAb (10 µg/mL) significantly decreased the promoter luciferase activity of the deleted region B (ΔB, -1100 to -600) and C (ΔC, -1600 to -1100) in hAR promoter reporter constructs but did not affect the luciferase activity of the ΔA construct. Isotype control IgG (10 µg/mL) did not significantly change the promoter reporter activity of all deletion constructs. **C**, The DNA sequence of region A (-600 to -40, 560 bp) contains early growth response gene-1 (EGR-1) binding site (-181 to -170), sterol regulatory element-binding protein-1 (SREBP-1) binding site (-347 to -336) and activator protein-1 (AP-1) binding site (-475 to -465). Among the three deletion constructs (ΔEGR-1, ΔSREBP-1 and ΔAP-1 binding sites), the promoter luciferase activities of ΔEGR-1 and ΔAP-1 binding site constructs were significantly inhibited by β2M mAb in LNCaP and C4-2B cells. Only a slight drop of promoter luciferase activity was observed in a ΔSREBP-1 binding site construct while treated with β2M mAb in prostate cancer cells. Control IgG did not change the promoter reporter activities of these three deletion constructs. All promoter luciferase activity data were normalized by internal control β-galactosidase activity and expressed as the mean ± S.D. of three independent duplicate experiments. ***, P < 0.005.
transfected into LNCaP and C4-2B cells, β2M mAb significantly inhibited the original hAR, ΔB (deletion of -1100 to -600) and ΔC (deletion of -1600 to -1100) promoter luciferase activities (Fig. 4B). However, β2M mAb did not affect the promoter luciferase activity of the ΔA construct (deletion of -600 to -40, Fig. 4B), and a decrease in the basal promoter luciferase activity was observed only the ΔA construct in LNCaP and C4-2B cells. Control IgG did not significantly change the promoter activities of all these vector constructs. These results suggested that the region A within the hAR promoter fragment may contain the potential cis-acting element mediated AR transcriptional activity by β2M mAb. Because the original hAR promoter reporter vector contains approximate 6 kb (from -5400 to +580) in length, we further used a restriction enzyme, SacI, to generate a shorter promoter luciferase construct, the hAR/Sacl vector (2 kb only, deletion of -4700 to -740), and tested this reporter vector activity in LNCaP and C4-2B cells exposed with β2M mAb or IgG. After luciferase activity assay, the basal promoter activity of this hAR/Sacl vector slightly decreased in LNCaP and C4-2B cells compared with the original hAR promoter (Fig. 4B and 4C), but no significant difference. It implied that the fragment, -4700 to -740, within the hAR promoter region, is not responsible for AR transcriptional activity regulated by β2M mAb in prostate cancer cells.

To further determine the β2M mAb-mediated cis-acting factor in the region A, based on the computer databank searched and analyzed, we predicted that three potential cis-acting elements in the region A may be responsible for AR transcription by β2M mAb, early growth response gene-1 (EGR-1) binding site (-181 to -170), sterol regulatory element-binding protein-1 (SREBP-1) binding site (-347 to -336) and activator protein-1 (AP-1) binding site (-475 to -465) (Fig. 4C). Subsequently, we generated three deletion constructs which are individually deleted these three transcription factor binding sites from the hAR/Sacl promoter luciferase vector and tested their reporter activity in prostate cancer cells. Among these three deletion constructs, the promoter luciferase activities of ΔEGR-1 and ΔAP-1 binding site constructs significantly inhibited by β2M mAb similar with the hAR/Sacl construct activity in LNCaP and C4-2B cells (Fig. 4C). Only a slight drop of promoter luciferase activity was observed in a ΔSREBP-1 binding site construct while treated with β2M mAb, and decreased the basal promoter luciferase activity of this construct (Fig. 4C). Control IgG did not significantly change the promoter reporter activities of all these deletion constructs. These hAR promoter deletion data, taken together, demonstrated that SREBP-1 binding site located within the 5’-flanking hAR promoter region is important for hAR promoter activity regulated by β2M mAb.

4) A transcription factor, SREBP-1, interacting with SREBP-1 binding site within the hAR promoter region mediated by β2M mAb: Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation Assay (ChIP)—To further determine whether a nuclear transcription factor, SREBP-1, is a key molecule to regulate AR transcriptional activity through β2M mAb in prostate cancer cells, we conducted EMSA and ChIP. As shown in Fig. 5A, nuclear extracts prepared from β2M mAb treated LNCaP cells greatly decreased the SREBP-1 oligo-DNA probe with nuclear protein complex in comparison with nuclear extracts prepared from cells without β2M mAb or control IgG treatment (lanes 2, 3 and 4). Purified β2M protein could abrogate the complex formation decreased by β2M mAb (lane 5). For the specificity of the SREBP-1 oligo-DNA probe, the 32P unlabeled SREBP-1 oligo-DNA showed to compete off the DNA-protein complex in LNCaP cells (lane 6).

To investigate if the interaction between nuclear SREBP-1 and its binding cis-acting element affected by β2M mAb in the chromatin environment, we performed ChIP in prostate cancer cells. An expected single DNA band (116 bp) was detected by using a PCR primer set to amplify a SREBP-1 binding region within hAR promoter in LNCaP and C4-2B cells while the chromatin DNA fragments immunoprecipitated by anti-SREBP-1 antibody were used as templates (Fig. 5B, top panel). This amplified PCR product amount was decreased by exposed with β2M mAb in prostate cancer cells. In addition, by utilizing real-time quantitative PCR as readout, we observed that β2M mAb caused significantly reduction of the interaction between SREBP-1 and its binding site within AR promoter region by 13.3- and 11.0-fold decrease in LNCaP and C4-2B cells, respectively (Fig. 5B, bottom panel). The results of EMSA and ChIP assays demonstrated that SREBP-1 plays a critical role in regulation of AR transcriptional activity through β2M mAb in prostate cancer cells.
The role of SREBP-1 in regulating AR expression and cell viability in prostate cancer cells—We have shown that β2M mAb inhibited AR expression through the interaction of SREBP-1 and SREBP-1 binding site in prostate cancer cells. To examine whether β2M mAb also affected endogenous SREBP-1 expression, we performed Western blot to evaluate precursor and nuclear SREBP-1 protein expression in LNCaP and C4-2B cells. As shown in Fig. 6A, β2M mAb (0 to 10 µg/mL) specifically inhibited expression of precursor (125 kDa) and mature nuclear (68 kDa) SREBP-1 proteins in a concentration-dependent manner but did not affect expression of SREBP-2, which is a SREBP-1 isoform. Purified β2M protein rescued the inhibitory effect of endogenous SREBP-1 expression by β2M mAb. Control IgG did not decrease SREBP-1 and SREBP-2 expression. Next, to investigate the role of SREBP-1 in regulating AR expression, we conducted the functional studies to knock-down and overexpress SREBP-1 in prostate cancer cells. A sequence-specific siRNA of SREBP-1 caused great decrease of both precursor and nuclear SREBP-1 proteins in LNCaP and C4-2B cells.
Due to downregulation of SREBP-1, we also observed that expression of total AR and nuclear AR proteins was inhibited by SREBP-1 siRNA in LNCaP and C4-2B cells (Fig. 6B). SREBP-2 expression was not affected by SREBP-1 siRNA. Control non-specific siRNA did not inhibit expression of SREBP-1, SREBP-2 and AR. Conversely, we overexpressed SREBP-1 by using a SREBP-1 expression vector in prostate cancer cells. As an expectation, overexpressing SREBP-1 affected increase of precursor and nuclear SREBP-1 as well as AR protein expression, but not SREBP-2 in LNCaP and C4-2B cells (Fig. 6C).

**Fig. 6. The role of SREBP-1 in regulating endogenous AR expression in prostate cancer cells.**

**A**. β2M mAb decreased the expression of both precursor SREBP-1 (125 kDa) and mature nuclear SREBP-1 (68 kDa) in a concentration-dependent pattern (0 to 10 µg/mL) in LNCaP and C4-2B cells determined by Western blot. The inhibitory effect of endogenous SREBP-1 expression was restored by pre-incubation of β2M mAb with purified β2M protein. Isotype control IgG (10 µg/mL) did not affect SREBP-1 expression. β2M mAb did not change the expression of SREBP-2 which is an isoform of SREBP-1. β-actin was used as a loading control. NE, nuclear extracts. **B**, A sequence-specific siRNA of SREBP-1 decreased the expression of precursor and nuclear SREBP-1 proteins in LNCaP and C4-2B cells. Due to downregulation of SREBP-1 by SREBP-1 siRNA, AR and nuclear AR (NE) expression was also inhibited in LNCaP and C4-2B cells. For the specificity of this siRNA, SREBP-2 expression did not change by SREBP-1 siRNA. Control non-specific siRNA (Con) did not affect SREBP-1, AR and SREBP-2 expression. **C**, Overexpressing SREBP-1 by using a SREBP-1 expression vector (EV) increased the expression of precursor and nuclear SREBP-1 as well as endogenous AR protein in LNCaP and C4-2B cells. SREBP-1 expression vector did not affect SREBP-2 expression in prostate cancer cells. Con, control empty expression vector.

**KEY RESEARCH ACCOMPLISHMENTS:**

- We identified a *cis*-acting element, SREBP-1 binding site, within the 5’-flanking promoter region of hAR gene is responsible for AR transcriptional activity regulated by β2M mAb.
- We further demonstrated that a transcription factor, SREBP-1, interacting with SREBP-1 binding site within the hAR promoter region mediated by β2M mAb in prostate cancer cells.
- SREBP-1 plays a key role in regulation of AR expression and cell viability in prostate cancer cells.
- Blockade of β2M using β2M mAb significantly downregulated AR and PSA expression and induced an apoptotic caspase-dependent pathway in prostate cancer cells.
REPORTABLE OUTCOMES:
During the first year of this DoD geant from May 1, 2008 to April 30, 2009, I have already published a peer-reviewed research article in *Clinical Cancer Research* (14: 5341-7, 2008) and presented a poster presentation in 2009 AACR annual meeting (# 849; title: Anti-β2-microglobulin monoclonal antibody inhibition of androgen receptor expression and survival through a lipogenic pathway in prostate cancer), please see the APPENDICES section.

CONCLUSION:
β2M is a signaling and growth-promoting factor inducing prostate cancer cell proliferation, survival and progression. Interrupting β2M and its related signaling pathways by a novel agent, β2M mAb resulted in the inhibition of AR and PSA expression and the induction of apoptosis of prostate cancer cells. The molecular mechanism of AR inhibitory expression by β2M mAb was through decreasing the interaction between a lipogenic transcription factor, SREBP-1, and its binding *cis*-acting element located in the 5'-flanking AR promoter region determined by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). The functional study of SREBP-1 revealed that knocked-down or overexpressed SREBP-1 by utilizing a sequence-specific siRNA or an expression vector showed to decrease or increase total and nuclear AR proteins and cell viability in prostate cancer cells. In summary, β2M mAb may be a potent and attractive pleiotropic therapeutic agent to inhibit AR expression, cell proliferation, survival and fatty acid and lipid metabolism through down-regulation of a lipogenic transcription factor, SREBP-1, in prostate cancer cells. I will continuously determine and investigate the signaling molecular mechanism of β2M/SREBP-1 regulated AR expression in prostate cancer cells and the anti-tumor efficacy of β2M mAb on prostate cancer cells in pre-established prostate tumors in mice *in vivo* which proposed in my approved DoD Statement of Work.
References:


APPENDICES: (including a 2009 AACR annual meeting abstract and a copy of a peer-reviewed research article in Clinical Cancer Research)

2009 AACR Annual Meeting Abstract (#849)

Anti-β2-microglobulin monoclonal antibody inhibition of androgen receptor expression and survival through a lipogenic pathway in prostate cancer

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β2-Microglobulin (β2M) plays critical roles in promoting osteomimicry and cell proliferation through a cAMP/protein kinase A (PKA)/cAMP-responsive element binding protein (CREB) signaling pathway in human prostate cancer cells (Huang et al., Cancer Res., 65:2303-13, 2005). Blockade of β2M and its related signaling axis by using a new agent, anti-β2M monoclonal antibody (β2M mAb), resulted in inhibition of mRNA and protein expression of the androgen receptor (AR) and its target gene, prostate-specific antigen (PSA), in prostate cancer cells and induction of programmed death of prostate cancer cells via activation of a caspase-9 dependent pathway in vitro and in vivo. The molecular mechanism of AR inhibitory expression by β2M mAb was through decreasing the interaction between a lipogenic transcription factor, sterol regulatory element-binding protein-1 (SREBP-1), and its binding cis-acting element located in the 5’-flanking AR promoter region determined by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). The functional study of SREBP-1 revealed that knocked-down or overexpressed SREBP-1 by utilizing sequence-specific siRNA or an expression vector showed to decrease or increase total and nuclear AR proteins and cell viability in prostate cancer cells. SREBP-1 has been identified and reported as a key regulator to control genes involved in fatty acid and cholesterol biosynthesis. Accompanying inhibition of AR expression by β2M mAb, we also observed that β2M mAb significantly decreased SREBP-1 expression and fatty acid contents, which are important for cell membrane construction and energy storage, in prostate cancer cells. In summary, β2M mAb may be a potent and attractive pleiotropic therapeutic agent to inhibit AR expression, cell proliferation, survival and lipid metabolism through down-regulation of a lipogenic pathway in prostate cancer cells.
Abstract

Purpose: β2-Microglobulin (β2M) has been shown to promote osteomimicry and the proliferation of human prostate cancer cells. The objective of this study is to determine the mechanism by which targeting β2M using anti-β2M antibody inhibited growth and induced apoptosis in prostate cancer cells.

Experimental Design: Polyclonal and monoclonal β2M antibodies were used to interrupt β2M signaling in human prostate cancer cell lines and the growth of prostate tumors in mice. The effects of the β2M antibody on a survival factor, androgen receptor (AR), and its target gene, prostate-specific antigen (PSA) expression, were investigated in cultured cells and in tumor xenografts.

Results: The β2M antibody inhibited growth and promoted apoptosis in both AR-positive and PSA-positive, and AR-negative and PSA-negative, prostate cancer cells via the down-regulation of the AR in AR-positive prostate cancer cells and directly caused apoptosis in AR-negative prostate cancer cells in vitro and in tumor xenografts. The β2M antibody had no effect on AR expression or the growth of normal prostate cells.

Conclusions: β2M downstream signaling regulates AR and PSA expression directly in AR-positive prostate cancer cells. In both AR-positive and AR-negative prostate cancer cells, interrupting β2M signaling with the β2M antibody inhibited cancer cell growth and induced apoptosis. The β2M antibody is a novel and promising therapeutic agent for the treatment of human prostate cancers.

β2-Microglobulin (β2M) is produced by all nucleated cells as a 119-amino-acid residue protein and, after processing, is secreted in a 99-amino-acid form (11,800 Da; refs. 1, 2). The most common known function of β2M, a light-chain antigen-presenting molecule, is to serve as a coreceptor for the presentation of the MHC class I in nucleated cells for cytotoxic T-cell recognition (3). However, cancer cells frequently down-regulate the expression of MHC class I to evade recognition by the immune system (4–7), presumably allowing the secretion of free β2M into circulation or in the tumor microenvironment. Our laboratory first identified β2M, an active component secreted by prostate cancer, and prostate and bone stromal cells, as a major growth factor and signaling molecule (8). β2M conferred osteomimicry, the ability of cancer cells to mimic gene expression by bone cells, in prostate cancer cells through the activation of a cyclic AMP (cAMP)–dependent protein kinase A (PKA) and cAMP-responsive element binding (CREB) protein signaling pathway (9). The use of a sequence-specific small interfering RNA (siRNA) targeting β2M and its signaling resulted in extensive prostate cancer cell death in vitro and greatly promoted prostate tumor regression in immunocompromised mice (8). We also showed that interrupting β2M signaling similarly blocked human renal cell carcinoma growth (10). β2M has recently been shown to be a useful biomarker for advanced human prostate cancer (11). β2M seems to be a downstream androgen target gene, more specific than prostate-specific antigen (PSA), under the control of the androgen receptor (AR), in a human LNCaP prostate cancer cell line (11).

Anti-β2M antibody is a potent interrupter of β2M-mediated signaling (8, 12). The β2M antibody was shown to be a highly cytotoxic reagent against the growth of solid tumors like renal cell carcinoma (13) as well as liquid tumors, such as leukemia, lymphoma, and multiple myeloma (12). We showed here that the β2M antibody inhibited the expression of a survival factor, AR, and its target gene, PSA, in AR-positive and PSA-positive human prostate cancer cell lines, including androgen-dependent LNCaP and androgen-independent C4-2B cells (14),...
and in androgen-independent C4-2 tumor xenograft models. The β2M antibody also suppressed growth and induced apoptosis in both AR-positive and PSA-positive, and AR-negative and PSA-negative human prostate cancer cells and in xenograft tumors in mice. Moreover, our studies showed that the β2M antibody induced prostate cancer cell death through an activation of a caspase-9–mediated apoptotic cascade pathway without affecting normal or nontumorigenic prostatic epithelial and stromal cells. These results support the idea that targeting β2M signaling via the external application of the β2M antibody can profoundly alter intracellular cell signaling networks, including, but not limited to, the AR downstream signaling axis. Effective β2M antibody–mediated targeting of the growth of both AR-positive and PSA-positive, and AR-negative and PSA-negative human prostate cancer cells may prove to be an attractive and safe therapeutic approach for the treatment of human prostate cancer and its lethal progression.

Materials and Methods

Cell lines, cell culture, and β2M antibody. The human prostate cancer cell line LNCaP (androgen dependent), the LNCaP lineage–derived bone metastatic subline C4-2B (androgen independent; ref. 14), DU-145 (brain metastatic, androgen independent), PC3 (bone metastatic, androgen independent), and ARCaP (ascites-fluid–derived, androgen repressive; refs. 15, 16) were cultured in T-medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. A human normal/nontumorigenic prostatic epithelial cell line, RWPE-1 (American Type Culture Collection), was cultured in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract (Invitrogen). These prostate cancer and normal cell lines were maintained in 5% CO2 at 37°C. The anti-β2M antibody, a polyclonal antibody, was obtained from Santa Cruz Biotechnology, Inc. (sc-15366), for in vitro cell culture studies and in vivo animal experiments. We also tested the β2M monoclonal antibody (Santa Cruz Biotechnology; sc-13565) and found it to have similar inhibitory effects on the growth of human prostate cancer cells in vitro (data not shown).

Reverse transcription-PCR. LNCaP and C4-2B cells were plated on six-well dishes at 3 × 104 cells per well and grown to 70% confluence in T-medium with 5% fetal bovine serum. The cells were gently washed with PBS and incubated in T-medium plus 5% dextran-coated, charcoal-treated fetal bovine serum for overnight incubation. The cells were then treated with 0, 1, 5, or 10 μg/mL of β2M antibody; the β2M antibody was preincubated for 30 min with the same amounts of purified human β2M protein (Sigma) or 10 μg/mL of isotype control IgG for 24 h. The total RNA was isolated from these treated cells using a RNeasy Mini Kit (Qiagen) and subjected to reverse transcription according to the manufacturer’s instructions (Invitrogen). The primer sequences used for PCR analysis were AR [5′-ATGGCTGT-CATTCAGTACTCCTGGAGA-3′ (forward) and 5′-AGATGGGCTGT-GACTTCCACCAAGA-3′ (reverse)], PSA [5′-ATGGGCTGTCCGGTC-GCTCTCCTACCCGGCTC-3′ (forward) and 5′-TACCGCGTGGCCAC-GATGGTGCTCCATCG-3′ (reverse)], and glyceraldehyde-3-phosphate dehydrogenase [5′-ACCACGACATCATCATGCA-3′ (forward) and 5′-TCCACACCCCTGCTGCTG-3′ (reverse)]. The thermal profiles for AR, PSA, and glyceraldehyde-3-phosphate dehydrogenase cDNA amplification are 25 cycles, starting with denaturation for 1 min at 94°C, followed by 1 min of annealing at 61°C (for AR), 55°C (for PSA), and 60°C (for glyceraldehyde-3-phosphate dehydrogenase), and 1 min of extension at 72°C. The reverse transcription-PCR products were analyzed by agarose gel electrophoresis.

Western blot analysis and ELISA. Cell lysates were prepared from β2M monoclonal antibody–treated or IgG-treated prostate cells using a lysis buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.02% NaN3, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] containing 1 mmol/L phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined by the Bradford assay using the Coomassie Plus Protein Reagent (Pierce). Western blot was done with the Novex system (Invitrogen) as described previously (8, 10). The primary antibodies anti-AR (1:500 dilution) and PSA (1:1,000 dilution; Santa Cruz Biotechnology), anti–caspase-9, caspase-3, and poly[ADP]ribose polymerase (PARP; 1:1,000 dilution; Cell Signaling Technology); and the secondary antibodies that were conjugated with horseradish peroxidase (1:5,000 dilution; GE Healthcare) were used. The detection of protein bands was done with the use of enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare). The soluble PSA levels were determined by microparticle ELISA with the Abbott Immx machine (Abbott Laboratories).

Cell proliferation assay. LNCaP (6,000 cells per well), C4-2B (6,000 cells per well), DU-145 (3,000 cells per well), PC3 (3,000 cells per well), ARCaP (5,000 cells per well), and RWPE-1 (6,000 cells per well) cells were plated on 96-well plates and treated with the β2M antibody or control IgG for a 3-d incubation. The cell numbers were measured every 24 h by mitochondrial 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), assay with the use of the CellTiter 96 Aquoese One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions.

Sub-G1 DNA content measurement. LNCaP and C4-2B prostate cancer cells were plated on six-well plates at 3 × 105 per well in T-medium containing 5% dextran-coated, charcoal-treated fetal bovine serum and exposed to 10 μg/mL of β2M monoclonal antibody or control IgG for 48-h incubation. The treated cells were collected by trypsinization and fixed in 70% ice-cold ethanol, incubated with RNase A (100 μg/mL; Sigma) for 30 min, and stained with propidium iodide (25 μg/mL; Chemicon) for 30 min. The cell cycle was determined by a FACScan flow cytometer and CellQuest software (Becton Dickinson Labware) for analysis of sub-G1 DNA content.

In vivo animal experiments. All the animal experiments were approved and done in accordance with institutional guidelines. The mice were maintained at the Animal Research Facility in Emory University. To test the antitumor efficacy and AR expression regulated by the β2M antibody in vivo, 4-wk-old male athymic nu/nu mice (National Cancer Institute) were inoculated s.c. with C4-2 or PC3 prostate cancer cells with 2 × 106 cells per mouse. After 3 wk (PC3 tumor) or 4 wk (C4-2 tumor) of inoculation, 10 μg of β2M monoclonal antibody mixed with Surgifom (Ethicon Inc.) to keep and slow release the β2M antibody around the tumors were given by intratumoral implantation, one shot per mouse. The control group mice received equal doses of isotype IgG or placebo (saline) implanted the same way and in the same volume. We previously showed that the β2M antibody decreased AR and PSA expression in human prostate cancer cells. We previously showed that β2M is a novel signaling and growth-regulating molecule capable of...
promoting cell proliferation and survival in human prostate and renal cancer cells (8, 10). Interrupting β2M and its downstream signaling by β2M siRNA induced cell death in both human prostate and renal carcinoma models (8, 13). Because the downstream targets for β2M signaling interruption are not completely clear in human prostate cancer cells, we conducted a cDNA microarray study (17) comparing β2M siRNA stably transfected AR-positive and PSA-positive C4-2B prostate cancer cells with their scramble stably transfected control clones. The results of these studies showed a 4-fold and 16-fold decreased expression of AR and PSA mRNA, respectively, in C4-2B cells, and these data were confirmed by reverse transcription-PCR and Western blot.5 To test the hypothesis that blocking β2M-mediated signaling pathways may affect AR gene expression and transactivation, which are involved in prostate cancer cell growth, survival, and progression, we tested the effect of a new reagent, β2M polyclonal antibody, on AR and PSA expression in AR-positive and PSA-positive LNCaP (androgen dependent) and C4-2B (androgen independent) cells. Consistent with cDNA microarray data, interrupting β2M by the β2M antibody decreased endogenous AR and PSA mRNA expression as determined by reverse transcription-PCR (Fig. 1A). The inhibitory effect of the β2M antibody (0-10 μg/mL) was concentration dependent, and the addition of purified β2M protein rescued the decreased AR and PSA mRNA expression that had been inhibited by the β2M antibody in LNCaP and C4-2B cells. Isotype-matched control IgG (10 μg/mL) did not suppress AR and PSA mRNA expression. In parallel, the β2M antibody (0-10 μg/mL) also inhibited AR and PSA protein levels in a concentration-dependent manner as analyzed by Western blot (Fig. 1B), and this inhibition can also be rescued by the addition of purified β2M protein to the cultured LNCaP and C4-2B cells. The control IgG did not change AR and PSA protein expression. Consistent with the blockade of AR expression, we found that secreted soluble PSA levels, assayed by ELISA, were also decreased by the β2M antibody, but not the control IgG, in LNCaP and C4-2B cells (Fig. 1C). These results indicate that the β2M antibody diminished AR and PSA mRNA and protein expression in both androgen-dependent and androgen-independent human prostate cancer cells.

**β2M antibody inhibited cell proliferation in human prostate cancer cell lines.** Because β2M stimulated prostate and renal cancer cell growth through the promotion of cAMP/PKA/CREB signaling pathway and the activation of cyclins and cell cycle progression (8, 10), we investigated the possibility that interrupting the β2M-mediated signaling axis may be cytotoxic to prostate cancer cells. When the LNCaP and C4-2B cells were exposed to the β2M antibody (0-20 μg/mL) for a 2-day incubation, the growth of these two prostate cancer cell lines was inhibited in a concentration-dependent manner, with an IC50 of 10.3 and 7.4 μg/mL, respectively (Fig. 2A). The purified β2M protein was shown to rescue the β2M antibody–induced inhibition of prostate cancer cell proliferation, whereas the control IgG did not affect the growth of the LNCaP and C4-2B cells (Fig. 2A). Because of the AR heterogeneity in human prostate cancer cells (18), we compared the effects of the β2M antibody on the cell proliferation of AR-positive (LNCaP, C4-2B, and ARCaP) and AR-negative (PC3 and DU-145) human prostate cancer cell lines. Figure 2B shows that the β2M antibody (10 μg/mL) inhibited the proliferation of these prostate cancer cells at day 3 by 57% (LNCaP), 82% (C4-2B), 91% (DU-145), 93% (PC3), and 94% (ARCaP). These data suggest that the β2M antibody significantly inhibited cell proliferation in a broad range of human prostate cancer cell lines.

**β2M antibody induced apoptotic death and inhibited AR expression of prostate cancer cells in vitro and in mouse xenograft models.** To determine the molecular mechanism by which the β2M antibody inhibited the growth of prostate cancer cells, we first examined apoptotic death in LNCaP and C4-2B cells, including sub-G1 DNA content analysis and activation of caspase (19) and PARP expression. The results of flow cytometric analysis revealed that the β2M antibody greatly increased sub-G1 DNA contents in LNCaP (% sub-G1 = 82.49) and C4-2B (% sub-G1 = 79.45) cells compared with the control IgG-treated LNCaP (% sub-G1 = 0.86) and C4-2B (% sub-G1 = 0.54) cells (Fig. 3A). Western blot analysis of

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5 Unpublished data.
caspases showed that cleaved caspase-9, caspase-3, and PARP, a downstream factor of caspases, were increased by exposing the LNCaP and C4-2B cells to the β2M antibody, but not the control IgG, for a 48-h incubation (Fig. 3B). The induction of cleaved caspases and PARP was attenuated by the preincubation of the β2M antibody with purified β2M protein. In addition, cell death induced by the β2M antibody was also confirmed at the level of light microscopy in LNCaP and C4-2B cells (Fig. 3C).

Next, we examined the effects of the β2M antibody on cell death and/or the status of AR in preexisting C4-2 (AR positive) and PC3 (AR negative) prostate tumors grown in mice as subcutaneous xenografts, with the antibody delivered as Surgifoam implants, and isotype-matched IgG and saline delivered similarly as controls. After 1-week treatment, tumor tissues were harvested from the euthanized mice and subjected to immunohistochemical staining of the AR and a commercially available cell death marker, M30 CytoDeath. Figure 4A and B shows that the β2M antibody dramatically inhibited AR expression in C4-2 tumors and induced cell death in both C4-2 and PC3 tumors in mice compared with the IgG-treated and saline-treated (C4-2, 10 ± 3 cells per 100 cells; PC3, 13 ± 2 cells per 100 cells) control groups.

We further investigated whether the β2M antibody may be a safe reagent to selectively kill cancer but not normal or nonneoplastic immortalized cell lines. A human nonneoplastic prostatic epithelial cell line, RWPE-1, was exposed to the β2M antibody and the control IgG. In contrast to human prostate cancer cells, the β2M antibody did not inhibit RWPE-1 cell growth (Fig. 5A), did not decrease its endogenous AR expression (Fig. 5B), and did not activate apoptotic marker expression as assayed by Western blot (Fig. 5B). While the β2M antibody showed low cytotoxicity in RWPE-1 cells, it also did not affect the growth of P69, a SV40-immortalized human normal prostatic epithelial cell line (20), and human normal prostatic stromal cells (data not shown).

In summary, our results collectively indicate that the β2M antibody effectively induced human prostate cancer, but not normal prostate, cell apoptosis in culture. The β2M antibody induced cell death in prostate tumor xenografts in mice regardless of their AR status. The β2M antibody was also shown to down-regulate AR and PSA expression in AR-positive and PSA-positive human prostate cancer cells grown in culture and as subcutaneous xenografts in mice.

**Discussion**

Prostate cancer progression from an androgen-dependent to an androgen-independent state symbolizes its hormone-refractory status and occurs in patients clinically. Because there is currently no effective therapy for the management of hormone-refractory prostate cancer, we undertook the investigation of the molecular mechanisms and effects of a recently identified novel molecular target, β2M, using β2M antibody as a single agent in experimental models of human prostate cancer. Our results showed that the β2M antibody exerted growth inhibitory and apoptotic action in AR-positive and PSA-positive human prostate cancer cells. The β2M antibody was also shown to induce similar apoptotic death in AR-negative and PSA-negative, and androgen-unresponsive human prostate cancer cells. Because aberrant androgen signaling mediated by the AR, a ligand-activated transcription factor and a survival factor, plays a key role in regulating prostate cancer growth and survival even in cells that are considered as androgen refractory (21, 22), we investigated the effects of the β2M antibody on the AR-signaling axis based on a cDNA microarray study, in which targeting β2M was shown to markedly down-regulate AR and PSA in AR-positive human prostate cancer cells C4-2B. Our results confirmed that the β2M antibody blocked AR signaling and PSA production in a series of AR-positive and PSA-positive, and lineage-related LNCaP (androgen dependent), C4-2 (androgen independent), and C4-2B (androgen independent) cells in a β2M-dependent manner (i.e., β2M protein could rescue the inhibitory effects of the β2M antibody).
We previously reported that a small protein, β2M, which was considered as a “housekeeping” gene product (23), was a key growth and signaling molecule regulating osteomimicry and promoting growth and survival in prostate cancer cells (8, 9). Targeting β2M and its signaling by β2M siRNA greatly induced prostate cancer cell death both in cultured cells and in mice with preestablished human prostate tumors (8). In the present study, we used the β2M antibody to block β2M-related signaling pathways, hoping to induce apoptosis in prostate tumors and rationalize the exploration of the β2M antibody as a novel agent for clinical trial in men with hormone-refractory cancer. We showed that the β2M antibody as a single agent significantly inhibited AR and PSA mRNA and protein expression in both LNCaP and C4-2B cells and induced apoptotic cell death in prostate tumor cells in vitro and in mouse xenografts (C4-2 and PC3 tumors) in vivo regardless of their AR status. The selective ability of the β2M antibody to block prostate tumor growth without affecting normal or nontumorigenic cells, including human normal prostatic epithelial and stromal cells, and normal hematopoietic cells in vitro or other normal tissues in vivo (12), suggests that the β2M antibody is a cancer-specific targeting agent that can be applied in the treatment of human prostate cancers. This conclusion is supported by previous studies in which immune intact mice with β2M knockdown survived and developed mild degrees of iron overload and arthritis without compromising their life expectancy (24–26). In addition, during a 10-week observation period, we have not noted any toxicity in mice treated intratumorally with the β2M antibody as evaluated by their body weights and physical appearance (data not shown). This observation is concurred by the early report of Yang et al. (12) although additional work is warranted to test the potential cytotoxicity of this antibody in immune intact hosts. We envision, nevertheless, that the β2M antibody can be applied in a cyclic manner to patients with prostate cancer, allowing
Fig. 5. β2M antibody did not affect cell proliferation and endogenous AR expression; it also did not induce apoptotic death in human normal/nontumorigenic prostatic epithelial cells. A. β2M antibody (0-20 μg/mL, 3-d incubation) did not significantly affect cell proliferation of human normal prostatic epithelial cells, RWPE-1, as determined by mitochondrial MTS assay. Control IgG (20 μg/mL) also did not affect the growth of RWPE-1 cells. The relative fold was assigned as 1.0 in the absence of β2M antibody treatment. Columns, mean of five replicate experiments; bars, SD. B. β2M antibody (0-10 μg/mL, 24-h treatment) did not inhibit AR nor activate cleaved caspase-9, caspase-3, and PARP protein expression in RWPE-1 cells assayed by Western blot. Control IgG (10 μg/mL) also did not affect AR, cleaved caspase, or PARP protein expression.

the immune system to return to normal function during the off-cycle of the β2M antibody application.

Other than the blockade of the β2M antibody on AR survival factor expression, the detailed molecular mechanisms by which the β2M antibody induced prostate cancer apoptosis are unclear. We previously showed that β2M promoted the expression of cell cycle markers, cyclin D1 and cyclin A, and cell growth in prostate cancer cells through the activation of a cAMP/PKA/CREB signaling pathway (8). We also showed that β2M stimulated renal cancer cell proliferation via the induction of phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and cAMP/PKA/CREB pathways (10). This pleiotropic cell signaling network activated by β2M is likely to be the target for the β2M antibody. It has been amply documented that the activation of AR, PI3K/Akt, and MAPK pathways are important features contributing to uncontrolled prostate cancer cell growth and survival (22, 27, 28). Indeed, we observed that the β2M antibody blocked not only the AR (Fig. 1A and B) but also the cell signaling network mediated by PI3K/Akt and MAPK pathways in LNCaP and C4-2B cells (Supplementary Fig. S1). These results are consistent with previous presentations that blocking β2M-mediated signaling pathways can interrupt the PI3K/Akt and MAPK signaling pathways and induce c-Jun-NH2-kinase phosphorylation, resulting in the activation of a caspase-9-dependent apoptotic cascade in human renal cell carcinoma (13) and hematologic cancer cells (12). The constitutive activation of a PI3K/Akt signaling pathway has been shown in prostate cancer cell lines by the inactivation of the PTEN tumor suppressor (29). Because the PI3K/Akt signaling pathway has been reported to mediate AR mRNA and protein expression through AR promoter regulation (30), we anticipated that the β2M antibody inhibition of the PI3K/Akt and MAPK signaling pathways would cause growth retardation, apoptosis, and down-regulation of AR expression and activity in AR-positive and PSA-positive LNCaP/C4-2/C4-2B cells. Likewise, because of the blockade of these critical signaling pathways, we also expected diminished growth and induced apoptosis in AR-negative prostate cancer cells in vitro and in vivo. These results could have significant clinical implications. For example, the β2M antibody could be superior to other antiandrogenic therapies with actions that rely on intrinsic AR expression by prostate cancer cells. The β2M antibody could be used either as a single reagent or in combination with other therapeutic modalities for the treatment of both hormone-dependent and hormone-refractory prostate cancers because these have been shown to exhibit marked heterogeneity of AR expression (31). This approach is promising, considering recent success in the development of therapeutic antibodies (32), such as trastuzumab, a HER2/erbB2 antibody for breast cancers; bevacizumab, a vascular endothelial growth factor antibody; and cetuximab, an epidermal growth factor receptor antibody for metastatic colon cancers.

In summary, our investigation revealed for the first time that (a) the β2M antibody inhibited the expression of the AR and PSA in both androgen-dependent and androgen-independent AR-positive and PSA-positive human prostate cancer cells; (b) the β2M antibody has a broad spectrum of growth-inhibitory effects in both AR-positive and AR-negative prostate cancer cells; and (c) although the β2M antibody has been shown to be a potent pleiotropic signaling and growth inhibitor and to induce programmed cell death through a caspase-9–dependent pathway in prostate cancer cells, this antibody exhibited low cytotoxicity in human normal prostatic epithelial and stromal cells, which make it an attractive and safe therapeutic agent for future clinical application to treat prostate cancer and its progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


