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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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| 14. ABSTRACT<br>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 <i>in vitro</i> and <i>in vivo</i> . 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  $\beta$ 2-microglobulin ( $\beta$ 2M).  $\beta$ 2M 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).  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M during prostate cancer progression. We also focus on the  $\beta$ 2M-mediated signaling and AR as a therapeutic target using a novel anti- $\beta$ 2M monoclonal antibody ( $\beta$ 2M 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  $\beta$ 2M-mediated signaling regulates AR expression in prostate cancer cells. **Specific Aim 2:** To determine the anti-tumor efficacy of  $\beta$ 2M Ab on prostate cancer cells *in vitro* and pre-established prostate tumors in mice *in vivo*.

## BODY:

**1) Blockade of  $\beta$ 2M downregulated AR and PSA expression in human prostate cancer cells**—We previously reported that  $\beta$ 2M is a growth and signaling-promoting factor for human prostate cancer cells (8). Target  $\beta$ 2M using a sequence specific  $\beta$ 2M siRNA (8) or  $\beta$ 2M 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  $\beta$ 2M and its related signaling interruption in cell cultures and an animal model, we established permanent  $\beta$ 2M knocking-down prostate cancer cells, C4-2B, by  $\beta$ 2M

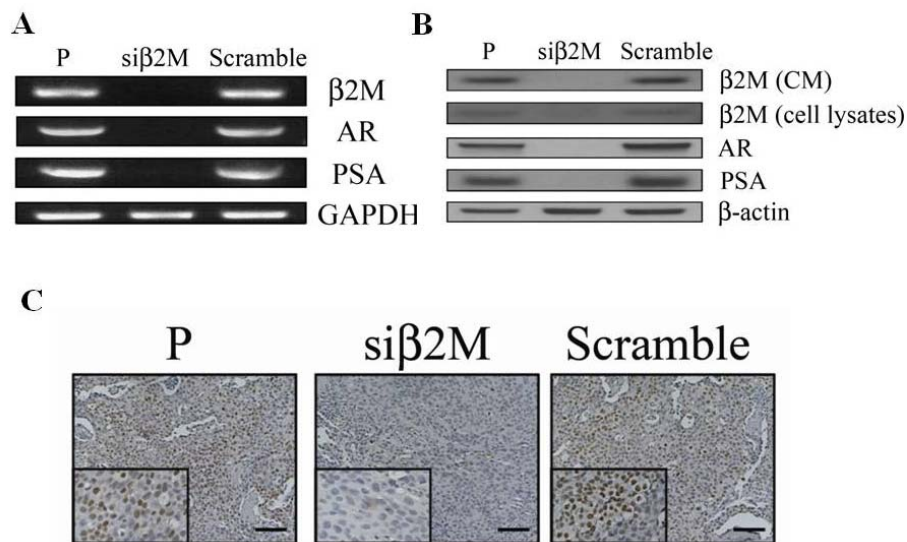


**Fig. 1.  $\beta$ 2M siRNA inhibited expression of AR and PSA mRNA and protein in prostate cancer cells *in vitro* and *in vivo*.** A,  $\beta$ 2M siRNA (si $\beta$ 2M) dramatically decreased  $\beta$ 2M, AR and PSA mRNA expression in stably transfected si $\beta$ 2M C4-2B prostate cancer cells analyzed by RT-PCR. Expression of GAPDH was used as a loading control. P: parental non-transfected C4-2B cells; Scramble: control scramble siRNA transfected C4-2B cells B, si $\beta$ 2M also markedly inhibited expression of secreted  $\beta$ 2M (from conditioned media, CM), soluble  $\beta$ 2M (from cell lysates), AR and PSA protein in si $\beta$ 2M C4-2B cells compared with P and Scramble C4-2B cells assayed by Western blot.  $\beta$ -actin was used as an internal loading control. C, Immunohistochemical staining analysis of AR in P (parental, untreated), si $\beta$ 2M ( $\beta$ 2M siRNA treated) and Scramble (control scramble siRNA treated) C4-2B tumor specimens from a subcutaneous xenograft mouse model. The result revealed that si $\beta$ 2M greatly inhibited AR expression compared with P and Scramble C4-2B subcutaneous tumors *in vivo*. Scale bars = 100  $\mu$ m.

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 ( $1.03 \pm 0.52$  ng, N=5, after 28-day treatment) compared with Scramble siRNA-treated C4-2B tumors ( $19.70 \pm 9.04$  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

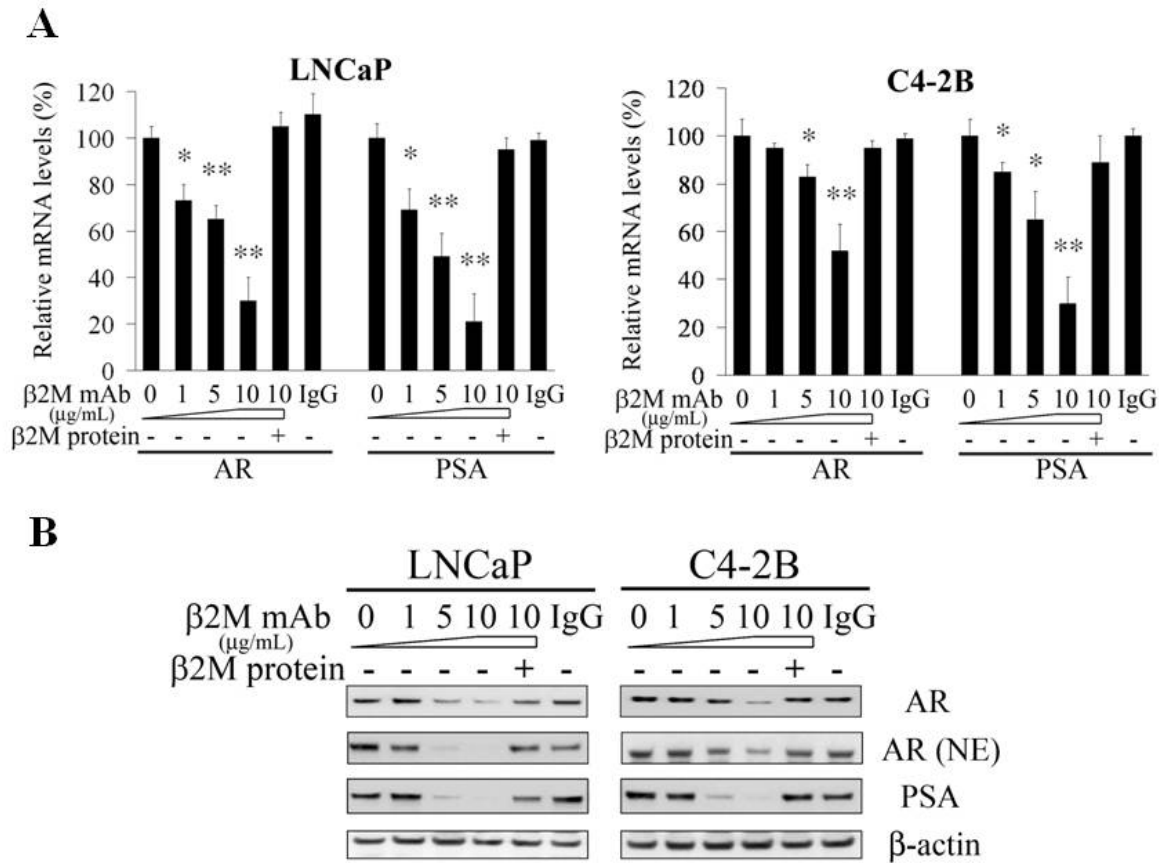
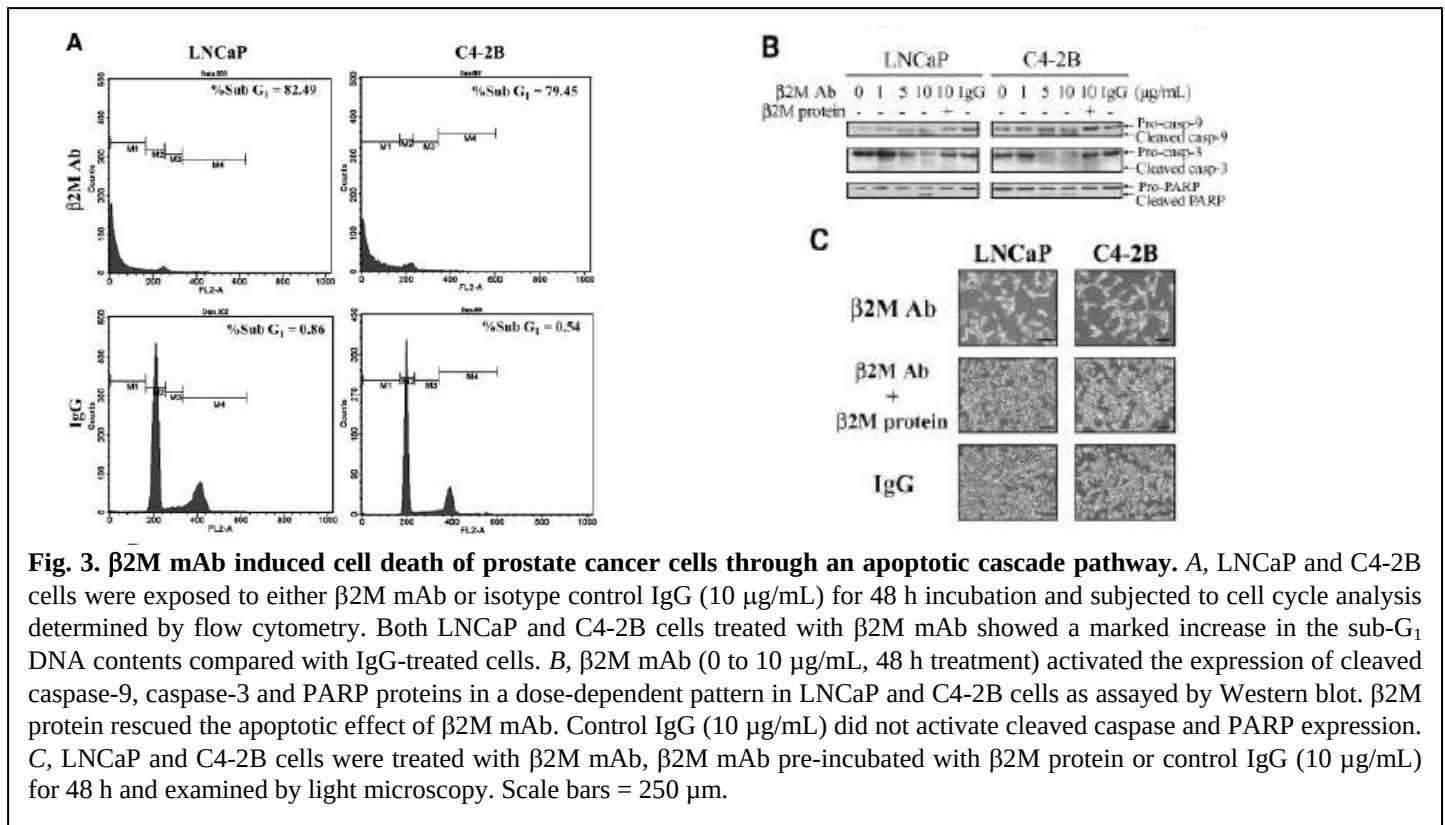


**Fig. 2. 2M mAb decreased AR and PSA expression in prostate cancer cells.** A,  $\beta$ 2M mAb decreased AR and PSA mRNA expression in a dose-dependent manner (0 to 10  $\mu$ g/mL) in both LNCaP (AD) and C4-2B (AI) prostate cancer cells determined by semi-quantitative RT-PCR. The inhibitory effect was restored by pre-incubation of  $\beta$ 2M mAb with the same amounts of purified  $\beta$ 2M protein. Isotype control IgG (10  $\mu$ g/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  $\pm$  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  $\mu$ g/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  $\mu$ g/mL) did not change AR and PSA protein expression.  $\beta$ -actin was used as an internal loading control.

to 10  $\mu\text{g/mL}$ ) 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  $\beta 2\text{M}$  mAb inhibitory effect, purified  $\beta 2\text{M}$  protein could rescue AR and PSA inhibition by  $\beta 2\text{M}$  mAb in prostate cancer cells. Control IgG did not affect AR and PSA expression. Not only decreased endogenous total AR protein,  $\beta 2\text{M}$  mAb also showed to inhibit nuclear AR protein expression in LNCaP and C4-2B cells (Fig. 2B). These data demonstrated that antagonizing extracellular  $\beta 2\text{M}$  by  $\beta 2\text{M}$  mAb also reduced AR and PSA expression at transcriptional and translational levels in prostate cancer cells.

**2)  $\beta 2\text{M}$  mAb induced apoptotic death in prostate cancer cells**—To determine the molecular mechanism by which  $\beta 2\text{M}$  mAb inhibited the growth of prostate cancer cells, we first examined apoptotic death in LNCaP and C4-2B cells, including sub- $G_1$  DNA content analysis and activation of caspase (19) and PARP expression. The results of flow cytometric analysis revealed that  $\beta 2\text{M}$  mAb greatly increased sub- $G_1$  DNA contents in LNCaP (%sub  $G_1$  = 82.49) and C4-2B (%sub  $G_1$  = 79.45) cells compared to control IgG-treated LNCaP (%sub  $G_1$  = 0.86) and C4-2B (%sub  $G_1$  = 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  $\beta 2\text{M}$  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  $\beta 2\text{M}$  mAb with purified  $\beta 2\text{M}$  protein. In addition, cell death induced by  $\beta 2\text{M}$  mAb was also confirmed at the level of light microscopy in LNCaP and C4-2B cells (Fig. 3C).



**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  $\beta$ 2M mAb**—Subsequently, we sought to characterize the transcriptional mechanism of AR expression regulated by  $\beta$ 2M 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),  $\beta$ 2M mAb (0 to 10  $\mu$ g/mL) significantly decreased hAR promoter luciferase activity in a concentration-dependent pattern (Fig. 4A). Purified  $\beta$ 2M protein could restore the inhibition of hAR promoter reporter activity by  $\beta$ 2M 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 ( $\Delta$ A,  $\Delta$ B and  $\Delta$ C, Fig. 4B) were generated and confirmed the DNA sequence. After

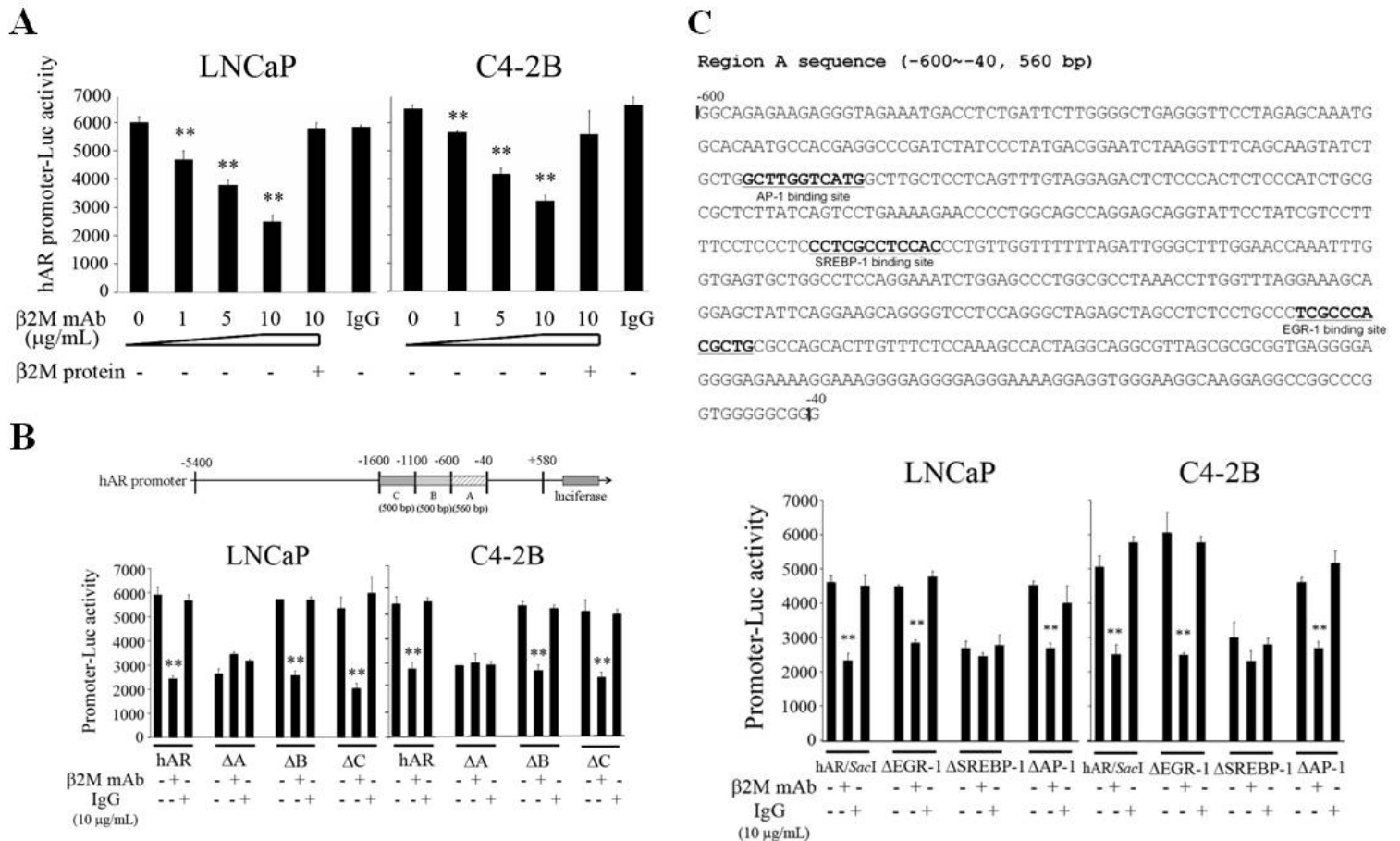


**Fig. 4. Sterol regulatory element-binding protein-1 (SREBP-1) binding site within the 5'-flanking promoter region of human AR (hAR) gene is responsible for AR transcriptional activity mediated by  $\beta$ 2M mAb.** A,  $\beta$ 2M mAb decreased hAR promoter (-5400 to +580) luciferase activity with a concentration-dependent pattern (0 to 10  $\mu$ g/mL) in LNCaP and C4-2B cells. Purified  $\beta$ 2M protein could restore the inhibitory effect of hAR promoter activity regulated by  $\beta$ 2M 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  $\beta$ 2M mAb in LNCaP and C4-2B cells.  $\beta$ 2M mAb (10  $\mu$ g/mL) significantly decreased the promoter luciferase activity of the deleted region B ( $\Delta$ B, -1100 to -600) and C ( $\Delta$ C, -1600 to -1100) in hAR promoter report constructs but did not affect the luciferase activity of the  $\Delta$ A construct. Isotype control IgG (10  $\mu$ g/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 ( $\Delta$ EGR-1,  $\Delta$ SREBP-1 and  $\Delta$ AP-1 binding sites), the promoter luciferase activities of  $\Delta$ EGR-1 and  $\Delta$ AP-1 binding site constructs were significantly inhibited by  $\beta$ 2M mAb in LNCaP and C4-2B cells. Only a slight drop of promoter luciferase activity was observed in a  $\Delta$ SREBP-1 binding site construct while treated with  $\beta$ 2M 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  $\beta$ -galactosidase activity and expressed as the mean  $\pm$  S.D. of three independent duplicate experiments. \*\*,  $P < 0.005$ .

transfected into LNCaP and C4-2B cells,  $\beta$ 2M mAb significantly inhibited the original hAR,  $\Delta$ B (deletion of -1100 to -600) and  $\Delta$ C (deletion of -1600 to -1100) promoter luciferase activities (Fig. 4B). However,  $\beta$ 2M mAb did not affect the promoter luciferase activity of the  $\Delta$ A construct (deletion of -600 to -40, Fig. 4B), and a decrease in the basal promoter luciferase activity was observed only the  $\Delta$ A 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  $\beta$ 2M mAb. Because the original hAR promoter reporter vector contains approximate 6 kb (from -5400 to +580) in length, we further used a restriction enzyme, *Sac*I, to generate a shorter promoter luciferase construct, the hAR/*Sac*I vector (2 kb only, deletion of -4700 to -740), and tested this reporter vector activity in LNCaP and C4-2B cells exposed with  $\beta$ 2M mAb or IgG. After luciferase activity assay, the basal promoter activity of this hAR/*Sac*I 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  $\beta$ 2M mAb in prostate cancer cells.

To further determine the  $\beta$ 2M 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  $\beta$ 2M 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/*Sac*I promoter luciferase vector and tested their reporter activity in prostate cancer cells. Among these three deletion constructs, the promoter luciferase activities of  $\Delta$ EGR-1 and  $\Delta$ AP-1 binding site constructs significantly inhibited by  $\beta$ 2M mAb similar with the hAR/*Sac*I construct activity in LNCaP and C4-2B cells (Fig. 4C). Only a slight drop of promoter luciferase activity was observed in a  $\Delta$ SREBP-1 binding site construct while treated with  $\beta$ 2M 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  $\beta$ 2M mAb.

**4) A transcription factor, SREBP-1, interacting with SREBP-1 binding site within the hAR promoter region mediated by  $\beta$ 2M 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  $\beta$ 2M mAb in prostate cancer cells, we conducted EMSA and ChIP. As shown in Fig. 5A, nuclear extracts prepared from  $\beta$ 2M 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  $\beta$ 2M mAb or control IgG treatment (lanes 2, 3 and 4). Purified  $\beta$ 2M protein could abrogate the complex formation decreased by  $\beta$ 2M mAb (lane 5). For the specificity of the SREBP-1 oligo-DNA probe, the  $^{32}$ P 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  $\beta$ 2M 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  $\beta$ 2M mAb in prostate cancer cells. In addition, by utilizing real-time quantitative PCR as readout, we observed that  $\beta$ 2M 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  $\beta$ 2M mAb in prostate cancer cells.

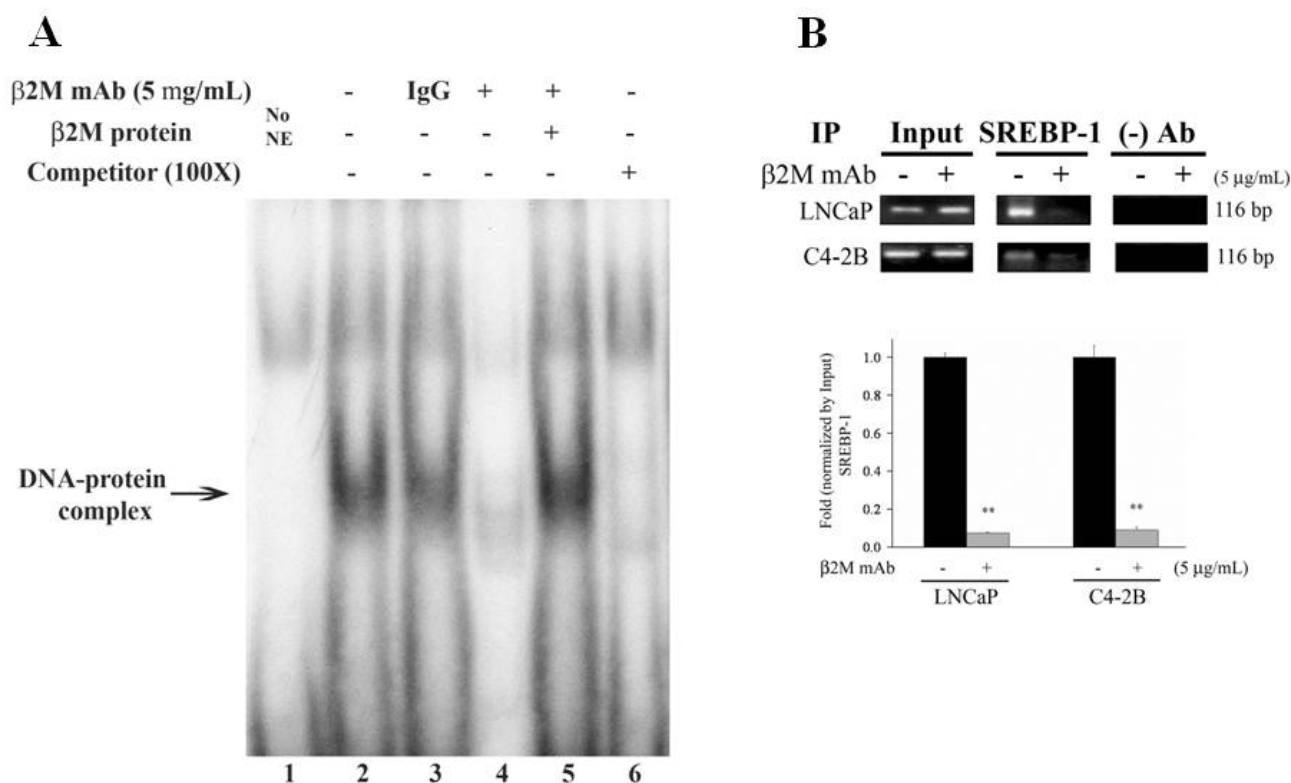
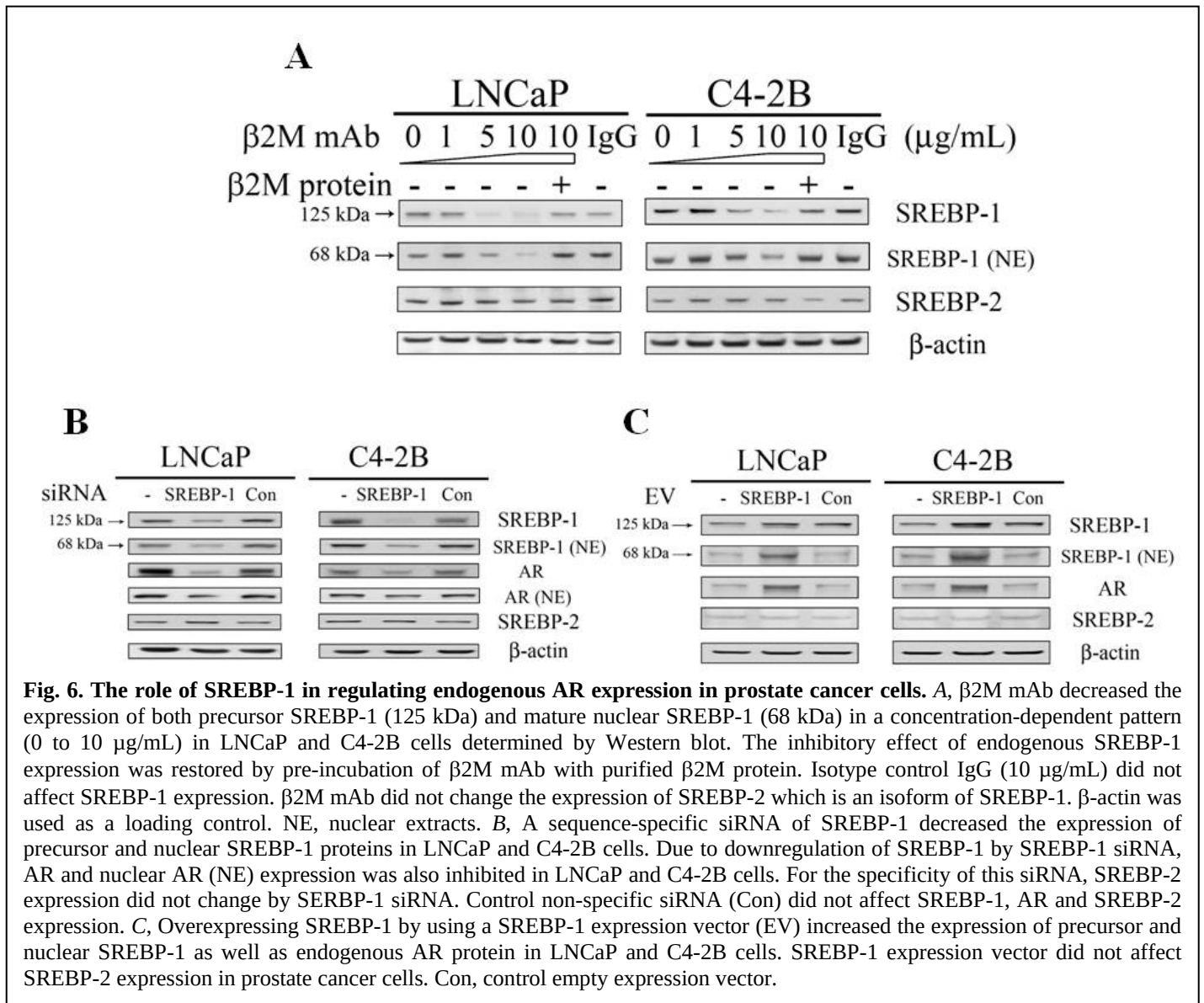


**Fig. 5.  $\beta$ 2M mAb inhibited the interaction between SREBP-1 and its binding *cis*-acting element located in the 5'-flanking hAR promoter region in prostate cancer cells.** A, Electrophoretic Mobility Shift Assay (EMSA). LNCaP cells were exposed to  $\beta$ 2M mAb, control IgG (5  $\mu$ g/mL) or vehicle for 24 h in a serum-free condition. Cells were harvested and nuclear extracts were prepared. EMSA was performed by incubating nuclear extracts with the  $^{32}$ P-labeled SREBP-1 oligo-DNA probe and Novex TBE system (Invitrogen). Lane 1 was no nuclear extracts added. Lanes 2 and 3 showed the nuclear protein-SREBP-1-oligo-DNA complex was formed and this complex did not affect by control IgG. Lane 4 showed  $\beta$ 2M mAb greatly inhibited this nuclear protein-DNA complex formation. Purified  $\beta$ 2M protein could rescue the inhibitory effect by  $\beta$ 2M mAb (lane 5). This complex was competed off by adding 100-fold of the  $^{32}$ P-unlabeled specific SREBP-1 oligo-DNA probe (lane 6). B, Chromatin Immunoprecipitation Assay (ChIP). LNCaP and C4-2B cells were treated with or without  $\beta$ 2M mAb (5  $\mu$ g/mL) for 24 h. The chromatin and nuclear proteins were cross-linked by formaldehyde and sheared by Enzymatic Shearing Cocktail (ChIP-IT kit, Active Motif, Inc.), and subjective to immunoprecipitation assay by using anti-SREBP-1 antibody or without antibody as a negative control. Analysis of the PCR product from chromatin DNA fragments immunoprecipitated by anti-SREBP-1 antibody as templates, a predicted single DNA band (116 bp, top panel) was amplified and visualized in LNCaP and C4-2B cells. This PCR product was decreased by treated with  $\beta$ 2M mAb in prostate cancer cells. In addition, quantitative real-time PCR of ChIP was performed (bottom panel). Quantitative PCR showed that  $\beta$ 2M mAb significantly caused downregulation of the interaction between SREBP-1 and SREBP-1 binding site at the 5'-flanking region of hAR promoter by 13.3 and 11.0-fold decrease in LNCaP and C4-2B cell, respectively. The quantitative PCR data were normalized by input and assigned as 1.0-fold without treating  $\beta$ 2M mAb for each prostate cancer cell line. \*\*,  $P < 0.005$ .

**5) The role of SREBP-1 in regulating AR expression and cell viability in prostate cancer cells**—We have shown that  $\beta$ 2M mAb inhibited AR expression through the interaction of SREBP-1 and SREBP-1 binding site in prostate cancer cells. To examine whether  $\beta$ 2M 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,  $\beta$ 2M mAb (0 to 10  $\mu$ g/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  $\beta$ 2M protein rescued the inhibitory effect of endogenous SREBP-1 expression by  $\beta$ 2M 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

(Fig. 6B). 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).



## 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 grant 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- $\beta$ 2-microglobulin monoclonal antibody inhibition of androgen receptor expression and survival through a lipogenic pathway in prostate cancer), please see the **APPENDICES** section.

## CONCLUSION:

$\beta$ 2M is a signaling and growth-promoting factor inducing prostate cancer cell proliferation, survival and progression. Interrupting  $\beta$ 2M and its related signaling pathways by a novel agent,  $\beta$ 2M 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  $\beta$ 2M 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,  $\beta$ 2M 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  $\beta$ 2M/SREBP-1 regulated AR expression in prostate cancer cells and the anti-tumor efficacy of  $\beta$ 2M mAb on prostate cancer cells in pre-established prostate tumors in mice *in vivo* which proposed in my approved DoD Statement of Work.

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**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- $\beta$ 2-microglobulin monoclonal antibody inhibition of androgen receptor expression and survival through a lipogenic pathway in prostate cancer**

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$\beta$ 2-Microglobulin ( $\beta$ 2M) 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  $\beta$ 2M and its related signaling axis by using a new agent, anti- $\beta$ 2M monoclonal antibody ( $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M mAb, we also observed that  $\beta$ 2M 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,  $\beta$ 2M 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.

## $\beta$ 2-Microglobulin Signaling Blockade Inhibited Androgen Receptor Axis and Caused Apoptosis in Human Prostate Cancer Cells

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**Abstract** **Purpose:**  $\beta$ 2-Microglobulin ( $\beta$ 2M) 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  $\beta$ 2M using anti- $\beta$ 2M antibody inhibited growth and induced apoptosis in prostate cancer cells.

**Experimental Design:** Polyclonal and monoclonal  $\beta$ 2M antibodies were used to interrupt  $\beta$ 2M signaling in human prostate cancer cell lines and the growth of prostate tumors in mice. The effects of the  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M antibody had no effect on AR expression or the growth of normal prostate cells.

**Conclusions:**  $\beta$ 2M 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  $\beta$ 2M signaling with the  $\beta$ 2M antibody inhibited cancer cell growth and induced its apoptosis. The  $\beta$ 2M antibody is a novel and promising therapeutic agent for the treatment of human prostate cancers.

$\beta$ 2-Microglobulin ( $\beta$ 2M) 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  $\beta$ 2M, 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  $\beta$ 2M into circulation or in the tumor microenvironment. Our laboratory first identified  $\beta$ 2M, an active component secreted by prostate cancer, and prostate and bone stromal cells, as a major growth factor and signaling molecule (8).  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M signaling similarly blocked human renal cell carcinoma growth (10).  $\beta$ 2M has recently been shown to be a useful biomarker for advanced human prostate cancer (11).  $\beta$ 2M 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- $\beta$ 2M antibody is a potent interrupter of  $\beta$ 2M-mediated signaling (8, 12). The  $\beta$ 2M 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  $\beta$ 2M 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),

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and in androgen-independent C4-2 tumor xenograft models. The  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M signaling via the external application of the  $\beta$ 2M antibody can profoundly alter intracellular cell signaling networks, including, but not limited to, the AR downstream signaling axis. Effective  $\beta$ 2M 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  $\beta$ 2M 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  $\mu$ g/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% CO<sub>2</sub> at 37°C. The anti- $\beta$ 2M 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  $\beta$ 2M 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  $3 \times 10^5$  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  $\mu$ g/mL of  $\beta$ 2M antibody; the  $\beta$ 2M antibody was preincubated for 30 min with the same amounts of purified human  $\beta$ 2M protein (Sigma) or 10  $\mu$ g/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'-ATGGCTGTCATTCACTACTCCTGGA-3' (forward) and 5'-AGATGGGCTTGACITTTCCAGAAAG-3' (reverse)], PSA [5'-ATGTGGGTCCCGGTTGTCTTCCTCACCCTGTC-3' (forward) and 5'-TCAGGGGTGGCCACGATGGTGTCTCTGATC-3' (reverse)], and glyceraldehyde-3-phosphate dehydrogenase [5'-ACCACAGTCCATGCCATCA-3' (forward) and 5'-TCCACACCTGTGTCTGT-3' (reverse)], respectively. 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  $\beta$ 2M monoclonal antibody-treated or IgG-treated prostate cells using a

lysis buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.02% NaN<sub>3</sub>, 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 IMx 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  $\beta$ 2M 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 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

**Sub-G<sub>1</sub> DNA content measurement.** LNCaP and C4-2B prostate cancer cells were plated on six-well plates at  $3 \times 10^5$  per well in T-medium containing 5% dextran-coated, charcoal-treated fetal bovine serum and exposed to 10  $\mu$ g/mL of  $\beta$ 2M 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  $\mu$ g/mL; Sigma) for 30 min, and stained with propidium iodide (25  $\mu$ g/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-G<sub>1</sub> 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  $\beta$ 2M 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  $2 \times 10^6$  cells per mouse. After 3 wk (PC3 tumor) or 4 wk (C4-2 tumor) of inoculation, 10  $\mu$ g of  $\beta$ 2M monoclonal antibody mixed with Surgifoam (Ethicon Inc.) to keep and slow release the  $\beta$ 2M 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 as the  $\beta$ 2M antibody. After 1 wk of treatment, tumor tissues were harvested from the euthanized mice and fixed in 10% formalin, dehydrated in ethanol, embedded in paraffin, and sectioned in slides. The blank tissue slides were subjected to immunohistochemical staining with anti-AR antibody (Santa Cruz Biotechnology) and M30 CytoDeath marker (DiaPharma Group, Inc.), and detected by the Dako Autostainer Plus system (Dako Corp.). For quantification of AR and M30 CytoDeath staining, 100 cells at five randomly selected areas were counted and the positive-staining cells were recorded.

**Statistical analysis.** Statistical analyses were done as described previously (9). Student's *t* test and two-tailed distribution were applied in the analysis of statistical significance.

## Results

**$\beta$ 2M antibody decreased AR and PSA expression in human prostate cancer cells.** We previously showed that  $\beta$ 2M 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  $\beta$ 2M and its downstream signaling by  $\beta$ 2M siRNA induced cell death in both human prostate and renal carcinoma models (8, 13). Because the downstream targets for  $\beta$ 2M signaling interruption are not completely clear in human prostate cancer cells, we conducted a cDNA microarray study (17) comparing  $\beta$ 2M 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.<sup>5</sup> To test the hypothesis that blocking  $\beta$ 2M-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,  $\beta$ 2M 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  $\beta$ 2M by the  $\beta$ 2M antibody decreased endogenous AR and PSA mRNA expression as determined by reverse transcription-PCR (Fig. 1A). The inhibitory effect of the  $\beta$ 2M antibody (0-10  $\mu$ g/mL) was concentration dependent, and the addition of purified  $\beta$ 2M protein rescued the decreased AR and PSA mRNA expression that had been inhibited by the  $\beta$ 2M antibody in LNCaP and C4-2B cells. Isotype-matched control IgG (10  $\mu$ g/mL) did not suppress AR and PSA mRNA expression. In parallel, the  $\beta$ 2M antibody (0-10  $\mu$ g/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  $\beta$ 2M 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  $\beta$ 2M antibody, but not the control IgG, in LNCaP and C4-2B cells (Fig. 1C). These results indicate that the  $\beta$ 2M antibody diminished AR and PSA mRNA and protein expression in both androgen-dependent and androgen-independent human prostate cancer cells.

**$\beta$ 2M antibody inhibited cell proliferation in human prostate cancer cell lines.** Because  $\beta$ 2M 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  $\beta$ 2M-mediated signaling axis may be cytotoxic to prostate cancer cells. When the LNCaP and C4-2B cells were exposed to the  $\beta$ 2M antibody (0-20  $\mu$ g/mL) for a 2-day incubation, the growth of these two prostate cancer cell lines was inhibited in a concentration-dependent manner, with an IC<sub>50</sub> of 10.3 and 7.4  $\mu$ g/mL, respectively (Fig. 2A). The purified  $\beta$ 2M protein was shown to rescue the  $\beta$ 2M 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  $\beta$ 2M antibody on the cell proliferation of AR-positive (LNCaP,

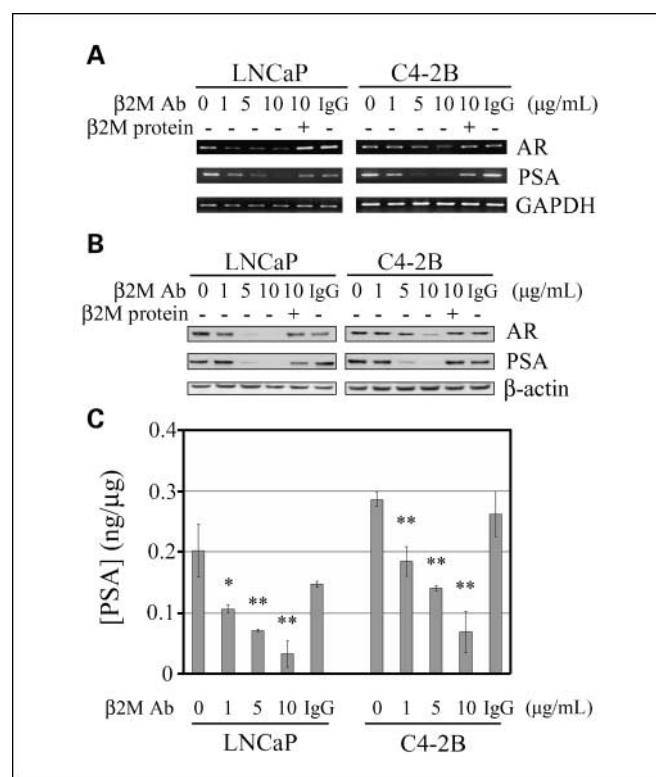


**Fig. 1.**  $\beta$ 2M antibody inhibited AR and PSA mRNA and protein expression in human prostate cancer cells. **A**,  $\beta$ 2M antibody ( $\beta$ 2M Ab) decreased AR and PSA mRNA expression in a dose-dependent manner (0-10  $\mu$ g/mL, 24-h treatment) in both LNCaP (androgen dependent) and C4-2B (androgen independent) prostate cancer cell lines detected by reverse transcription-PCR. The inhibitory effect was restored by the preincubation of the  $\beta$ 2M antibody with purified  $\beta$ 2M protein. Isotype control IgG (10  $\mu$ g/mL) did not affect AR and PSA mRNA expression. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **B**,  $\beta$ 2M antibody inhibited AR and PSA protein expression in a dose-dependent pattern (0-10  $\mu$ g/mL, 24-h treatment) in LNCaP and C4-2B cells assayed by Western blot. The inhibitory effect was abrogated by the preincubation of  $\beta$ 2M antibody with  $\beta$ 2M protein. Control IgG (10  $\mu$ g/mL) did not change AR and PSA protein expression.  $\beta$ -Actin was used as an internal loading control. **C**, secreted soluble PSA levels were also decreased by the  $\beta$ 2M antibody (0-10  $\mu$ g/mL), but not the control IgG, in a concentration-dependent inhibition in LNCaP and C4-2B cells determined by ELISA. The concentrations of PSA (ng) were normalized by total proteins ( $\mu$ g). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ , significant differences from the  $\beta$ 2M-antibody – untreated group. Columns, mean; bars, SD.

C4-2B, and ARCaP) and AR-negative (PC3 and DU-145) human prostate cancer cell lines. Figure 2B shows that the  $\beta$ 2M antibody (10  $\mu$ g/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  $\beta$ 2M antibody significantly inhibited cell proliferation in a broad range of human prostate cancer cell lines.

**$\beta$ 2M 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  $\beta$ 2M antibody inhibited the growth of prostate cancer cells, we first examined apoptotic death in LNCaP and C4-2B cells, including sub-G<sub>1</sub> DNA content analysis and activation of caspase (19) and PARP expression. The results of flow cytometric analysis revealed that the  $\beta$ 2M antibody greatly increased sub-G<sub>1</sub> DNA contents in LNCaP (% sub-G<sub>1</sub> = 82.49) and C4-2B (% sub-G<sub>1</sub> = 79.45) cells compared with the control IgG-treated LNCaP (% sub-G<sub>1</sub> = 0.86) and C4-2B (% sub-G<sub>1</sub> = 0.54) cells (Fig. 3A). Western blot analysis of

<sup>5</sup> Unpublished data.

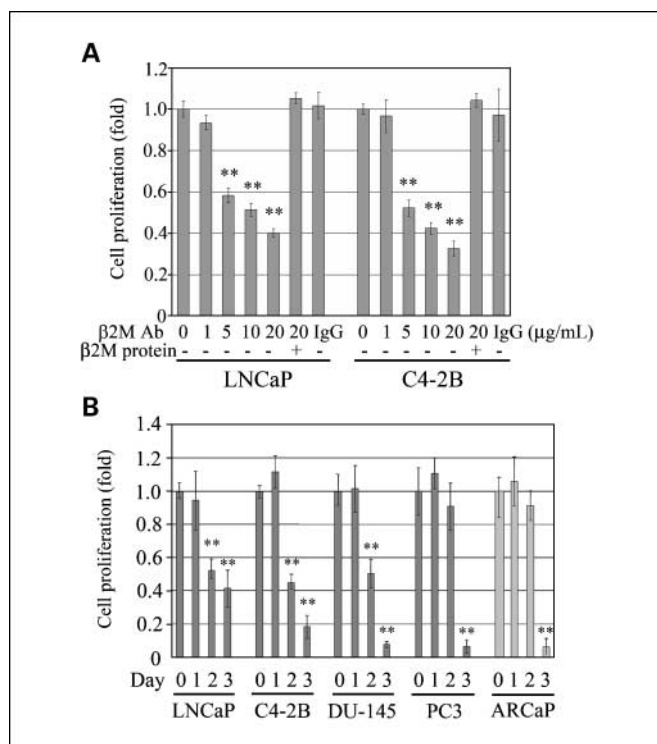


**Fig. 2.**  $\beta$ 2M antibody inhibited the growth of prostate cancer cell lines. **A**,  $\beta$ 2M antibody significantly affected the cell proliferation of LNCaP and C4-2B prostate cancer cells, with a dose-dependent inhibition (0–20  $\mu$ g/mL) after 2-d incubation determined by mitochondrial MTS assay (Promega). Purified  $\beta$ 2M protein rescued the inhibitory effect on cell growth regulated by the  $\beta$ 2M antibody. IgG (20  $\mu$ g/mL) did not decrease the growth of LNCaP and C4-2B cells. The relative fold was assigned as 1.0 in the absence of  $\beta$ 2M antibody treatment. \*\*,  $P < 0.005$ , significant differences from the  $\beta$ 2M-antibody – untreated group. Columns, mean of five replicate experiments; bars, SD. **B**,  $\beta$ 2M antibody (10  $\mu$ g/mL) inhibited the cell proliferation of a broad range of human prostate cancer cell lines, LNCaP, C4-2B, DU-145, PC3, and ARCaP, during 3-d treatment. The cell numbers were measured daily with a mitochondrial MTS method. The relative fold was assigned as 1.0 at day 0 for each prostate cancer cell line. \*\*,  $P < 0.005$ , significant differences from day 0 for each cell line. Columns, mean of four or five replicate experiments; bars, SD.

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  $\beta$ 2M 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  $\beta$ 2M antibody with purified  $\beta$ 2M protein. In addition, cell death induced by the  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M 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 controls. The cell numbers of positive AR staining

in the  $\beta$ 2M-antibody-treated C4-2 tumor xenografts were greatly decreased from  $81 \pm 6$  per 100 cells (IgG controls) and  $76 \pm 4$  per 100 cells (saline controls) to  $10 \pm 3$  per 100 cells. Markedly increased prostate cancer death from the  $\beta$ 2M antibody was observed in both C4-2 (the positive M30 CytoDeath staining cells were  $36 \pm 8$  cells per 100 cells) and PC3 ( $55 \pm 15$  cells per 100 cells) tumor specimens compared with the IgG-treated (C4-2,  $9 \pm 2$  cells per 100 cells; PC3,  $16 \pm 3$  cells per 100 cells) and saline-treated (C4-2,  $10 \pm 3$  cells per 100 cells; PC3,  $13 \pm 2$  cells per 100 cells) control groups.

We further investigated whether the  $\beta$ 2M antibody may be a safe reagent to selectively kill cancer but not normal or nontumorigenic immortalized cell lines. A human nontumorigenic prostatic epithelial cell line, RWPE-1, was exposed to the  $\beta$ 2M antibody and the control IgG. In contrast to human prostate cancer cells, the  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M antibody effectively induced human prostate cancer, but not normal prostate, cell apoptosis in culture. The  $\beta$ 2M antibody induced cell death in prostate tumor xenografts in mice regardless of their AR status. The  $\beta$ 2M 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,  $\beta$ 2M, using  $\beta$ 2M antibody as a single agent in experimental models of human prostate cancer. Our results showed that the  $\beta$ 2M antibody exerted growth inhibitory and apoptotic action in AR-positive and PSA-positive human prostate cancer cells. The  $\beta$ 2M 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  $\beta$ 2M antibody on the AR-signaling axis based on a cDNA microarray study, in which targeting  $\beta$ 2M was shown to markedly down-regulate AR and PSA in AR-positive human prostate cancer cells C4-2B. Our results confirmed that the  $\beta$ 2M 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  $\beta$ 2M-dependent manner (i.e.,  $\beta$ 2M protein could rescue the inhibitory effects of the  $\beta$ 2M antibody).

We previously reported that a small protein,  $\beta 2M$ , 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  $\beta 2M$  and its signaling by  $\beta 2M$  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  $\beta 2M$  antibody to block  $\beta 2M$ -related signaling pathways, hoping to induce apoptosis in prostate tumors and rationalize the exploration of the  $\beta 2M$  antibody as a novel agent for clinical trial in men with hormone-refractory cancer. We showed that the  $\beta 2M$  antibody as a single agent significantly inhibited AR and PSA mRNA and protein

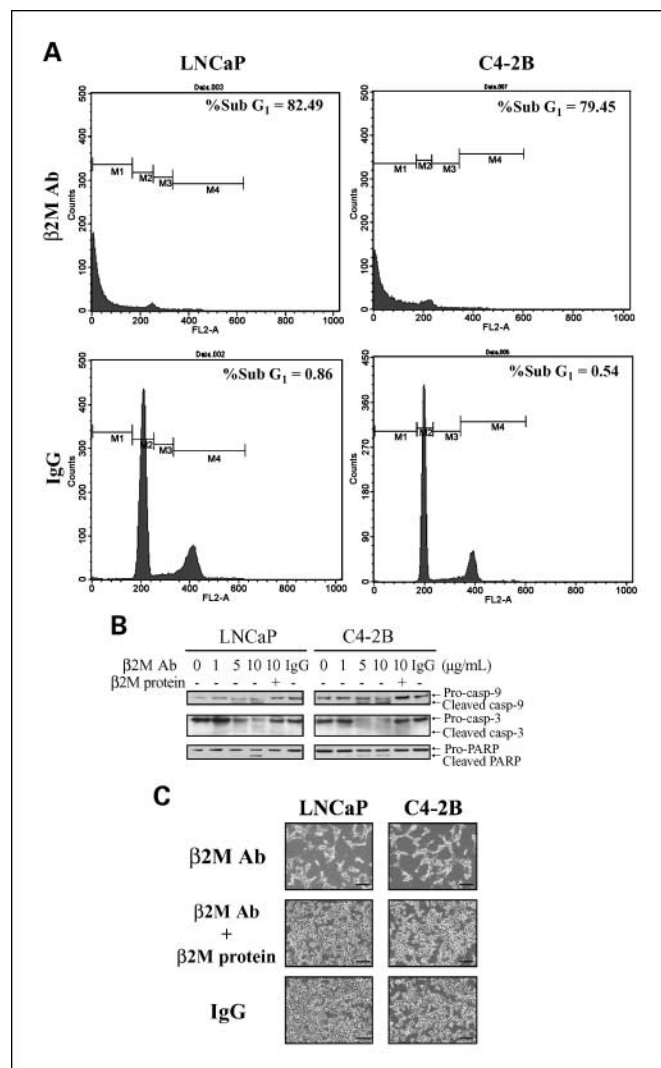


**Fig. 3.**  $\beta 2M$  antibody induced the cell death of prostate cancer cells through an apoptotic cascade pathway. **A**, LNCaP and C4-2B cells were exposed to either the  $\beta 2M$  antibody or isotype control IgG (10  $\mu\text{g}/\text{mL}$ ) for 48-h incubation and subjected to cell cycle analysis determined by flow cytometry. Both LNCaP and C4-2B cells treated with the  $\beta 2M$  antibody showed a marked increase in the sub-G<sub>1</sub> DNA contents compared with IgG-treated cells. **B**,  $\beta 2M$  antibody (0-10  $\mu\text{g}/\text{mL}$ , 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.  $\beta 2M$  protein rescued the apoptotic effect of the  $\beta 2M$  antibody. Control IgG (10  $\mu\text{g}/\text{mL}$ ) did not activate cleaved caspase and PARP expression. **C**, LNCaP and C4-2B cells were treated with the  $\beta 2M$  antibody; the  $\beta 2M$  antibody was preincubated with  $\beta 2M$  protein or control IgG (10  $\mu\text{g}/\text{mL}$ ) for 48 h and examined by light microscopy. Bar, 250  $\mu\text{m}$ .

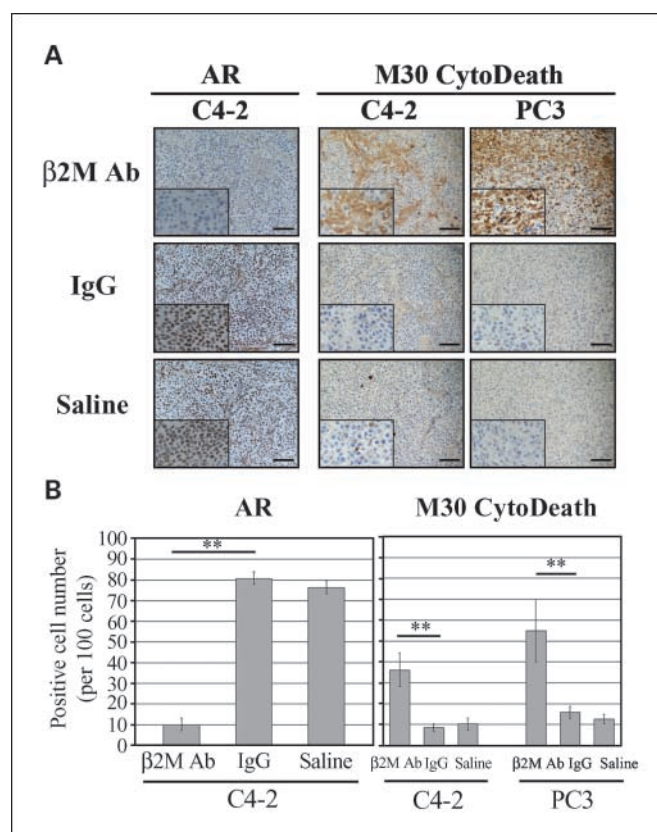


**Fig. 4.**  $\beta 2M$  antibody decreased AR expression and induced the cell death of subcutaneous C4-2 and PC3 prostate tumor growth in athymic *nu/nu* mice. **A**, immunohistochemical analysis showed dramatic down-regulation of AR expression in  $\beta 2M$  antibody-treated subcutaneous C4-2 tumor mouse xenografts ( $n = 4$ ) but not in the control IgG-treated ( $n = 4$ ) or saline-treated ( $n = 4$ ) C4-2 tumor-bearing mice. The  $\beta 2M$  antibody also markedly induced apoptotic death in both subcutaneous C4-2 ( $n = 4$ ) and PC3 ( $n = 4$ ) prostate tumors in xenograft mice assayed by M30 CytoDeath marker staining. Bar, 100  $\mu\text{m}$ . **B**, quantification of the positive AR and M30 CytoDeath marker staining cells in C4-2 and PC3 tumor specimens from the immunohistochemical analysis (**A**). One hundred cells at five randomly selected areas were counted. \*\*,  $P < 0.005$ , significant differences from the control IgG group. Columns, mean; bars, SD.

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  $\beta 2M$  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  $\beta 2M$  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  $\beta 2M$  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  $\beta 2M$  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  $\beta 2M$  antibody can be applied in a cyclic manner to patients with prostate cancer, allowing

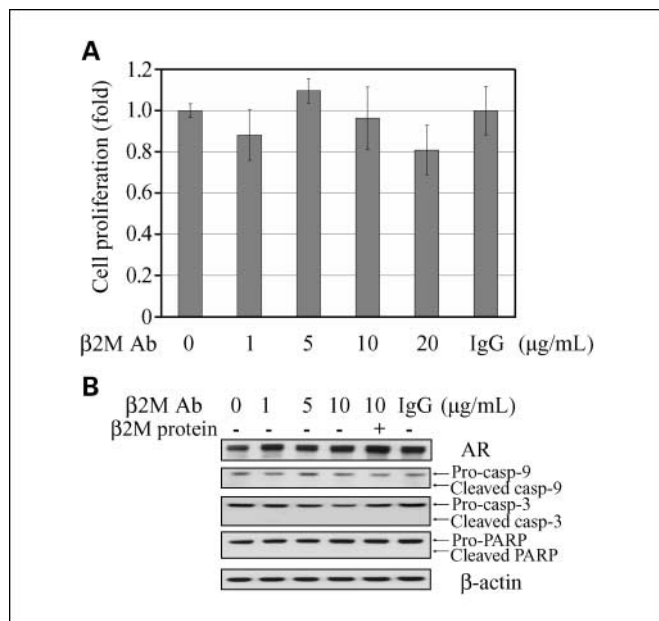


**Fig. 5.**  $\beta$ 2M antibody did not affect cell proliferation and endogenous AR expression; it also did not induce apoptotic death in human normal/nontumorigenic prostatic epithelial cells. **A.**  $\beta$ 2M antibody (0–20  $\mu$ g/mL, 3-d incubation) did not significantly affect cell proliferation of human normal prostatic epithelial cells, RWPE-1, as determined by mitochondrial MTS assay. Control IgG (20  $\mu$ g/mL) also did not affect the growth of RWPE-1 cells. The relative fold was assigned as 1.0 in the absence of  $\beta$ 2M antibody treatment. Columns, mean of five replicate experiments; bars, SD. **B.**  $\beta$ 2M antibody (0–10  $\mu$ g/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  $\mu$ g/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  $\beta$ 2M antibody application.

Other than the blockade of the  $\beta$ 2M antibody on AR survival factor expression, the detailed molecular mechanisms by which the  $\beta$ 2M antibody induced prostate cancer apoptosis are unclear. We previously showed that  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M is likely to be the target for the  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M-mediated signaling

pathways can interrupt the PI3K/Akt and MAPK signaling pathways and induce c-Jun-NH<sub>2</sub>-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  $\beta$ 2M 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  $\beta$ 2M antibody could be superior to other antiandrogenic therapies with actions that rely on intrinsic AR expression by prostate cancer cells. The  $\beta$ 2M 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  $\beta$ 2M 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  $\beta$ 2M antibody has a broad spectrum of growth-inhibitory effects in both AR-positive and AR-negative prostate cancer cells; and (c) although the  $\beta$ 2M 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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