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Award Number:
W81XWH-05-1-0598

TITLE:
New Strategy for Prostate Cancer Prevention Based on Selenium
Suppression of Androgen Receptor Signaling

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REPORT DATE:
April 2010

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-04-2010		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15-SEP-2005-31-MAR-2010	
4. TITLE AND SUBTITLE New Strategy for Prostate Cancer Prevention Based on Selenium Suppression of Androgen Receptor Signaling				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0598	
				5c. PROGRAM ELEMENT NUMBER	
				5d. PROJECT NUMBER	
6. AUTHOR(S) Zhang, Haitao Email: HZHANG@TULANE.EDU				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
				8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tulane University Health Sciences Center New Orleans, LA 70112				10. SPONSOR/MONITOR'S ACRONYM(S)	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command Fort Detrick, MD 21702-5012				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In this project, we investigated the potential of targeting the androgen receptor and the 5 ^α -reductase simultaneously in prostate cancer prevention. We report that this combined androgen receptor blockade approach produced a synergistic effect on tumor growth inhibition by in vitro and in vivo. We have also identified that methylseleninic acid (MSA) activates FOXO1 and FOXO1 activation plays an important role in mediating apoptosis induction and androgen receptor suppression by MSA. In summary, the research support by this grant suggests a novel and effective approach for managing early stage prostate cancer and contributes to a better understanding of the anticancer effects of MSA.					
15. SUBJECT TERMS selenium; 5α-reductase inhibitor; prostate cancer; chemoprevention; androgen receptor; FOXO1					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 20	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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A. INTRODUCTION

Androgen plays an important role in prostate carcinogenesis. Testosterone is the major androgen in circulation; it is converted to the more potent dihydrotestosterone in the prostate by the enzyme 5 α -reductase. The Prostate Cancer Prevention Trial (PCPT) demonstrated that treatment with finasteride, an inhibitor of 5 α -reductase, reduced prostate cancer incidence by 25%. Selenium, on the other hand, is shown to reduce prostate cancer risk by 50% by the Nutrition Prevention of Cancer (NPC) trial. In vitro studies have shown that selenium suppresses androgen signaling by downregulating expression of the androgen receptor (AR). This project consists of two specific aims: 1). To evaluate the combined use of selenium and a 5 α -reductase inhibitor in preventing prostate cancer; 2). To investigate the role of FOXO1 in mediating the anticancer effect of selenium. This report summarizes the research findings for the revised Statement of Work (SOW).

B. BODY

Task 1. Determine the optimal dose of finasteride to achieve growth inhibition of tumor xenografts in nude mice.

In the original proposal, we proposed to identify an optimal dose of dutasteride for the combination experiments. We have since switched to finasteride and we found in the literature doses of finasteride effective in inhibiting the growth of LNCaP xenografts in nude mice inhibiting LNCaP xenograft (1,2). In order to minimize the number of nude mice used in this study, we decided to use these doses in the following experiments.

Tasks 2. Assess the combinatorial effect of finasteride and selenium on growth of tumor xenografts in nude mice.

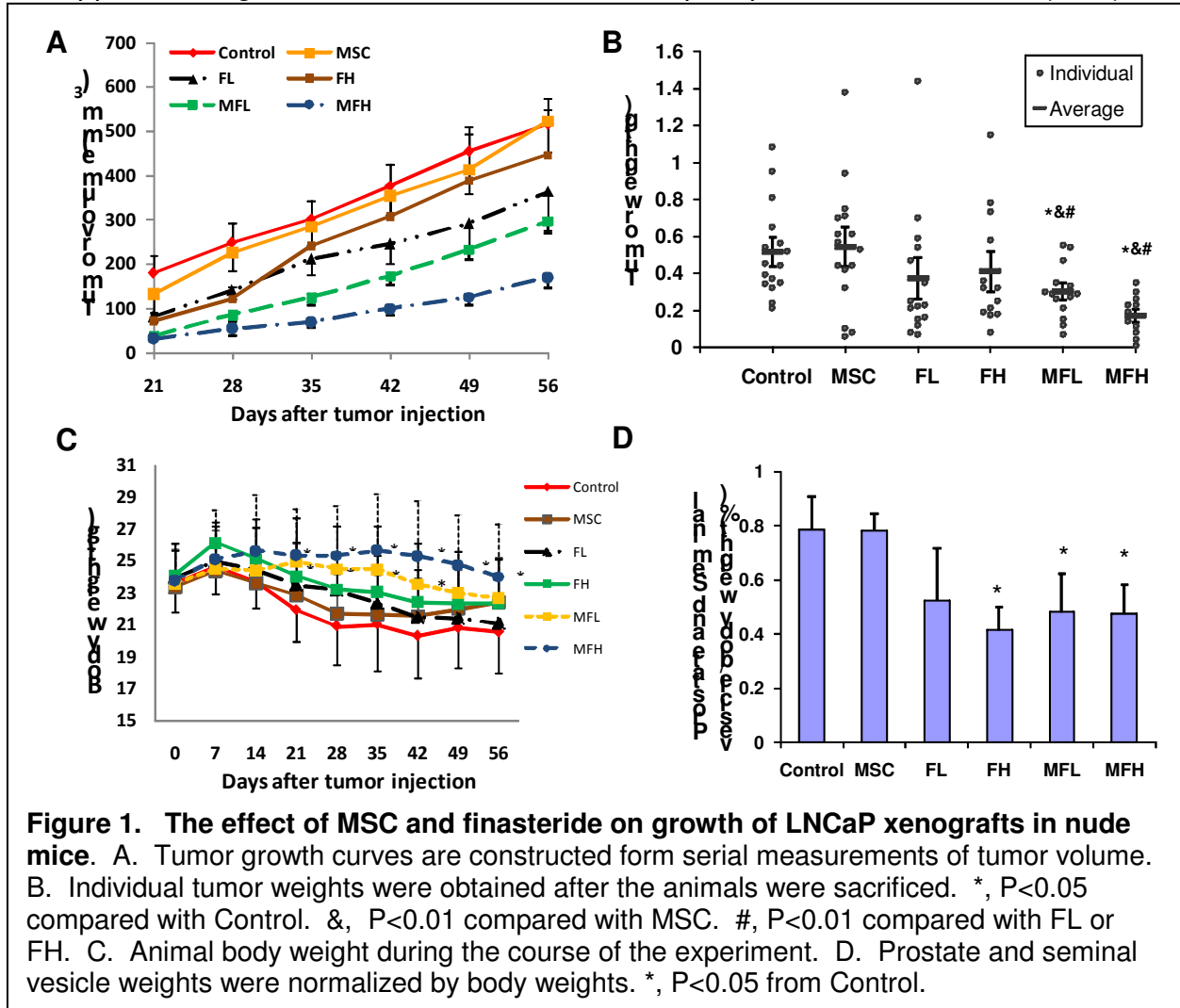
Based on the literature information, we decided to use finasteride at 5 and 50 mg/kg/day, in combination with MSC at 100 μ g/day. For xenografting, 4X10⁶ LNCaP cells were suspended in 50 μ l Matrigel (Becton Dickinson Labware) and injected subcutaneously to both sides of the dorsal flank. The Matrigel milieu is required for the formation of tumors in immunodeficient mice (3). Forty-eight mice were randomized to 6 groups, with 8 mice per group (Table 1). MSC and finasteride were administered the day after tumor implantation. Finasteride was prepared in a mixture of 10% ethanol/90% olive oil and given to the mice by oral gavage using a

Table 1. Treatment groups and tumor take rate.

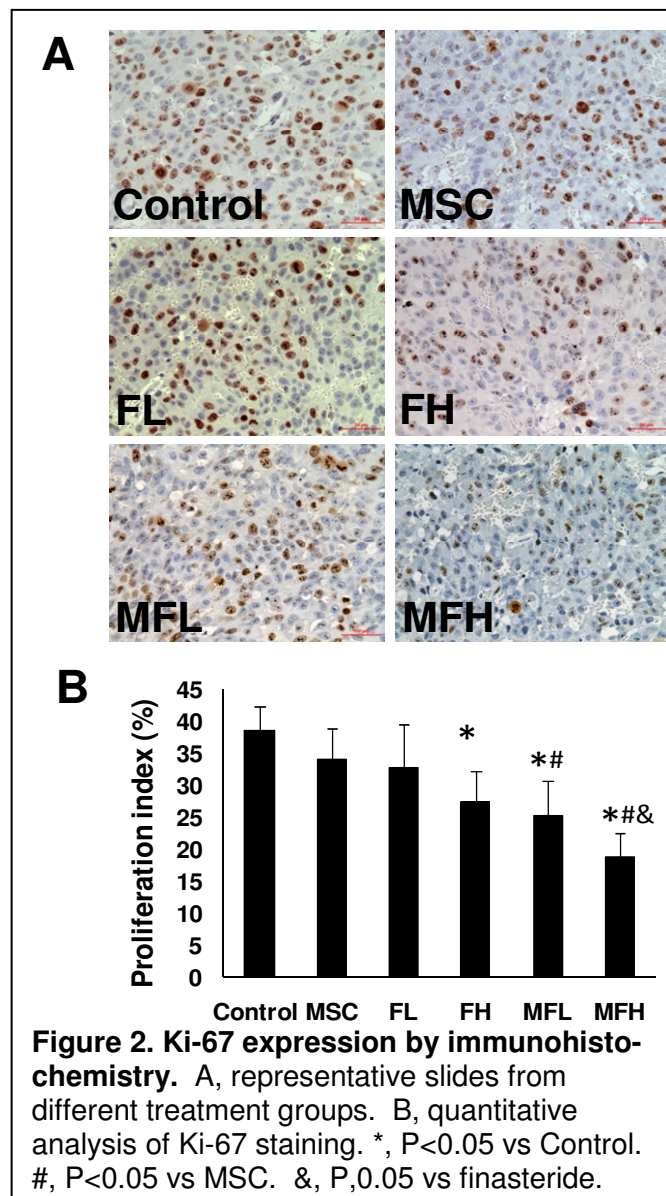
Group ID	# of Mice	MSC (g/kg)	Fin (mg/kg)	# of tumor	Tumor take rate
Control	8	-	-	16	100%
MSC	8	100	-	16	100%
FL	8	-	5	15	93.7%
FH	7*	-	50	13	92.8%
MFL	7*	100	5	14	100%
MFH	7*	100	50	13	92.8%

*One mouse in each of these groups died accidentally. FL, finasteride, low dose. FH, finasteride, high dose. MFL, MSC plus low dose finasteride. MFH, MSC plus high dose finasteride.

ball-tipped feeding needle. MSC was dissolved in phosphate-buffered saline (PBS) and

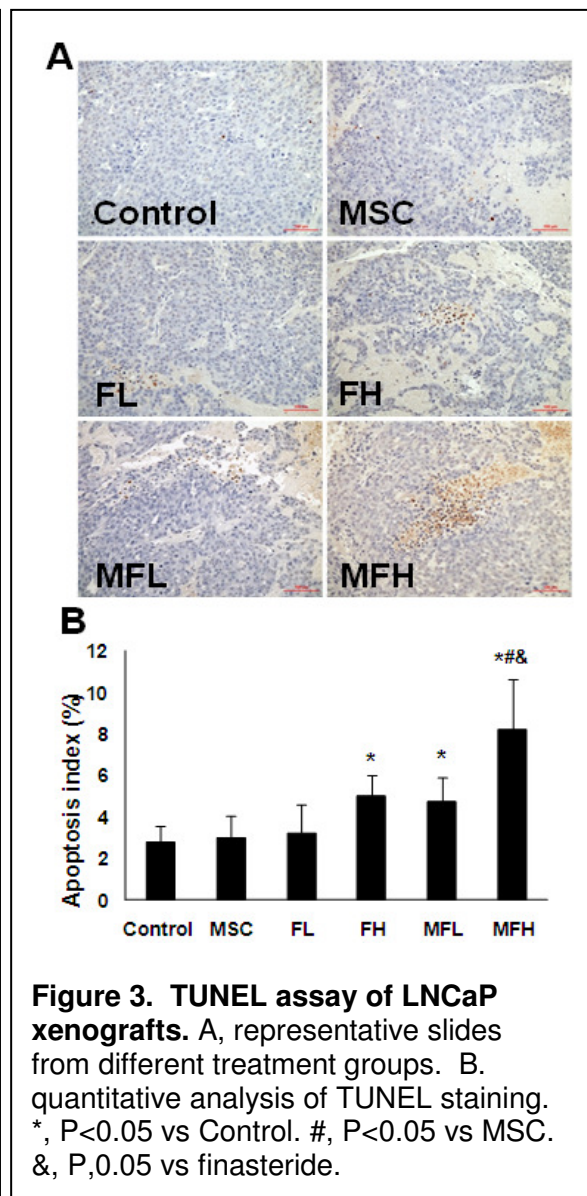


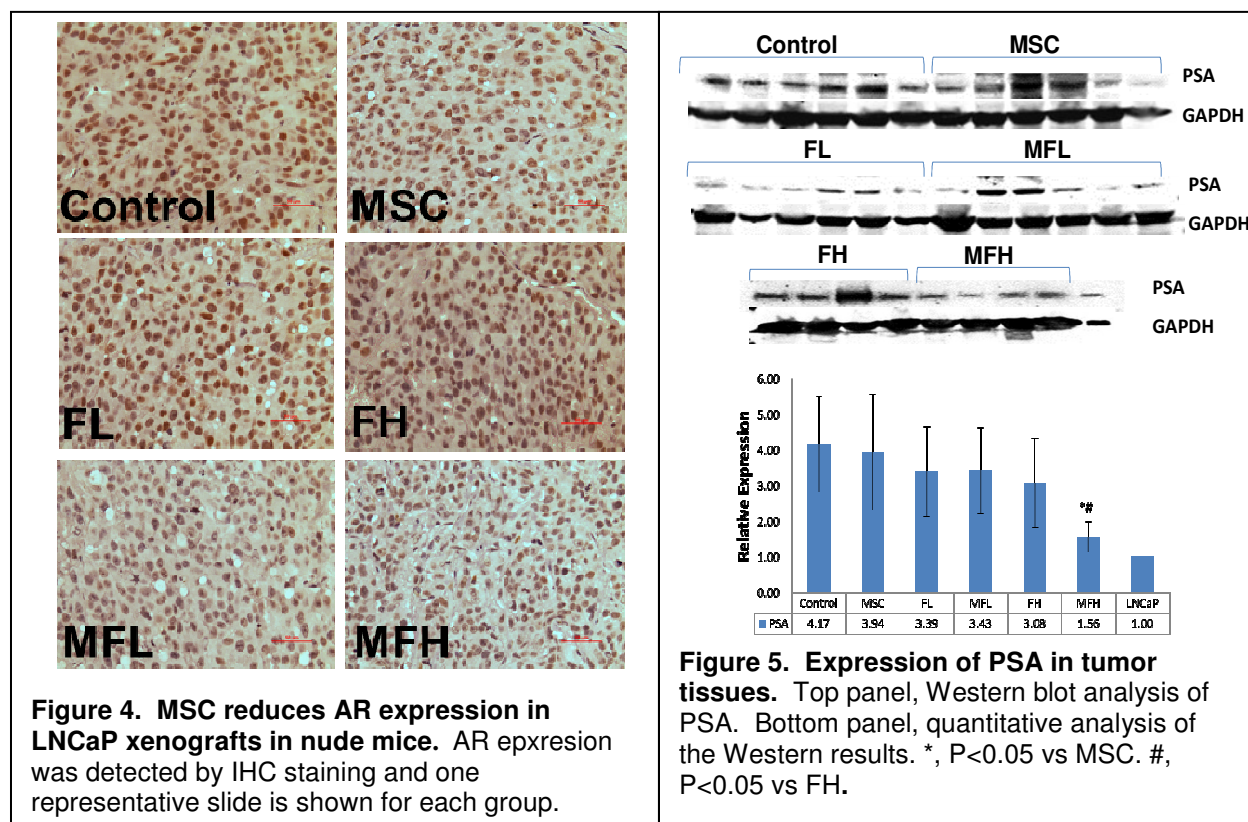
administered by intraperitoneal injection. Animals were observed daily, and tumor measurement was taken twice weekly. Tumor volumes were calculated by the following formula: length x width x height x 0.5236. Little change in tumor take rate was observed among different groups (Table 1). Tumor growth was monitored for 8 weeks. As shown in Figure 1A, treatment of MSC at this dose had very little impact on tumor growth, if any. Finasteride at the 5 mg/kg dose slowed down tumor growth, but the difference was not statistically significant ($p>0.05$). Interestingly, the higher dose of finasteride (50 mg/kg) appeared to be less effective than the lower dose in inhibiting tumor growth. In contrast, tumors in the combination groups grew at a much reduced rate, and the differences were statistically significant when compared with the control or the respective single treatment groups ($P<0.05$). The animals were sacrificed after 8 weeks, and the tumor weight correlated very closely with tumor volume (Figure 1B). No adverse effects were observed in animals receiving treatments. In fact, animals receiving the combination treatments had less weight loss than animals in the control group (Figure 1C), possibly due to reduced tumor burden in these animals. As expected, the weight of the prostate and the seminal vesicle was reduced by finasteride



but not MSC (Figure 1D), suggesting that MSC has no adverse effect on normal prostate. In summary, the tumor growth results showed that at the doses tested, neither MSC nor finasteride has a significant impact on inhibiting tumor growth. However, synergistic growth inhibitions were observed with two different combinations of MSC and finasteride.

Tumor cell proliferation was determined by immunohistochemistry using an anti-Ki-67 antibody. As shown in Figure 2, neither selenium nor finasteride at the lower dose affected cell proliferation. Despite having no effect on tumor volume, finasteride at the 50 mg/kg dose reduced cell proliferation by ~30%. Samples from the MFH group showed a dramatic decrease in cell proliferation. In addition, apoptosis was analyzed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Once again, the combination of MSC and finasteride at the higher dose induced apoptosis more effectively than either alone (Figure 3).





We next analyzed the expression of AR and PSA in the tumor tissues. The expression of AR was detected by immunohistochemistry (IHC) staining with an anti-AR antibody. As shown in Figure 4, the expression of AR was reduced by MSC treatment. Finasteride treatment did not seem to affect AR expression. In the combination groups (MFL and MFH), the AR expression level was reduced to a similar extent as that in the MSC group. PSA expression was analyzed by Western blotting using tumor tissues. The results show that the MSC and finasteride combination inhibited PSA expression more efficiently than either treatment alone (Figure 5, MFH vs MSC or FH).

In the SOW, we proposed to study PSA expression by analyzing PSA in the blood using an ELISA-based detection kit because we were concerned that we might not have enough tumor samples for Western analysis. However, we were able to detect PSA expression in the tumor tissues using Western analysis. Therefore, we decided not to proceed with the ELISA analysis.

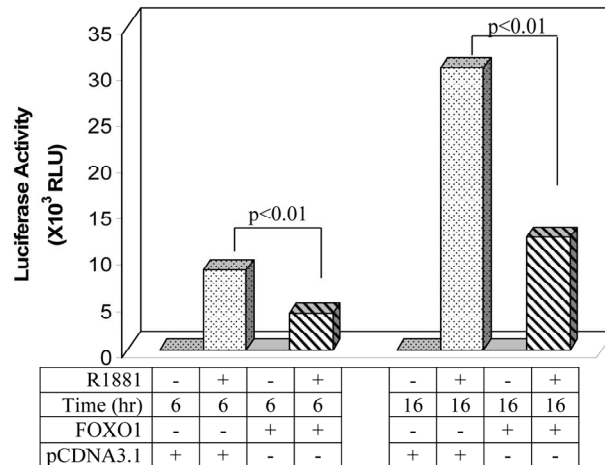
Task 3. To determine the effect of selenium on the interaction between AR and FOXO1.

FOXO1 activation suppresses AR *trans*-activation. To examine the effect of FOXO1 activation on the transcriptional activity of AR, we transiently co-transfected LNCaP cells with a reporter construct containing 3 repeats of the androgen response element (ARE) ligated in tandem to the luciferase reporter, together with a *FOXO1* expression vector,

pcDNA3-FKHR, or the empty vector. The ARE-luciferase reporter assay is commonly used to assess the *trans*-activating activity of AR. Following transfection, cells were exposed to 1 nM R1881, a synthetic androgen, for 6 or 16 hr before they were lysed for luciferase assay. As shown in Figure 6A, the AR transcriptional activity was greatly stimulated by the addition of the ligand. In the presence of ectopically expressed FOXO1, the induction was significantly diminished (Figure 6A, comparing columns 2 and 4 for both time points). Therefore, our results confirmed published studies showing that FOXO1 activation suppresses AR signaling (4,5).

FOXO1 induction contributes to AR suppression by MSA. It has been found previously that MSA is a potent suppressor of AR signaling (6-8). The mechanisms involved in suppression of AR signaling by MSA include reduction in AR mRNA transcription and stability, increase in AR protein turnover, reduction in AR translocation, inhibition of coactivator recruitment, and increased corepressor recruitment to the promoters of AR-regulated genes (6-8). The result from the previous section prompted us to investigate whether *FOXO1* induction is a contributing

a. FOXO1 overexpression on AR activity



b. FOXO1 knockdown on suppression of AR activity by MSA

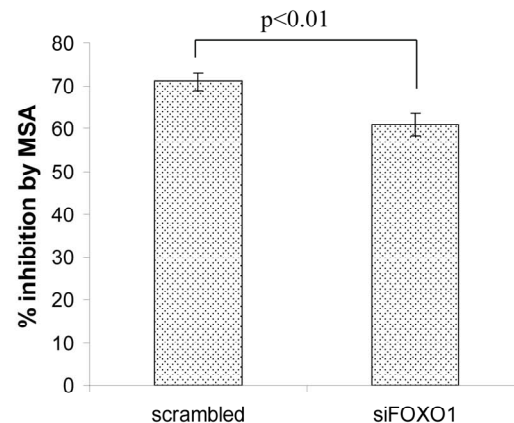


Figure 6. Induction of FOXO1 contributes to AR suppression by MSA. A. Increased expression of FOXO1 reduced the transcriptional activity of AR. LNCaP cells were co-transfected with the ARE-luciferase reporter construct and either the pcDNA3-FKHR or the pcDNA3 vector, and treated with 1 nM R1881 for the indicated times. B. FOXO1 knockdown attenuated the suppression of AR transactivation by MSA. LNCaP cells were co-transfected with the ARE-luciferase construct and either the scrambled control or siFOXO1, and treated with 10 μ M MSA for 24 hr. The luciferase reading was normalized by protein concentration. The experiment was done 3 times and the results were expressed as mean percent inhibition $\pm$ SEM.

factor for AR suppression by MSA. Once again, we employed the gene knockdown approach. LNCaP cells were co-transfected with the ARE-luciferase construct and siFOXO1, and treated with 0 or 10 μ M MSA. In the presence of the scrambled oligo, MSA suppressed AR activity by approximately 70% (Figure 6B). This is in line with our previous observations. However, when *FOXO1* was silenced, the suppression was attenuated to about 60% ($P<0.01$). This was further confirmed when we examined the modulation of PSA expression by MSA in the presence or absence of siFOXO1 (data not shown). These results are in agreement with previous studies showing that MSA suppresses AR signaling through a multitude of mechanisms and identified *FOXO1* activation as a novel mechanism contributing to the inhibition of AR *trans*-activation by MSA.

In task 3, we had tried to investigate how MSA modulate the physical interaction between AR and FOXO1 by co-immunoprecipitation, as described in the SOW. However, we encountered technical difficulties. Instead, we switched to the previous experiments to demonstrate that MSA modulates the crosstalk between AR and FOXO1 signaling pathways.

C. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that combined androgen signaling blockade by simultaneously targeting the androgen receptor and 5 α -reductase is a valid and effective strategy in prostate cancer prevention.
- Consistent with recent clinical and preclinical findings, the animal experiment demonstrated that selenium compound by itself is not an effective chemopreventive agent in prostate cancer. However, MSC synergized with finasteride, a 5 α -reductase inhibitor, and reduced the growth rate of tumor xenografts quite effectively. This suggests a new direction for using selenium compounds in prostate cancer prevention.
- Confirmed that FOXO1 and AR pathways counteract the action of each other in prostate cancer cells. Demonstrated MSA modulates the balance between AR and FOXO signaling pathways.

D. REPORTABLE OUTCOMES

Publications

Haitao Zhang, Jian Fang, Dian Yao, Yue Wu, Clement Ip, and Yan Dong. (2010) Activation of FOXO1 is critical for the anticancer effect of selenium in prostate cancer cells. *Prostate* 70:1265-1273.

Haitao Zhang, Xichun Liu, Dian Yao, Felicia Parker, and Clement Ip. Synergistic interaction between selenium and finasteride in prostate cancer chemoprevention. Manuscript in preparation.

Presentations

1. Haitao Zhang: Targeting androgen signaling axis for prostate cancer intervention. Invited seminar. Jilin University School of Medicine, Changchun, Jilin Province, China. July 27, 2009.
2. Haitao Zhang: New concepts of selenium in prostate cancer intervention. Invited oral presentation. The 3rd World Cancer Congress, Singapore, June 22 - 25, 2010.

E. CONCLUSIONS

The results from the current study demonstrated a synergism between 5 α -reductase inhibition and AR down-regulation in inhibiting the growth of prostate cancer cells both *in vitro* and *in vivo*. This finding has significant clinical implications. Since the induction of PSA screening, the majority of the prostate cancers diagnosed are asymptomatic, early-stage, small volume diseases. Current treatment options, including surgery and radiation therapy, are associated with serious quality-of-life complications. Our study suggests that the combination of finasteride and MSA could be used to prevent the clonal expansion of small-volume, low-grade prostate cancer cells, providing a novel disease management strategy.

We have successfully demonstrated that the combination of selenium and finasteride synergistically suppresses androgen signaling. The changes in AR-regulated genes, PSA and KLK2, could be detected in both mRNA and protein levels. This confirms the use of these AR targets to monitor the responsiveness to the combination in future clinical practices.

The findings presented above demonstrated that MSA activates FOXO1 signaling pathway. FOXO1 plays a critical role in mediating the apoptotic activity of MSA, and also contributes to the suppression of androgen signaling by MSA. This study enhances our understanding of the molecular mechanisms of the anticancer activity of MSA, which will be critical for designing future prostate cancer intervention studies with MSA.

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Activation of FOXO1 Is Critical for the Anticancer Effect of Methylseleninic Acid in Prostate Cancer Cells

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BACKGROUND. Previous studies have demonstrated that physiological concentrations of methylseleninic acid (MSA) inhibits the growth of prostate cancer cells. The growth inhibitory effect could be attributed to cell cycle block and apoptosis induction. The current study was designed to investigate the involvement of forkhead box O1 (FOXO1) in the anticancer effect of MSA.

METHODS. LNCaP and LAPC-4 cells were treated with 10 μ M MSA for various time points, and the expression of FOXO1 was analyzed by qRT-PCR and Western blotting. FOXO1 activity was determined by a luciferase construct containing FOXO binding sites. The *trans*-activation activity of the androgen receptor (AR) was determined by the ARE-luciferase assay. FOXO1 gene silencing was achieved by using a small interfering RNA (siRNA).

RESULTS. MSA treatment led to a rapid and robust increase of FOXO1 expression, as well as an increase of the FOXO1 transcriptional activity. Blocking FOXO1 activation by gene silencing abolished apoptosis induction by MSA, suggesting FOXO1 plays a critical role in mediating the apoptotic effect of MSA. Recent studies have shown that FOXO1 and AR antagonize the actions of each other. We examined the consequence of FOXO1 induction on AR activity. Consistent with previous reports, we found that ectopic expression of FOXO1 suppressed the transcriptional activity of AR. Furthermore, FOXO1 silencing attenuated MSA suppression of AR activity, suggesting that FOXO1 induction contributes to suppression of AR signaling by MSA.

CONCLUSIONS. In prostate cancer cells, MSA activates the FOXO1 signaling pathway. FOXO1 activation is critical for the anticancer effects of MSA. *Prostate* 70: 1265–1273, 2010.

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KEY WORDS: methylseleninic acid; FOXO1; apoptosis; androgen receptor

Abbreviations: AR, androgen receptor; ARE, androgen response element; FOXO1, forkhead box O1; MSA, methylseleninic acid; NPC, Nutritional Prevention of Cancer; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SELECT, Selenium and Vitamin E Chemoprevention Trial; siRNA, small interfering RNA.

Grant sponsor: Department of Defense Prostate Cancer Research Program; Grant number: W81XWH-05-1-0598; Grant sponsor: American Cancer Society; Grant number: RSG-07-218-01-TBE; Grant sponsor: National Cancer Institute; Grant numbers: K01CA114252, P01CA126804, P30CA16056; Grant sponsor: Louisiana Cancer Research Consortium.

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Received 6 January 2010; Accepted 9 February 2010

DOI 10.1002/pros.21162

Published online 26 March 2010 in Wiley InterScience (www.interscience.wiley.com).

INTRODUCTION

Prostate cancer (PCa) is a significant public health problem that engenders huge medical care and human suffering costs in the United States. A number of case-control studies have demonstrated an inverse relationship between selenium status and prostate cancer risk [1–5]. One of the more important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer (NPC) trial initiated by Larry Clark [6,7]. The study was a randomized, double-blind, placebo-controlled trial involving 1,312 patients (mostly men) who were recruited initially because of a history of basal cell or squamous cell carcinoma of the skin. Individuals in the treatment arm were given 200 μ g selenized yeast per day for a mean of 4.5 years. After a total follow-up of 8,271 person-years, selenium treatment did not decrease the recurrence of these non-melanoma skin cancers. However, patients receiving the supplement showed a much lower risk of developing total (HR=0.75) or prostate cancer (HR=0.48) [6,7].

Encouraged by the prostate cancer results of the NPC trial, the National Cancer Institute launched the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in 2001. An interim data analysis after a median follow-up of 5.46 years suggested selenium, either alone or in combination with vitamin E, did not lower prostate cancer risk in this study population [8]. The trial was halted in October 2008, but the follow-up will continue for 3 more years.

The results of the SELECT have caused much controversy. One hot topic of debate is the formulation of selenium used in the SELECT. Selenomethionine, which was used in the SELECT, can be incorporated non-specifically into proteins in place of methionine [9]. Compartmentation into tissue proteins limits selenomethionine from being further metabolized. On the other hand, monomethylated forms of selenium, including methylseleninic acid (MSA) and methylselenocysteine (MSC), can be easily metabolized to methylselenol, which is considered to be the critical metabolite for the anticancer activity of selenium [9,10]. By itself, methylselenol is highly reactive and difficult to prepare. Therefore, the proximal precursors including MSA and MSC are superior to selenomethionine with regard to providing a steady stream of methylselenol. In fact, studies published before and after the launch of the SELECT have showed that MSA and MSC have stronger anticancer activities than selenomethionine [11–14]. While the conversion of MSC to methylselenol requires the action of β -lyase, MSA can be easily reduced to methylselenol through non-enzymatic reactions involving glutathione (GSH) or NADPH [15]. Due to the fact that epithelial cells express low

level of β -lyase, MSA is 10 times more potent than MSC in affecting biological processes *in vitro* [13]. MSA is widely accepted to be the best reagent for delineating the molecular action of selenium in cell culture studies [16–19]. It also has excellent anticancer activity in animals [13,20,21].

In view of the above information, we believe that the potential of selenium compounds as chemopreventive agents for prostate cancer should not be dismissed. Unraveling the mechanisms of action for these agents is urgent and will no doubt be helpful in rational design of future intervention trials. We and others have previously profiled selenium-induced gene expression changes in prostate cancer cells [17,22,23]. Based on the datasets generated from the microarray studies, we conducted a systematic data mining analysis, taking advantage of several publicly available clinical prostate cancer datasets, in order to gain new insights into novel molecular targets that may be relevant to the anticancer activity of MSA [24]. The analysis drew our attention to forkhead box O1 (FOXO1). We found that the expression of FOXO1 is consistently decreased in a large number of prostate cancer specimens, and the microarray analyses showed MSA up-regulates the expression of FOXO1 [24]. FOXO1 is a member of the FOXO family of transcription factors that induces the expression of pro-apoptotic genes including Fas ligand [25,26], bcl-2 family proteins [27–29], and TRAIL [30]. FOXO1 is also involved in cell cycle regulation [31]. FOXO1 is phosphorylated and suppressed by AKT [32,33], which is an important survival molecule for prostate cancer. In prostate cancer cells, androgen receptor (AR) interacts with FOXO1 and inhibits its activation of downstream targets [34]. The current study was designed to examine the role of FOXO1 in mediating the anticancer effect of MSA.

MATERIALS AND METHODS

Materials

MSA was purchased from PharmaSe (Lubbock, TX). Fetal bovine serum, RPMI 1640, and the Lipofectamine PLUS transfection reagents were purchased from Invitrogen (Carlsbad, CA). Immobilon PVDF membrane was purchased from Millipore (Bedford, MA) and ECL Western blotting detection reagent from Amersham Pharmacia Biotech (Arlington Heights, IL). For Western blotting analysis, the antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (Temecula, CA) and anti-FOXO1 was from Cell Signaling (Danvers, MA). The Cell Death Detection ELISA kit was purchased from Roche Applied Science (Indianapolis, IN). The p3XIRS-luc reporter construct was kindly provided

by Dr. Kun-Liang Guan at the University of Michigan, and the pcDNA3-FKHR expression vector was obtained from Dr. Frederic G. Barr at the University of Pennsylvania. The pcDNA3-AR-FL expression vector was a gift from Dr. Shuyun Yeh at the University of Rochester.

Cell Culture and Treatment

The human LNCaP prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. Both LNCaP and LAPC-4 express AR and require androgen for their growth. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM of glutamine. In some experiments, cells were cultured in an androgen-defined condition by using charcoal-stripped FBS in the presence of 1 nM R1881 (a potent synthetic androgen). Treatment with MSA usually began at 72 hr after seeding, when the cultures were 60–80% confluent.

Quantitative Reverse transcription-Polymerase Chain Reaction (qRT-PCR)

The PCR primers and Taqman probes for β -actin, FOXO1, and AR were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative quantitation of gene expression was done using the comparative CT ($\Delta\Delta C_T$) method [35]. Details of the procedure were described in our previous publication [22].

Transient Transfection and Reporter Gene Assay

Supercoiled plasmid DNAs were prepared by the Qiagen column procedure (Qiagen, Valencia, CA). Twenty-four hours before transfection, cells were trypsinized and seeded at a density to reach 90–95% confluency at the time of transfection. Transient transfection was carried out by using the LipofectamineTM and PlusTM reagents (Invitrogen) per instruction of the manufacturer. After incubating with the transfection mixture for 3 hr, the cells were trypsinized and replated in triplicate into 6-well plates to achieve equal transfection efficiency. The cells were allowed to attach overnight before 10 µM MSA was added to the culture medium. At 6 or 16 hr following treatment, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison, WI), and the luciferase activity was assayed by using

the Luciferase Assay System (Promega). Protein concentration in the cell extract was determined by using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized to the protein concentration of the same sample. The transfection experiments were repeated three times.

Gene Silencing With siRNA

A small interfering RNA (siRNA) designed to target FOXO1 (Cat. # HSS103719) and a matching negative control oligonucleotide were purchased from Invitrogen. These oligonucleotides were transiently transfected into LNCaP cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 hr posttransfection, 10 µM MSA was added to the culture medium and the cells were treated for an additional 24 hr. RNA was prepared from the cells and qRT-PCR was performed to determine the efficiency of gene silencing.

Statistical Analysis

The Student's *t*-test was used to determine significant differences between different groups. Unless otherwise indicated, $P < 0.05$ was considered statistically significant. All analyses were two-tailed.

RESULTS

MSA Induces FOXO1 Expression

We first performed qRT-PCR and Western blotting to confirm the modulation of FOXO1 by MSA in LNCaP cells, as first noted from our microarray analysis [24]. Cells were treated with 10 µM MSA for various lengths of time before they were lysed for RNA and protein purification. The qRT-PCR results are shown in Figure 1a. Induction of FOXO1 mRNA was observed as early as 1 hr after exposure to MSA, suggesting that FOXO1 is a proximal target of MSA. The mRNA level peaked at 2 hr, then declined gradually with time, but still remained elevated at 24 hr. Western blotting of FOXO1 was carried out in LNCaP and LAPC-4 cells (Fig. 1b). No change in protein level was detected until at least after 3 hr. Thus the increases of FOXO1 protein appeared to lag behind the increases of the message, although the protein signal was decidedly stronger by 6 hr in cells treated with MSA.

MSA Induces the Transcriptional Activity of FOXO

As mentioned in the Introduction section, FOXO1 is a transcription factor. In order to study the effect of MSA on the activity of FOXO1 as a transcription factor, we transiently transfected LNCaP and LAPC-4 cells with a luciferase reporter construct, p3XIRS-luc. This

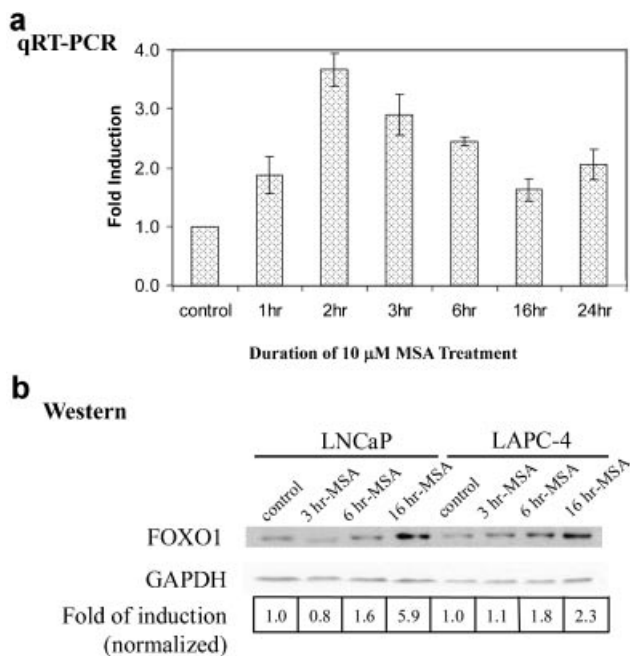


Fig. 1. Effect of methylseleninic acid (MSA) on FOXO1 expression. **a:** Change of FOXO1 mRNA in LNCaP cells as a function of time of MSA treatment, determined by qRT-PCR. The results are shown as mean $\pm$ standard error of mean (SEM). **b:** Western analysis of FOXO1 protein level as a function of time of MSA treatment, in both LNCaP and LAPC-4 cells. The band intensity was quantified by volume densitometry and normalized to that of GAPDH. The results were expressed as fold induction over untreated.

construct has three tandem repeats of a FOXO1 binding element, the insulin-responsive sequence (IRS), inserted upstream of the minimal thymidine kinase promoter [33]. It is widely used as an indicator of the transcriptional activity of FOXO proteins. As shown in Figure 2a, the transcriptional activity of this reporter

construct was induced by approximately two-fold in LNCaP cells after 6 hr of treatment with 10 μ M MSA. A pronounced induction (>5 -fold) was observed in LNCaP after 16 hr of treatment ($P < 0.01$). Nearly identical results were obtained in LAPC-4 cells (Fig. 2b).

FOXO1 Gene Silencing Blocks MSA-Induced Apoptosis

MSA has been shown to induce apoptosis in prostate cancer cells by several groups, including ours [17–19,36]. The experiments described above suggested that MSA induces the FOXO1 signaling pathway, which is known to positively regulate apoptosis. To establish the role of FOXO1 in MSA-induced apoptosis, we employed the RNA interference technique to knockdown the expression of FOXO1. A commercially available siRNA targeting FOXO1 was obtained. To confirm the specificity of the siRNA, we performed a Local Alignment Search Tool (BLAST) against the entire human transcriptome using the sequence provided by the manufacturer. With the exception of FOXO1, the search identified no other homology with the siRNA sequence, including other FOXO members. When introduced into LNCaP cells, the FOXO1 siRNA, named siFOXO1 hereafter, was able to decrease the baseline expression of FOXO1 by approximately 50% (Fig. 3a). Consistent with our previous finding, a two-fold induction of FOXO1 was observed when the cells were treated with 10 μ M MSA for 24 hr (comparing columns 1 and 3). siFOXO1 was able to abolish this induction by MSA (comparing columns 3 and 4).

Apoptosis was quantitated in siRNA-transfected and MSA-treated cells by using an ELISA-based

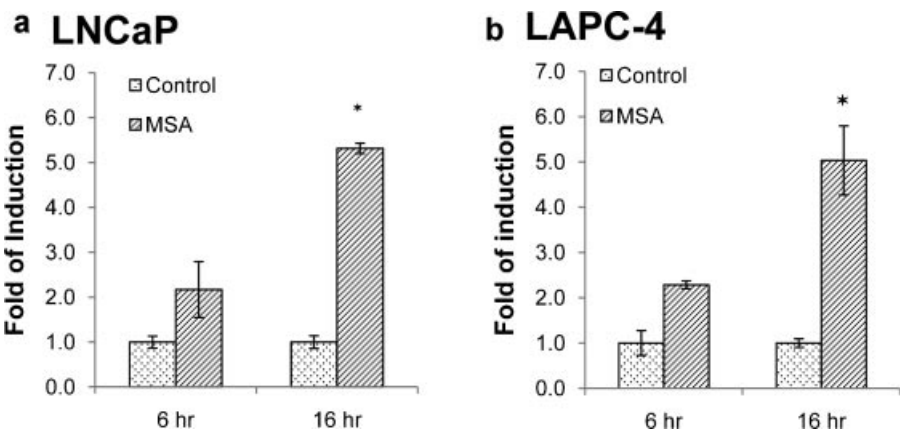


Fig. 2. Induction of FOXO transcriptional activity by MSA. LNCaP (**a**) and LAPC-4 (**b**) cells were transfected with the p3XIRS-luc construct and treated with 10 μ M MSA for the indicated times. At the end of treatment, cells were lysed for luciferase assay. Total protein concentration was also determined and used to normalize the luciferase reading. The results were expressed as mean $\pm$ SEM; *, $P < 0.05$.

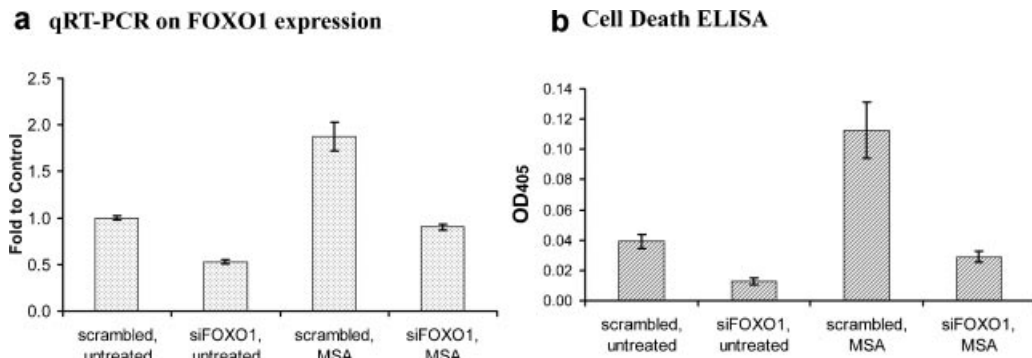


Fig. 3. Effect of FOXO1 gene silencing on MSA-induced apoptosis. **a:** qRT-PCR analysis of FOXO1 expression in cells transfected with small interference RNAs (siRNAs) and treated with or without MSA. The data were expressed as fold relative to the scrambled, untreated control. **b:** Quantitation of apoptotic cell death by an ELISA method.

method. The result is shown in Figure 3b. In general, the level of apoptosis in these cells correlated well with the expression level of FOXO1 (Fig. 3a), confirming that FOXO1 plays an important role in apoptosis regulation. More importantly, when the induction of FOXO1 was blocked by the addition of siFOXO1, no induction of apoptosis was observed (Fig. 3b, comparing columns 1 and 4). These results suggest that FOXO1 is a key mediator of apoptosis induction by MSA.

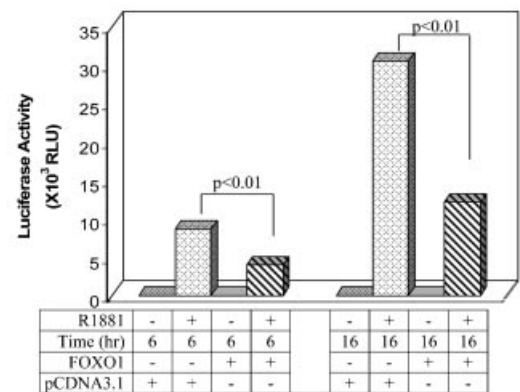
FOXO1 Activation Suppresses AR *trans*-Activation

To examine the effect of FOXO1 activation on the transcriptional activity of AR, we transiently co-transfected LNCaP cells with a reporter construct containing three repeats of the androgen response element (ARE) ligated in tandem to the luciferase reporter, together with a FOXO1 expression vector, pcDNA3-FKHR, or the empty vector. The ARE-luciferase reporter assay is commonly used to assess the *trans*-activating activity of AR. Following transfection, cells were exposed to 1 nM R1881, a synthetic androgen, for 6 or 16 hr before they were lysed for luciferase assay. As shown in Figure 4a, the AR transcriptional activity was greatly stimulated by the addition of the ligand. In the presence of ectopically expressed FOXO1, the induction was significantly diminished (Fig. 4a, comparing columns 2 and 4 for both time points). Therefore, our results confirmed published studies showing that FOXO1 activation suppresses AR signaling [37–40].

FOXO1 Induction Contributes to AR Suppression by MSA

It has been found previously that MSA is a potent suppressor of AR signaling [22,23,41]. The mechanisms involved in suppression of AR signaling by MSA include reduction in AR mRNA transcription [22,41] and stability, increase in AR protein turnover,

a FOXO1 overexpression on AR activity



b FOXO1 knockdown on suppression of AR activity by MSA

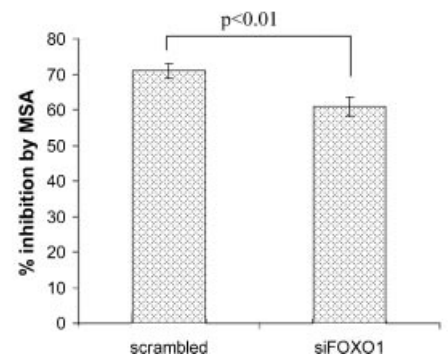


Fig. 4. Induction of FOXO1 contributes to AR suppression by MSA. **a:** Increased expression of FOXO1 reduced the transcriptional activity of AR. LNCaP cells were co-transfected with the ARE-luciferase reporter construct and either the pcDNA3-FKHR or the pcDNA3 vector, and treated with 1 nM R1881 for the indicated times. **b:** FOXO1 knockdown attenuated the suppression of AR *trans*-activation by MSA. LNCaP cells were co-transfected with the ARE-luciferase construct and either the scrambled control or siFOXO1, and treated with 10 μ M MSA for 24 hr. The luciferase reading was normalized by protein concentration. The experiment was done three times and the results were expressed as mean percent inhibition $\pm$ SEM.

reduction in AR translocation, inhibition of co-activator recruitment, and increased co-repressor recruitment to the promoters of AR-regulated genes [22,41,42]. The result from the previous section prompted us to investigate whether FOXO1 induction is a contributing factor for AR suppression by MSA. Once again, we employed the gene knockdown approach. LNCaP cells were co-transfected with the ARE-luciferase construct and siFOXO1, and treated with 0 or 10 μ M MSA. In the presence of the scrambled oligo, MSA suppressed AR activity by approximately 70% (Fig. 4b). This is in line with our previous observations [22]. However, when FOXO1 was silenced, the suppression was attenuated to about 60% ($P < 0.01$). This was further confirmed when we examined the modulation of prostate-specific antigen (PSA) expression by MSA in the presence or absence of siFOXO1 (data not shown). These results are in agreement with previous studies showing that MSA suppresses AR signaling through a multitude of mechanisms and identified FOXO1 activation as a novel mechanism contributing to the inhibition of AR *trans*-activation by MSA.

DISCUSSION

Despite the protective effect of selenium against prostate cancer demonstrated by the NPC study and several studies which showed selenium is very effective in switching off androgen signaling, recent results from the SELECT showed that selenium, alone or in combination with vitamin E, did not prevent prostate cancer in a randomized trial of 33,000 men at average risk [8]. Several potential reasons have been discussed to explain the discrepancy of the findings in SELECT and the NPC trial. In addition to the dose and formulation of selenium used in the trial, one important consideration is the baseline selenium level. The NPC trial showed that the protective effect of selenium was limited to patients with baseline serum selenium in the lower two tertiles [7]. The average baseline selenium level of the participants in SELECT was much higher than that observed in the NPC study. In fact, 78% of men in SELECT had baseline selenium above the range that selenium provided protection in the NPC trial (<121.6 ng/ml) [8]. Another important consideration is how selenium exerts its anticancer activity. The Physicians' Health Study demonstrated an inverse association of plasma selenium level with risk of advanced prostate cancer, not localized prostate cancer, suggesting selenium might function by slowing down tumor progression [5]. In view of the above information, we believe that the negative finding by SELECT should not be simply interpreted as selenium is ineffective against prostate cancer. Instead, the outcome of this trial, as well as those of several recently

published clinical trials [43–45], may indicate that it is difficult to find a single chemoprevention strategy which can benefit the general population. There is an urgent need to re-evaluate all the pre-clinical and clinical evidence to identify the subset of patients that are most likely to benefit from selenium supplementation.

This report is the first to show that MSA induces the expression of FOXO1. The elevated expression is accompanied by an increase of the FOXO transcriptional activity. We further demonstrated that FOXO1 is a key mediator of apoptosis induction by MSA. The above conclusion is supported by the following observations. First, FOXO1 induction occurred very early following MSA treatment, suggesting that FOXO1 is a proximal target of MSA. Second, MSA failed to induce apoptosis when FOXO1 stimulation was abolished by the addition of a FOXO1-specific siRNA. There are two major cell death signaling pathways, one triggered through death receptors (the extrinsic pathway), and the other through the mitochondria (the intrinsic pathway). A signature of the intrinsic pathway is the release of cytochrome *c* from the mitochondria, which is regulated by the Bcl-2 family of proteins. As a pro-apoptotic member of the Bcl-2 family, Bim functions by antagonizing the actions of the anti-apoptotic Bcl-2 and Bcl-XL. Both TRAIL and TRADD are associated with the extrinsic pathway. MSA has been shown to activate caspases that are involved in both the intrinsic and extrinsic apoptosis signaling pathways [18,36]. We are currently working on identifying the pro-apoptotic targets of FOXO1 that are induced by MSA. In addition to its role in regulating apoptosis, FOXO1 also plays an important role in cell cycle control. It up-regulates the expression of p27 [31,46] and down-regulates the expression of cyclins D1 and D2 [47,48], a pattern consistent with the G1 cell cycle block by MSA [17,49]. Therefore, it is possible that FOXO1 also mediates the cell cycle effects of MSA. Research along this line is currently ongoing in our laboratories.

Several mechanisms could account for the induction of FOXO1 signaling by MSA. One is through the induction of FOXO1 expression, as evidenced by the increased transcript and protein levels following MSA treatment. It has been shown that AR interacts and suppresses the activity of FOXO1 in prostate cancer cells [34,50]. Another potential mechanism of MSA activation of FOXO1 is through decreasing AR expression and thereby relieving the inhibition of FOXO1 by AR. This is supported by the fact that ectopic expression of AR could attenuate the induction of FOXO1 activity by MSA (data not shown). Yet, there might be a third mechanism by which MSA induces FOXO1. A key regulator of cellular FOXO1 activity is

Akt, an important survival molecule for many cancer types, including prostate cancer. Akt phosphorylates FOXO1, which leads to nuclear exclusion and proteosomal degradation of FOXO1 [33]. MSA has been shown to suppress the PI3K-Akt signaling pathway [19,51–53]. Therefore, it is possible that relieving the suppression by Akt may contribute to MSA induction of FOXO1. Further experimental evidence is needed to support this hypothesis.

In agreement with previous reports [37,38,40,54], our data showed increased abundance of FOXO1 leads to decreased AR activity. Together with the well-documented AR inhibition of FOXO1 activity, it appears that in prostate cancer cells, the AR and FOXO1 signaling pathways antagonize the action of each other. The outcome is likely determined by the relative abundance of AR and FOXO1 proteins. When AR signaling dominates, the growth inhibitory signals conveyed by FOXO1 are muted, and the cells undergo proliferation. On the other hand, when FOXO1 signaling dominates, the antiproliferative and pro-apoptotic signaling prevail. When prostate cancer cells are exposed to MSA, AR signaling is suppressed whilst FOXO1 signaling is stimulated. By doing so, MSA could shift the balance heavily in favor of FOXO1, leading to cell cycle arrest and apoptosis. Therefore, modulating the crosstalk between AR and FOXO1 could be the key mechanism underlining the anticancer effect of MSA in the prostate.

CONCLUSIONS

The work described herein demonstrates that MSA activates FOXO1 signaling pathway. FOXO1 plays a critical role in mediating the apoptotic activity of MSA, and also contributes to the suppression of androgen signaling by MSA. This study enhances our understanding of the molecular mechanisms of the anticancer activity of MSA, which will be critical for designing future prostate cancer intervention studies with MSA.

ACKNOWLEDGMENTS

This work was supported by DOD New Investigator grant no. W81XWH-05-1-0598 (H. Zhang), American Cancer Society grant no. RSG-07-218-01-TBE (Y. Dong), National Cancer Institute grant nos. K01 CA114252 (Y. Dong), P01 CA126804 (C. Ip, H. Zhang, and Y. Dong), start-up funds from the Louisiana Cancer Research Consortium (H. Zhang and Y. Dong).

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