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14. ABSTRACT We hypothesized that semenogelins, especially semenogelin I (Sgl) in the presence of zinc, promote prostate cancer growth via functioning as androgen receptor (AR) co-activators. Using cell lines stably expressing Sgl, we investigated biological functions of Sgl in prostate cancer. Zinc, without Sgl, inhibited cell growth of both AR-positive and AR-negative lines. Co-expression of Sgl prevented zinc inhibiting androgen-mediated proliferation of AR-positive cells, whereas Sgl and/or androgen showed marginal effects in AR-negative cells. Culture in the conditioned medium containing secreted forms of Sgl failed to significantly increase cell viability with or without zinc. Similar effects of Sgl overexpression on androgen-induced cell invasion, such as its significant enhancement with zinc, were seen. Overexpression of Sgl also augmented androgen-mediated prostate-specific antigen (mRNA, protein) in the presence of zinc. In luciferase assays, Sgl showed even slight inhibitory effects at 0 µM zinc and significant stimulatory effects at 100 µM zinc on androgen-enhanced AR transactivation. Using co-immunoprecipitation, we demonstrated androgen-induced physical interactions between AR and Sgl. These results suggest that intracellular Sgl, together with zinc, functions as an AR co-activator and thereby promotes androgen-mediated prostate cancer progression. We further found that Sgl did not interact with other steroid hormone receptors, including estrogen receptors and glucocorticoid receptor, and did not significantly affect AR N/C-terminus interactions. More importantly, the LxxLL motif (L=leucine; x=any amino acids) present in Sgl is likely to be essential and sufficient for mediating the interaction with AR.					
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1. INTRODUCTION

Semenogelins, mainly expressed and secreted by the seminal vesicle, are the major structural proteins in human semen containing a high concentration of Zn^{2+} , and their physiological functions have been well characterized. Specifically, semenogelins, upon binding to Zn^{2+} , play an important role in gel-like formation of the semen. After ejaculation, these proteins are degraded into smaller fragments by prostate-specific antigen (PSA), resulting in clotted gel liquefaction and release of the encased spermatozoa. Semenogelins are shown to be expressed in other male genital organs, such as the vas deferens, epididymis, and prostate, as well as in non-genital organs, suggesting their physiological role as modulators of zinc-dependent proteases throughout the body. Semenogelin I (SgI) expression has been detected in an androgen-sensitive prostate cancer line LNCaP, which is enhanced by zinc treatment, but not in other prostate cancer lines such as VCAP, CWR22Rv1, DU145, and PC3. We additionally demonstrated significantly higher levels of nuclear SgI expression in prostatic carcinoma than in non-neoplastic prostatic epithelium or high-grade prostatic intraepithelial neoplasia, which could also predict biochemical recurrence after radical prostatectomy. However, no functional analyses of semenogelins in pathological conditions have been reported and their roles in prostate cancer outgrowth remain uncertain. In this project, we aim to determine biological functions of SgI in prostate cancer, using preclinical models. In particular, we hypothesized that SgI could function as an androgen receptor (AR) co-activator in the presence of zinc and could thereby promote prostate cancer progression.

2. KEYWORDS

Androgen receptor; Co-activator; Prostate cancer; Prostate-specific antigen; Semenogelin; Seminal plasma protein; Seminal vesicle; Zinc

3. ACCOMPLISHMENTS

Major Goals

Task 1: To characterize semenogelins by testing their effects on the progression of prostate cancer *in vitro* (months 1-12: 100% completed)

Task 2: To assess the interactions between AR and semenogelins and the outcomes of their disruptions (months 13-36: 50% completed)

Task 3: To characterize semenogelins by testing their effects on the development and progression of prostate cancer *in vivo* (months 13-30: 20% completed)

Task 4: To assess the outcomes of disruption of AR-semenogelin interactions *in vivo* (months 25-36: 5% completed)

Accomplishments under the goals

(for Task 1) We performed additional experiments required for publication as an original research article and finalized the data. This resulted in a publication in March of 2015 (please see section 6). As we anticipated in this task, this article included the data showing that: 1) prostate cancer cells did not normally secrete detectable amounts of SgI (Figure 1); 2) SgI

protected AR-positive prostate cancer cells, but not AR-negative cells, from zinc that could strongly inhibit cell proliferation (Figure 2) and invasion (Figure 3); 3) Sgl increased the levels of PSA expression in prostate cancer cells (Figure 4); and 4) Sgl significantly induced androgen-mediated AR transactivation in the presence of a high level of zinc in prostate cancer cells but rather marginally inhibited it in the presence of low levels of zinc (Figure 5). These findings, together with co-immunoprecipitation assay data described below (also see Figure 6), suggest that Sgl promotes prostate cancer cell growth via functioning as an AR co-activator and protecting against zinc toxicity.

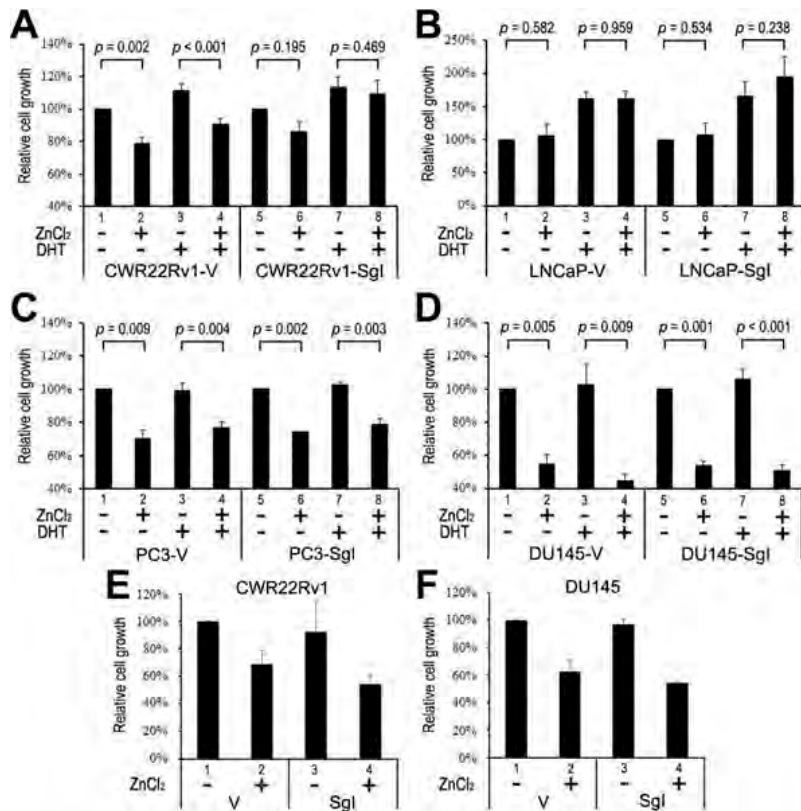
Figure 1. Sgl protein

expression and secretion in prostate cancer lines stably expressing Sgl. Cell

extracts (A) or acetone-precipitated proteins in conditioned media (B) from LNCaP-V/Sgl, DU145-V/Sgl, PC3-V/Sgl, and CWR22Rv1-V/Sgl were analyzed on western blots, using an antibody to Sgl (52 kDa) or β -actin (42 kDa). Fresh human seminal vesicle (SV) tissue was used as a positive control.



Figure 2. Cell viability of prostate cancer lines stably expressing Sgl. CWR22Rv1-V/Sgl (A), LNCaP-V/Sgl (B), PC3-V/Sgl (C), and DU145-V/Sgl (D) were cultured in phenol red-free medium supplemented with 5% charcoal-stripped fetal bovine serum (FBS) in the presence or absence of 100 μ M zinc and 1 nM dihydrotestosterone (DHT) for 96 hours. CWR22Rv1 (E) and DU145 (F) were cultured in conditioned medium (containing 10% normal FBS) derived from CWR22Rv1-V/Sgl culture in the presence or absence of 100 μ M zinc for 96 hours. Proliferation was assayed with MTT, and growth rates are presented relative to cell number in respective lines with mock treatment [lanes 1 (A-F) and 5 (A-D); set as 100%]. Each value represents the mean \pm SD of at least three determinations.



(for Task 2a: Nuclear receptor-semenogelin interactions) We first assessed the interactions between AR and Sgl. Co-immunoprecipitation assay clearly demonstrated a formation of AR (wild-type, T877A mutant)-Sgl complex especially in the presence of androgen (Figure 6). Nonetheless, we failed to confirm AR-Sgl interactions by mammalian two-hybrid assay in cells transfected with Gal4-DBD-AR, VP16-Sgl, and pG5-Luc reporter and treated with androgen. Additionally, in these assays, Sgl did not interact with estrogen receptor- α , estrogen receptor- β , or glucocorticoid receptor.

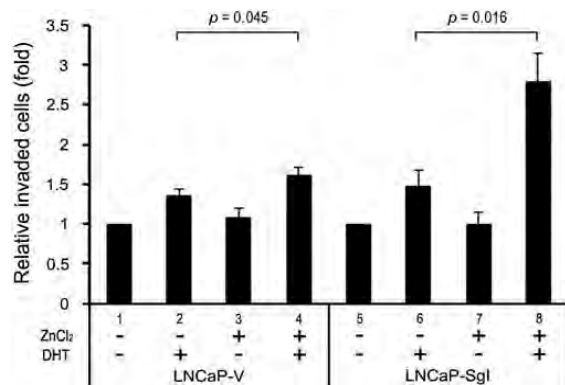


Figure 4. PSA expression in prostate cancer lines expressing SgI. (A) LNCaP-V/SgI cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 300 μ M zinc and 1 nM DHT for 48 hours were subjected to a quantitative reverse transcription-polymerase chain reaction. Expression of *PSA* gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock treatment in each cell line (lane 1 or 5; set as 1-fold). Each value represents the mean + SD from at least three independent experiments. CWR22Rv1 cells (B) transiently transfected with pSG5 or pSG5-SgI were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 100 μ M zinc and 1 nM DHT for 48 hours, and LNCaP-V/SgI cells (C) were similarly cultured with 300 μ M zinc \pm 1 nM DHT for 48 hours, as indicated. Cell extracts were then analyzed on western blots, using an antibody to PSA (33 kDa) or β -actin.

Figure 3. Cell invasion of prostate cancer lines stably expressing SgI. LNCaP-V/SgI cells cultured in the Matrigel-coated transwell chamber for 36 hours in the presence or absence of 300 μ M zinc and 1 nM DHT were used for transwell assay. The number of invaded cells in five random fields was counted under a light microscope, using a 40x objective. Invasion ability is presented relative to that in each cell line with mock treatment (lane 1 or 5; set as 1-fold). Each value represents the mean + SD of at least three independent experiments.

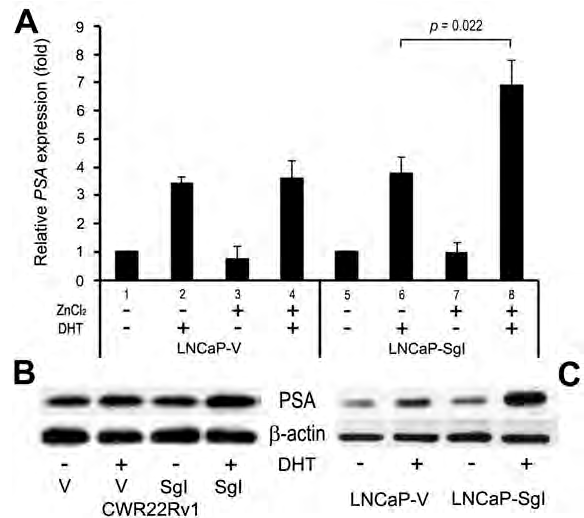


Figure 5. The effects of SgI on AR transcriptional activity in prostate cancer cells. PC3 cells were co-transfected with pSG5-AR, MMTV-Luc, pRL-TK, and either pSG5 or pSG5-SgI (AR:SgI = 1:5), and cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS along with mock (ethanol), zinc [(A) 0 μ M; (B) 15 μ M; (C) 100 μ M], and/or 1 nM DHT for 24 hours. Similarly, CWR22Rv1 cells were co-transfected with MMTV-Luc, pRL-TK, and either pSG5 or pSG5-SgI, and treated with mock (ethanol) or 1 nM DHT in the absence (D) or presence (E) of 100 μ M zinc for 24 hours. The luciferase activity is presented relative to that of mock treatment (first lanes; set as 1-fold). Each value represents the mean + SD of at least three determinations.

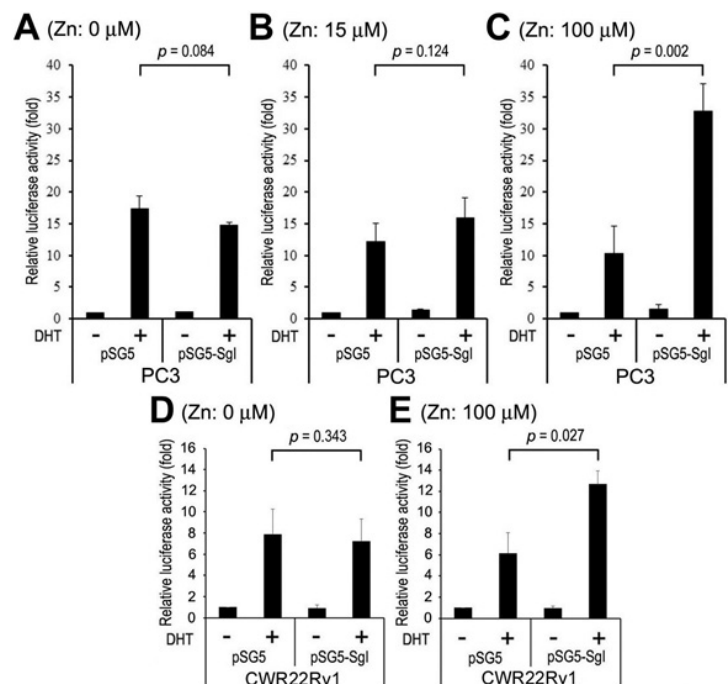
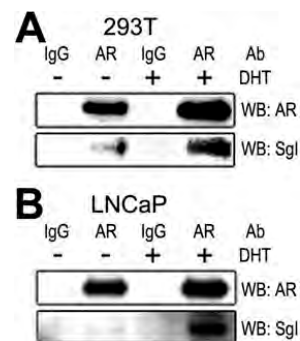


Figure 6. Co-precipitation of AR and SgI. Cell lysates from 293T transfected with pSG5-AR and pSG5-SgI (A) or LNCaP (B) treated with mock (ethanol) or 1 nM DHT were incubated with an anti-AR polyclonal antibody or normal rabbit IgG and then with A/G-agarose beads. The complex was resolved on a 10% SDS-polyacrylamide gel and blotted with an anti-AR or anti-SgI antibody.



(for Task 2b: AR N/C-terminus interaction) Mammalian two-hybrid assay was again used to determine the influence of SgI on AR N/C interaction. While AR N/C interactions were confirmed, no significant effect of SgI on these interactions was seen (Figure 7). Furthermore, SgI overexpression in LNCaP and VCAP cells did not significantly affect the expression and nuclear translocation of AR detected by Western blotting and immunofluorescence, respectively.

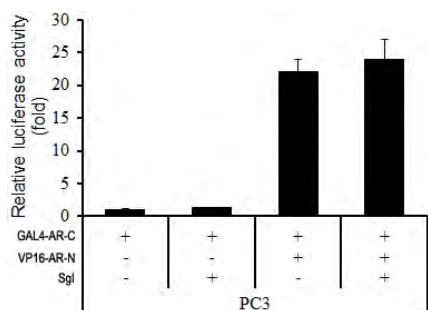


Figure 7. The effects of SgI on AR N/C interactions in prostate cancer cells. PC3 cells were co-transfected with GAL4-AR-C, VP16-AR-N or VP16-vector, pG5-Luc, pRL-TK, and pSG5 or pSG5-SgI, and cultured for 24 hours. The luciferase activity is presented relative to that of VP16-vector (no VP16-AR-N/SgI; first lane, set as 1-fold). Each value represents the mean + SD of at least three determinations.

(for Task 2d: Identification of AR-interaction domain in semenogelins) To identify minimal AR-interaction domain of SgI, the LxxLL (L = leucine; x = any amino acids) motif that has been reported to be essential and sufficient for mediating the interactions between AR and some AR co-regulators was mutated to LxxAA (A = alanine) by a site-directed mutagenesis method (Figure 8). This construct was used for further analyses.



Figure 8. Sequencing of wild-type SgI containing the LxxLL motif and a mutant SgI containing the LxxAA motif.

Co-immunoprecipitation assay in prostate cancer cells revealed that the mutant SgI containing the LxxAA motif no longer interacted with AR in the presence or absence of androgen (Figure 9). Mammalian two-hybrid assay also showed no interactions between AR and the mutant SgI, while there was no significant increase in luciferase activity in cells transfected with Gal4-DBD-AR, VP16-SgI (wild-type), and pG5-Luc.



Figure 9. Co-immunoprecipitation of AR and wild-type SgI or a mutant SgI. Cell lysates from PC3 transfected with AR and either wild-type or mutant SgI and treated with ethanol (mock) or 1 nM DHT was incubated with an anti-SgI antibody and was then immunoblotted with an anti-AR or anti-SgI antibody.

These findings suggest that the motif in SgI is critical for in AR-SgI interactions.

(for Task 3) We established a LNCaP subline stably expressing SgI-short hairpin RNA (shRNA)

(LNCaP-Sgl-shRNA) and a VCAP subline stably expressing Sgl (VCAP-Sgl), as well as their control lines, all of which will be used for the proposed animal study.

(for Task 4) As described above, we established the VCAP-Sgl and VCAP-vector cell lines both of which will be used for the proposed animal study.

Opportunities for training and professional development

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

Plan to do during the next reporting period to accomplish the goals

We will perform the remaining of the proposed experiments (Tasks 2, 3, and 4) to accomplish the goals.

4. IMPACT

Principal disciplines

Androgens act upon their binding to androgen receptor whose signals are further activated by co-activators. It is also well known that androgens play a key role in the development and progression of prostate cancer. The impact of our findings may be two-fold. First, a seminal plasma protein, semenogelin I, was found to serve as an androgen receptor co-activator only in the presence of zinc and could thereby promote prostate cancer outgrowth. Second, we demonstrated molecular evidence to answer why prostate cancer tissue contains high levels of zinc which by itself was known to have a strong inhibitory effect on tumor growth. These findings, together with our further work on this project, will help provide an effective treatment strategy for advanced prostate cancer.

Other disciplines

Nothing to report.

Technology transfer

Nothing to report.

Society beyond science and technology

Nothing to report.

5. CHANGES/PROBLEMS

Nothing to report.

6. PRODUCTS

Journal publications

Ishiguro H, Izumi K, Kashiwagi E, Zheng Y, Li Y, Kawahara T, Miyamoto H: Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity. *Am J Cancer Res* 5(2): 738-747, 2015 (please see APPENDICES).

Presentations

Kashiwagi E, Ishiguro H, Izumi K, Zheng Y, Li Y, Kawahara T, Miyamoto H: Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity.* 10th Annual Johns Hopkins Prostate Cancer Research Day at Mt. Washington Conference Center, Baltimore, Maryland (February 21, 2015).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project

Hiroshi Miyamoto, MD, PhD

Project role: PI

Person month worked: 1

Contribution to the project: As the PI Dr. Miyamoto has been responsible for overseeing all aspects of the project, including designing the experiments, analyzing the data, and writing project reports and manuscripts.

Other funding support: Department of Defense Prostate Cancer Research Program (W81XWH-10-2-0056 as a co-investigator); Astellas Scientific and Medical Affairs, Inc. (as a PI); NIH/NCI (R01 CA155477-01 as a consultant)

Eiji Kashiwagi, MD, PhD

Project role: Postdoctoral fellow

Person month worked: 6

Contribution to the project: Dr. Kashiwagi has performed most of the proposed experiments during months 13-18.

Other funding support: None

Hiroki Ide, MD

Project role: Postdoctoral fellow

Person month worked: 6

Contribution to the project: Dr. Ide has performed most of the proposed experiments during months 19-24.

Other funding support: None

Bin Han, MD, PhD

Project role: Postdoctoral fellow

Person month worked: 1

Contribution to the project: Dr. Han has performed co-immunoprecipitation assay and related experiments.

Other funding support: None

Change in the active other support of the PI

Department of Defense Prostate Cancer Research Program Physician Research Training Award (W81XWH-09-1-0305 to the PI) has closed on February 2, 2015.

Other organizations involved as partners

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

Ishiguro H, Izumi K, Kashiwagi E, Zheng Y, Li Y, Kawahara T, Miyamoto H: Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity. *Am J Cancer Res* 5(2): 738-747, 2015.

Original Article

Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity

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Abstract: A seminal plasma protein, semenogelin I (Sgl), contributes to sperm clotting, upon binding to Zn²⁺, and can be proteolyzed by prostate-specific antigen (PSA), resulting in release of the trapped spermatozoa after ejaculation. In contrast, the role of Sgl in the development and progression of any types of malignancies remains largely unknown. We previously demonstrated that Sgl was overexpressed in prostate cancer tissues and its expression was enhanced by zinc treatment in LNCaP cells. In the current study, using cell lines stably expressing Sgl, we investigated its biological functions, in conjunction with zinc, androgen, and androgen receptor (AR), in prostate cancer. Zinc, without Sgl, inhibited cell growth of both AR-positive and AR-negative lines. Co-expression of Sgl prevented zinc inhibiting dihydrotestosterone-mediated proliferation of AR-positive cells, whereas Sgl and/or dihydrotestosterone showed marginal effects in AR-negative cells. Similar effects of Sgl overexpression in LNCaP on dihydrotestosterone-induced cell invasion, such as its significant enhancement with zinc, were seen. Overexpression of Sgl in LNCaP and CWR22Rv1 cells also augmented dihydrotestosterone-mediated PSA expression (mRNA, protein) in the presence of zinc. However, culture in the conditioned medium containing secreted forms of Sgl failed to significantly increase cell viability with or without zinc. In luciferase reporter gene assays, Sgl showed even slight inhibitory effects (8% and 15% decreases in PC3 and CWR22Rv1, respectively) at 0 μ M zinc and significant stimulatory effects (2.1- and 3.2-fold) at 100 μ M zinc on dihydrotestosterone-enhanced AR transactivation. Co-immunoprecipitation then demonstrated dihydrotestosterone-induced physical interactions between AR and Sgl. These results suggest that intracellular Sgl, together with zinc, functions as an AR coactivator and thereby promotes androgen-mediated prostate cancer progression.

Keywords: Androgen receptor, prostate cancer, prostate-specific antigen, semenogelin, zinc

Introduction

The signaling pathway of androgen receptor (AR), a member of the nuclear receptor superfamily, plays a critical role in the growth of not only androgen-sensitive prostate cancer cells but also most cells from clinically defined androgen-independent prostate cancer. In particular, co-regulatory proteins that mediate receptor transcriptional activation or repression have been suggested to modulate the events of tumor progression. Various nuclear receptor coregulators as well as selective coactivators that enhance AR-mediated transcriptional activity have indeed been isolated [1-4].

The prostate accumulates the highest level of zinc (3,000-4,500 μ M in normal peripheral zone) in the body and secretes high amounts of zinc in the prostatic fluid (8,000-10,000 μ M) [5]. A significant decrease in zinc levels is seen in prostate cancer tissue, yet the concentrations (400-800 μ M) remain relatively high, compared with those in other soft tissue (200-400 μ M) or blood plasma (15 μ M) [5, 6]. Of note, however, zinc (e.g. 100 μ M in PC3 culture) has been shown to considerably inhibit the proliferation of prostate cancer cells [7-10]. To our knowledge, there is no definitive molecular evidence explaining the enigma of high concentrations of cytotoxic zinc in prostate cancer tissue. Furthermore, there are controversial epidemio-

logical data on the relationship between zinc intake and the risk of prostate cancer [5, 11].

Semenogelins, mainly expressed and secreted by the seminal vesicle, are the major structural proteins in human semen containing a high concentration of Zn^{2+} , and their physiological functions have been well characterized. Specifically, semenogelins, upon binding to Zn^{2+} , play an important role in gel-like formation of the semen [12]. After ejaculation, these proteins are degraded into smaller fragments by prostate-specific antigen (PSA), resulting in clotted gel liquefaction and release of the encased spermatozoa [13]. Semenogelins have also been shown to inhibit the protease activity of PSA [14]. Semenogelins are expressed in other male genital organs, such as the vas deferens, epididymis, and prostate, as well as in non-genital organs, suggesting their physiological role as modulators of zinc-dependent proteases throughout the body [15, 16]. Semenogelin I (Sgl) expression has been detected in an androgen-sensitive prostate cancer line LNCaP, which is enhanced by zinc treatment, but not in other prostate cancer lines such as CWR22Rv1, DU145, and PC3 [15, 17]. We additionally demonstrated significantly higher levels of nuclear Sgl expression in prostatic carcinoma than in non-neoplastic prostatic epithelium or high-grade prostatic intraepithelial neoplasia (PIN), which could also predict biochemical recurrence after radical prostatectomy [17, 18]. However, no functional analyses of semenogelins in pathological conditions have been reported and their roles in prostate cancer growth remain uncertain. In the current study, we aim to determine the biological significance of Sgl, in conjunction with zinc, androgen, and AR, in prostate cancer cells.

Materials and methods

Plasmids

The entire coding region of Sgl amplified using Phusion-High Fidelity DNA polymerase (Thermo Fisher Scientific) was subcloned into pSG5 [17] and lentivirus pWPI vector [19]. pSG5-AR, pGL3-MMTV-luciferase, and pRL-TK have been used in our previous studies [20, 21].

Antibodies and chemicals

Anti-AR (N-20), anti-Sgl (E-15), and anti- β -actin (R-22) antibodies were purchased from Santa

Cruz Biotechnology. An anti-PSA antibody (A0562) was purchased from Dako. Dihydrotestosterone (DHT) and ZnCl_2 were from Sigma-Aldrich and Alfa Aesar, respectively.

Cell lines

CWR22Rv1, LNCaP, PC3, and DU145 cell lines originally obtained from the American Type Culture Collection and recently authenticated by the institutional core facility were maintained with RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (FBS). To generate cell lines stably expressing Sgl, pWPI-Sgl, along with GFP expressing vector, was co-transfected, using GeneJuice transfection reagent (Novagen), and GFP expressing cells were selected.

MTT assay

Cell viability was assessed, using methylthiazolyl-diphenyl-tetrazolium bromide (MTT) assay. Cells ($1-3 \times 10^3$ /well) seeded in 96-well tissue culture plates were incubated in the presence or absence of zinc and DHT. The media were refreshed every 48 hours. After 96 hours of treatment, 10 μL MTT stock solution (5 mg/mL; Sigma) was added to each well with 100 μL of medium for 4 hours at 37°C . The medium was replaced with 100 μL dimethyl sulfoxide, followed by incubation for 5 minutes at room temperature. The absorbance at a wavelength of 570 nm with background subtraction at 655 nm was then measured.

Transwell invasion assay

Cell invasiveness was determined, using Matrigel-coated transwell chambers (Costar), as described previously [21]. Briefly, cells (5×10^4) in 100 μL of serum-free medium were added to the upper chamber of the transwell, while 600 μL of medium containing 5% FBS was added to the lower chamber. The media in both chambers contained ethanol, zinc, and/or DHT. After incubation for 36 hours at 37°C in a CO_2 incubator, invaded cells were fixed, stained with 0.1% crystal violet, and counted.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA (0.5 μg) was isolated from the cultured cells, using TRIzol (Invitrogen), and reverse transcribed with oligo (dT) primers and Omniscript reverse transcriptase (Qiagen), as

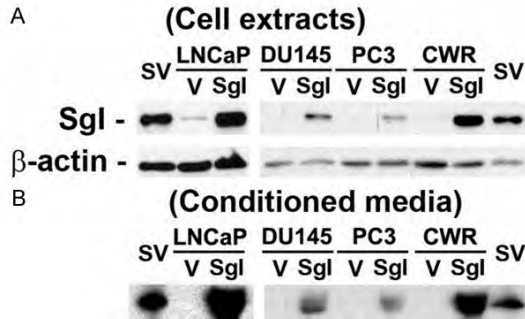


Figure 1. Sgl protein expression and secretion in prostate cancer lines stably expressing Sgl. Cell extracts (A) or acetone-precipitated proteins in conditioned media (serum-free, no additional zinc) (B) from LNCaP-V/Sgl, DU145-V/Sgl, PC3-V/Sgl, and CWR22Rv1-V/Sgl were analyzed on western blots, using an antibody to Sgl (52 kDa) or β-actin (42 kDa). Fresh human seminal vesicle (SV) tissue was used as a positive control.

described previously [19, 21, 22]. Real-time PCR was then performed, using RT² SYBR[®] Green FAST Mastermix (Qiagen) for iCycler (Invitrogen). The following primer pairs were used for RT-PCR: PSA (forward, 5'-GCAGTC-TGCGGCGGTGTTCT-3'; reverse, 5'-GCGGGTG-TGGGAAGGTGTGG-3'), and GAPDH (forward, 5'-CTCCTCCACCTTTGACGCTG-3'; reverse, 5'-C-ATACCAGGAAATGAGCTTGACAA-3').

Western blot

Protein extraction and western blotting were performed, as described previously [19-22] with minor modifications. Briefly, equal amounts of protein obtained from cell extracts were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes electronically, blocked, and incubated with a specific primary antibody. The membrane was then incubated with a HRP-conjugated secondary antibody, and specific signals were detected, using chemiluminescent substrate kit (Thermo Fisher Scientific).

Luciferase assay

Cells were transfected with an androgen response element-reporter (MMTV-Luc), pSG5 or pSG5-Sgl, and a control reporter (pRL-TK), using GeneJuice. pSG5-AR was also transfected into PC3 cells. Then, the cells were treated with zinc and/or DHT for 24 hours, and the luciferase activity was determined in the cell lysates, using a Dual-Luciferase Reporter Assay

kit (Promega) and luminometer (FLUOstar Omega, BMG Labtech).

Co-immunoprecipitation

The cell lysates (500 g) were incubated with 2 g anti-AR antibody or normal rabbit IgG for 16 hours at 4°C with agitation. Protein A/G-agarose beads were then added, and binding proteins were eluted. The eluted proteins were analyzed by western blot with an anti-AR or anti-Sgl antibody.

Statistical analysis

Student's *t*-test was used to analyze differences in variables with a continuous distribution. *P* values less than 0.05 were considered statistically significant.

Results

Expression of Sgl in prostate cancer cells and conditioned media

Using a lentivirus vector, we generated prostate cancer cell lines stably expressing Sgl. Overexpression of Sgl protein in these stable cell lines and relatively weak expression of endogenous Sgl in LNCaP were confirmed (**Figure 1A**). To detect a secreted form of Sgl, western blot was also performed in acetone-precipitated medium where each stable line was cultured under serum-free conditions for 24 hours. No signal was detected in conditioned medium after culturing Sgl-weakly positive (*i.e.* no additional zinc; RPMI 1640 with 10% FBS contains approximately 3.8 μM zinc [23]) LNCaP-Vector (V) as well as three Sgl-negative control lines (**Figure 1B**). In contrast, Sgl was found to be secreted in the supernates where Sgl-overexpressing cells were cultured. These results suggest that, in accordance with our immunohistochemistry data in radical prostatectomy specimens [18], prostate cancer cells do not normally secrete detectable amounts of Sgl.

Induction of prostate cancer progression by Sgl with zinc

To see if Sgl affects prostate cancer cell proliferation, we performed MTT assay in the stable cells. Each line was cultured for 4 days in the presence or absence of DHT (1 nM) and zinc (100 μM). As expected, zinc treatment signifi-

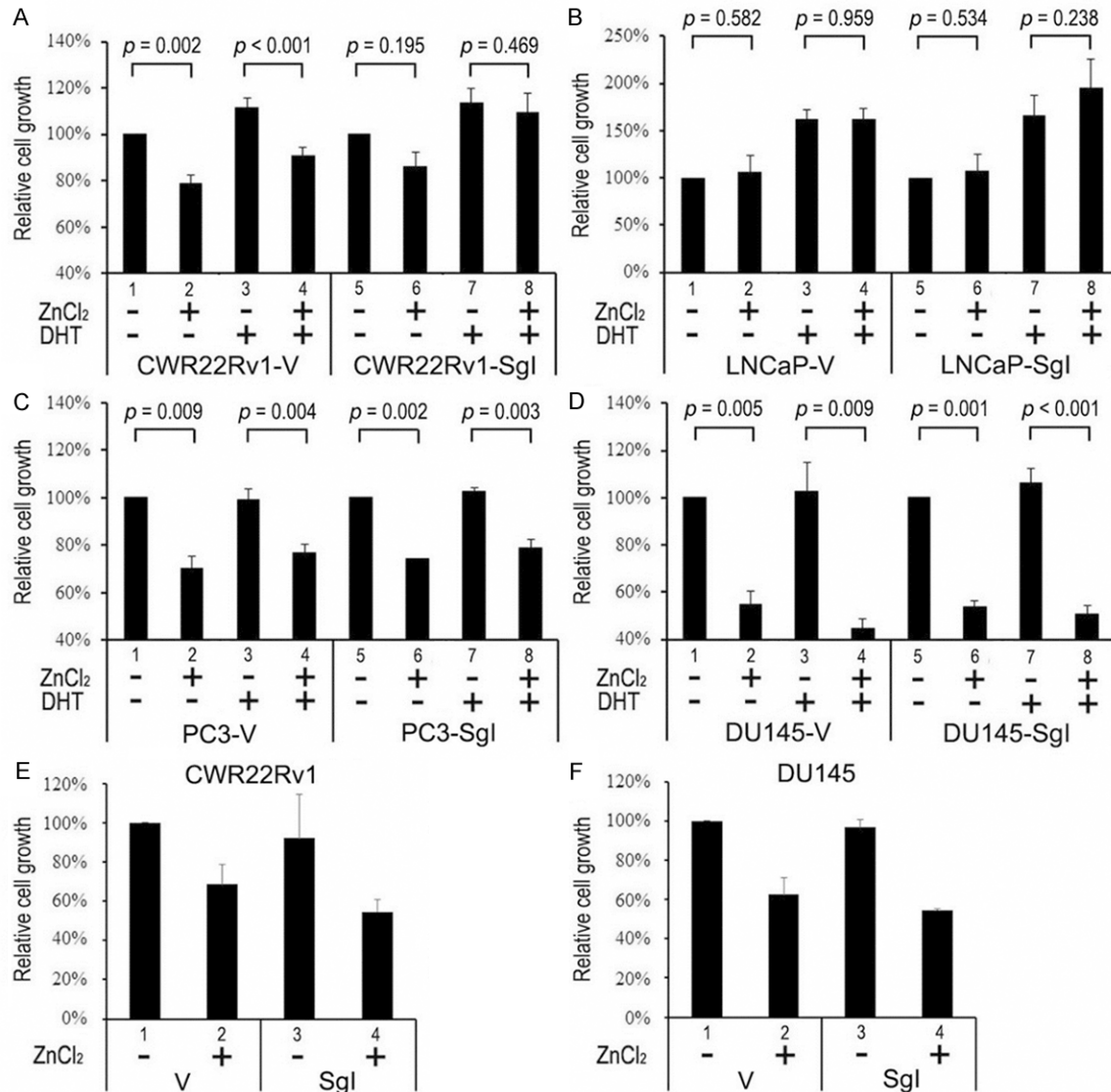


Figure 2. Cell viability of prostate cancer lines stably expressing Sgl. CWR22Rv1-V/Sgl (A), LNCaP-V/Sgl (B), PC3-V/Sgl (C), and DU145-V/Sgl (D) were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 100 μ M zinc and 1 nM DHT for 96 hours. CWR22Rv1 (E) and DU145 (F) were cultured in conditioned medium (containing 10% normal FBS) derived from CWR22Rv1-V/Sgl culture in the presence or absence of 100 μ M zinc for 96 hours. Proliferation was assayed with MTT, and growth rates are presented relative to cell number in respective lines with mock treatment [lanes 1 (A-F) and 5 (A-D); set as 100%]. Each value represents the mean \pm SD of at least three determinations.

cantly inhibited the growth of all control lines (Figure 2; 21-45% decrease; lanes 1 vs. 2) except LNCaP-V. In AR-positive CWR22Rv1-derived cells (Figure 2A), DHT increased the growth by 12-13% without zinc treatment (lanes 1 vs. 3 and 5 vs. 7). In the presence of zinc, DHT showed a similar induction rate in CWR22Rv1-V (14% increase; lanes 2 vs. 4), whereas overexpression of Sgl resulted in a statistically significant increase in the growth rate (27%; lanes 6 vs. 8; $p = 0.034$). Thus, zinc

only marginally decreased cell growth of CWR22Rv1-Sgl (lanes 5 vs. 6 and 7 vs. 8). In LNCaP cells with endogenous Sgl (LNCaP-V; Figure 2B), zinc treatment did not decrease, rather marginally increased, the growth in the absence (lanes 1 vs. 2) or presence (lanes 3 vs. 4) of DHT. DHT increased the growth of LNCaP-V without (62%; lanes 1 vs. 3; $p = 0.009$) or with (52%; lanes 2 vs. 4; $p = 0.014$) zinc as well as that of LNCaP-Sgl without (66%; lanes 5 vs. 7; $p = 0.036$) or with (82%; lanes 6 vs. 8; $p = 0.018$)

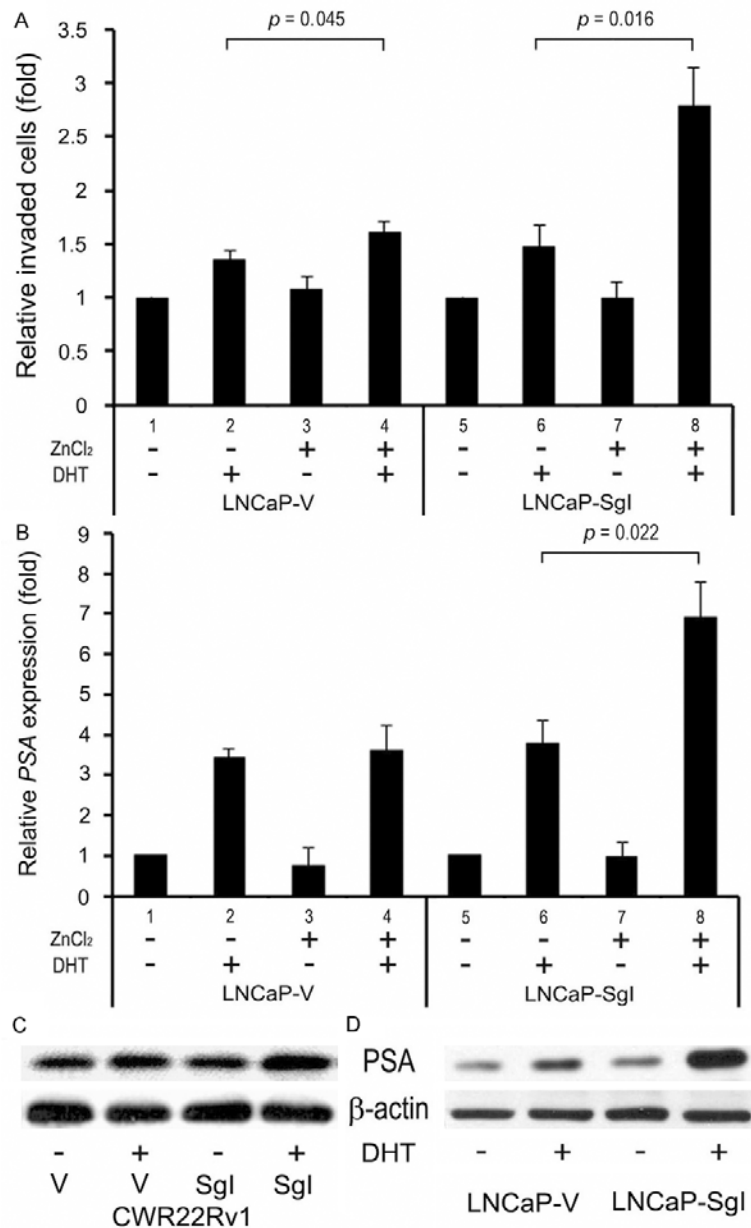


Figure 3. Progression of prostate cancer lines stably expressing Sgl. (A) LNCaP-V/Sgl cells cultured in the Matrigel-coated transwell chamber for 36 hours in the presence or absence of 300 μ M zinc and 1 nM DHT were used for transwell assay. The number of invaded cells in five random fields was counted under a light microscope, using a 40x objective. Invasion ability is presented relative to that in each cell line with mock treatment (lane 1 or 5; set as 1-fold). Each value represents the mean \pm SD of at least three independent experiments. (B) LNCaP-V/Sgl cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 300 μ M zinc and 1 nM DHT for 48 hours were subjected to a quantitative RT-PCR. Expression of *PSA* gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock treatment in each cell line (lane 1 or 5; set as 1-fold). Each value represents the mean \pm SD from at least three independent experiments. CWR22Rv1 cells (C) transiently transfected with pSG5 or pSG5-Sgl were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 100 μ M zinc and 1 nM DHT for 48 hours, and LNCaP-V/Sgl cells (D) were similarly cultured with 300 μ M zinc \pm 1 nM DHT for 48 hours, as

indicated. Cell extracts were then analyzed on western blots, using an antibody to PSA (33 kDa) or β -actin.

zinc. Thus, co-expression of Sgl in the presence of zinc appeared to induce androgen-mediated proliferation of AR-positive prostate cancer cells and, more importantly, protected the cells from cytotoxic effects of zinc. In AR-negative PC3-derived (Figure 2C) and DU145-derived (Figure 2D) cells, DHT treatment and Sgl overexpression showed only marginal effects on their growth ($< 10\%$ changes). Because semenogelins are secreted proteins [12], we further tested whether secreted forms of Sgl induced prostate cancer cell proliferation. MTT assay was again performed in CWR22Rv1 (Figure 2E) and DU145 (Figure 2F) cells incubated in the conditioned medium derived from CWR22Rv1-V/Sgl culture. In these parental lines, the secreted form of Sgl did not significantly affect cell viability in the absence (lanes 1 vs. 3) or presence (lanes 2 vs. 4) of zinc.

To investigate whether Sgl promotes tumor invasion, a transwell invasion assay was performed in the stable LNCaP lines (Figure 3A). DHT similarly induced cell invasion of LNCaP-V without (35% increase; lanes 1 vs. 2; $p = 0.042$) or with (48% increase; lanes 3 vs. 4; $p = 0.009$) zinc or LNCaP-Sgl without zinc (48% increase; lanes 5 vs. 6; $p = 0.026$). In contrast, in LNCaP-Sgl with zinc, the invasiveness was more significantly increased by DHT (2.8-fold over mock treatment; lanes 7 vs. 8; $p = 0.006$). Thus, significant induction of the DHT-mediated

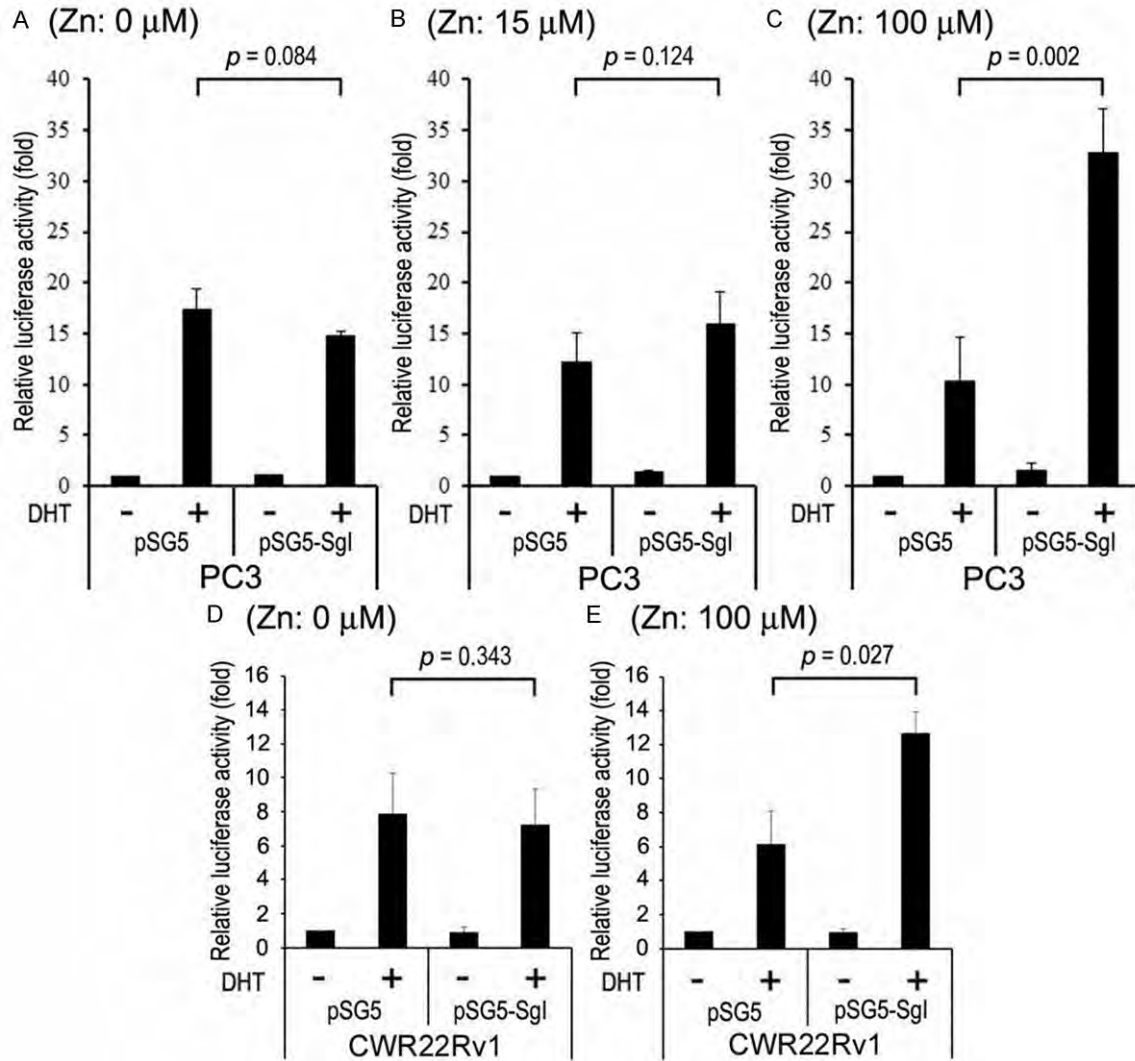


Figure 4. The effects of Sgl on AR transcriptional activity in prostate cancer cells. PC3 cells were co-transfected with pSG5-AR, MMTV-Luc, pRL-TK, and either pSG5 or pSG5-Sgl (AR:Sgl = 1:5), and cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS along with mock (ethanol), zinc [(A) 0 μ M; (B) 15 μ M; (C) 100 μ M], and/or 1 nM DHT for 24 hours. Similarly, CWR22Rv1 cells were co-transfected with MMTV-Luc, pRL-TK, and either pSG5 or pSG5-Sgl, and treated with mock (ethanol) or 1 nM DHT in the absence (D) or presence (E) of 100 μ M zinc for 24 hours. The luciferase activity is presented relative to that of mock treatment (first lanes; set as 1-fold). Each value represents the mean \pm SD of at least three determinations.

invasive properties by endogenous Sgl (lanes 2 vs. 4; 19% increase) or exogenous Sgl overexpression (lanes 6 vs. 8; 88% increase) with versus without addition of zinc was seen.

We next determined whether Sgl regulated the expression of PSA, an androgen-inducible AR target and also known to proteolyze Sgl in semen [12, 13], in prostate cancer cells. A quantitative RT-PCR showed that DHT treatment, in the absence of additional zinc, increased endogenous PSA expression over mock treatment by 3.4-fold (lanes 1 vs. 2; $p <$

0.001)/3.8-fold (lanes 5 vs. 6; $p = 0.009$) in LNCaP-V/Sgl (**Figure 3B**), respectively. In the presence of 300 μ M zinc, DHT increased PSA expression by 4.7-fold (lanes 3 vs. 4; $p = 0.004$)/7.1-fold (lanes 7 vs. 8; $p = 0.003$) in LNCaP-V/Sgl, respectively. The difference in DHT-mediated PSA expression in LNCaP-Sgl with versus without zinc was also statistically significant (lanes 6 vs. 8; 1.8-fold). Similarly, western blots in CWR22Rv1 cells cultured with 100 μ M zinc (**Figure 3C**) and LNCaP stable cells cultured with 300 μ M zinc (**Figure 3D**) showed that overexpression of Sgl resulted in consider-

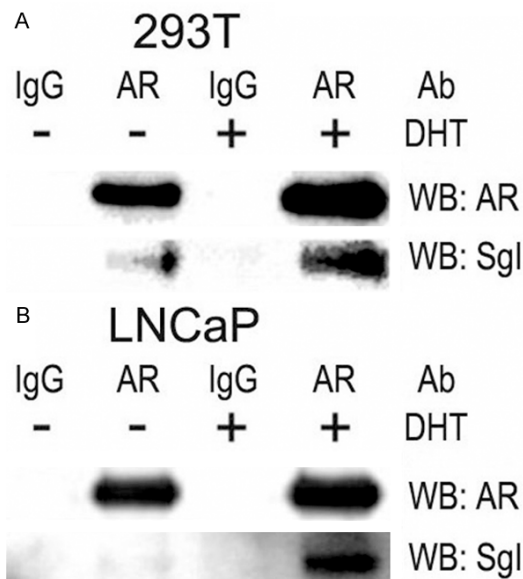


Figure 5. Co-precipitation of AR and Sgl. Cell lysates from 293T transfected with pSG5-AR and pSG5-Sgl (A) or LNCaP (B) treated with mock (ethanol) or 1 nM DHT were incubated with an anti-AR polyclonal antibody or normal rabbit IgG and then with A/G-agarose beads. The complex was resolved on a 10% SDS-polyacrylamide gel and blotted with an anti-AR or anti-Sgl antibody.

able increases in DHT-mediated PSA expression. However, no significant additive effects of Sgl on PSA protein expression were seen in these cell lines when cultured without additional zinc (figure not shown).

Enhancement of AR transcriptional activity by Sgl

To assess the effect of Sgl on androgen-mediated AR transactivation, luciferase activity was determined in PC3 cells transfected with AR, Sgl, and an androgen response element-reporter plasmid, and treated with different concentrations of zinc and 1 nM DHT. DHT increased AR transcription by 17-fold (0 μ M zinc; **Figure 4A**), 12-fold (15 μ M zinc; **Figure 4B**), and 10-fold (100 μ M zinc; **Figure 4C**), as compared with respective mock treatments. Thus, zinc reduced androgen-enhanced AR transactivation in a dose-dependent manner. Sgl showed a slight inhibitory effect (15% decrease at 0 μ M zinc; **Figure 4A**) or a slight stimulatory effect (31% increase at 15 μ M zinc; **Figure 4B**) on DHT-induced AR transcription. In contrast, in the presence of 100 μ M zinc, Sgl further induced DHT-mediated AR transcription by 3.2-

fold (**Figure 4C**). Induction of zinc/DHT-mediated AR transcription by Sgl (2.1-fold) was confirmed in CWR22Rv1, while Sgl did not significantly affect AR transactivation without additional zinc (8% decrease) (**Figure 4D, 4E**). These results suggest that Sgl functions as an AR coactivator in the presence of zinc in prostate cancer cells.

Interaction between AR and Sgl

AR coregulators modulate AR-mediated transcriptional activity by interacting with AR [1-3]. To verify the interaction between AR and Sgl, co-immunoprecipitation assay, using cell lysates with (293T) or without (LNCaP) transfection of AR and Sgl, was performed. Using an anti-AR antibody, we precipitated the AR binding protein complex in the protein lysate. We then proved that AR-Sgl form a complex, especially in the presence of DHT, in 293T (**Figure 5A**) and LNCaP (**Figure 5B**) cells.

Discussion

While functions of semenogelins have been thoroughly characterized in physiological environment especially in the male reproductive system, little is known about their roles in human malignancies. Our previous immunohistochemical studies showed that both Sgl and semenogelin II (SgII) were overexpressed in prostate cancer tissue specimens and that patients with Sgl-positive tumor, but not SgII-positive or SgII-negative tumor, had a significantly higher risk of recurrence following radical prostatectomy [17, 18]. Furthermore, transient transfection of Sgl, but not SgII, into AR-positive/semenogelin-negative CWR22Rv1 resulted in an increase in cell proliferation in the presence of a high level of zinc [17]. Based on these findings, we hypothesized that Sgl, in conjunction with zinc, androgen, and AR, promoted prostate cancer progression. In the present study, we tested our hypothesis in prostate cancer cell lines.

Experimental evidence indicates an inhibitory role of zinc in the development and progression of prostate cancer. However, it remains controversial whether zinc supplements reduce the risk of prostate cancer [5, 11]. In addition, the molecular basis for why prostate cancer tissue contains relatively high concentrations of cytotoxic zinc is poorly understood, although altera-

tions of zinc transporters in prostate cancer cells have been suggested to prevent zinc accumulation [24, 25]. Previous *in vitro* studies have shown that higher concentrations of zinc are required to inhibit cell proliferation of LNCaP (250-1000 μ M), compared with PC3 (100 μ M) [7, 8]. We confirmed these findings and further demonstrated that 100 μ M zinc could inhibit cell growth of other Sgl-negative prostate cancer lines. Thus, endogenous Sgl in LNCaP may protect the cells against inhibitory effects of zinc. Interestingly, co-expression of Sgl only in AR-positive CWR22Rv1 cells resulted in prevention from zinc cytotoxicity. Sgl also induced androgen-mediated prostate cancer cell invasion and PSA expression only in the presence of zinc. These results suggest that Sgl may require not only zinc, as in the case of its physiological action [12-16], but also AR to function as a modulator of prostate cancer outgrowth. Moreover, the presence of Sgl in prostate cancer cells can be a reason for zinc accumulation in tumors.

It is well documented that co-regulatory proteins modulate nuclear receptor-mediated transcriptional activity by interacting with the receptor [1-4]. We here showed that Sgl interacted with AR and enhanced androgen-induced AR transactivation in prostate cancer cells, indicating that Sgl is an AR coactivator. Again, a high level of zinc was most likely required for this newly recognized function of Sgl. Although a variety of general or specific AR coactivators have been identified, physiological functions of these coactivators are largely unknown and their characterization has not yet led to the development of new therapeutic options in patients with prostate cancer [26, 27]. It has been expected that suppression of coactivator actions or interruption of AR-coactivator interactions results in prostate cancer regression at any stages because castration-resistant tumors usually remain AR-dependent for their growth. Importantly, as aforementioned, physiological roles of semenogelins as seminal plasma proteins have been extensively studied. Sgl was also shown to be highly expressed in prostate cancer cells [15, 17, 18]. In addition, because PSA is known to physiologically degrade semenogelins [13], elevated Sgl may result in a further increase in PSA levels to attempt to target semenogelins. As a result, down-regulation of Sgl expression, compared with other AR coactivators, may more effectively inhibit prostate cancer progression that can be facilitated by PSA itself via enhancing an AR

coactivator ARA70-regulated AR transactivation [28]. The cytotoxic activity of zinc may also become distinct with Sgl down-regulation. Further analyses of Sgl in prostate cancer are necessary to credential a new therapeutic target.

The current results suggest that cellular Sgl, but not its secreted forms, plays an important role in prostate cancer outgrowth. However, semenogelins are essentially secreted proteins, mainly derived from the seminal vesicle. Indeed, we detected Sgl signals in secreted materials, in addition to cellular immunoreactivity, in prostatectomy specimens [18]. Although moderate to strong Sgl signals were seen in the majority of benign (97%) or PIN (98%) glands where the secretions were present, intraluminal secretions in carcinoma glands were uncommonly (13%) immunoreactive and their signals, if present, were mostly weak. These findings suggested that, in contrast to benign or PIN cells, carcinoma cells did not generally secrete a large amount of Sgl. We confirmed this by demonstrating the failure to detect Sgl signals in the conditioned medium after culturing control LNCaP with endogenous Sgl and other Sgl-negative prostate cancer cell lines in our western blotting. Instead, increased levels of serum semenogelins were detected in 4 of 13 patients with lung cancer, although their functions in lung carcinogenesis and tumor progression were not studied [29]. Again, in our assays, a secreted form of Sgl present in the conditioned medium where CWR22Rv1-Sgl was cultured failed to induce the proliferation of parental CWR22Rv1 cells even in the presence of zinc. It is still possible that Sgl secreted by benign prostate or PIN cells as well as tissues other than the prostate exerts an influence on prostate cancer growth with or without involving zinc and AR.

In conclusion, our current data indicating that intracellular Sgl in the presence of zinc functions as an AR coactivator and promotes the growth of prostate cancer cells provide its novel role in tumor progression. Particularly, Sgl protects the cells against zinc cytotoxicity, which may explain why prostate cancer tissue contains high levels of zinc.

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Disclosure of conflict of interest

None.

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