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# Abstract

## Background

Prostate cancer is a leading cause of cancer-related death in men worldwide. Survivin is a member of the inhibitor of apoptosis (IAP) protein family that is expressed in the majority of human tumors including prostate cancer, but is barely detectable in terminally differentiated normal cells. Downregulation of survivin could sensitize prostate cancer cells to chemotherapeutic agents *in vitro* and *in vivo*. Selenium is an essential trace element. Several studies have shown that selenium compounds inhibit the growth of prostate cancer cells. The objective of this study is to investigate whether survivin gene silencing in conjunction with selenium treatment could enhance the therapeutic efficacy for prostate cancer and to elucidate the underlying mechanisms.

## Methods

Expression of survivin was analyzed in a collection of normal and malignant prostatic tissues by immunohistochemical staining. *In vitro* studies were conducted in PC-3M, C4-2B, and 22Rv1 prostate cancer cells. The effect of selenium on survivin expression was analyzed by Western blotting and semi-quantitative RT-PCR. Survivin gene knockdown was carried out by transfecting cells with a short hairpin RNA (shRNA) designed against survivin. Cell proliferation was quantitated by the 3-(4,5-Dimethylthiazol-2-yl)- 2,5-Diphenyltetrazolium Bromide (MTT) assay and apoptosis by propidium iodide staining followed by flow cytometry analysis. Finally, *in vivo* tumor growth assay was performed by establishing PC-3M xenograft in nude mice and monitoring tumor growth following transfection and treatment.

**Results**

We found that survivin was undetectable in normal prostatic tissues but was highly expressed in prostate cancers.   Survivin knockdown or selenium treatment inhibited the growth of prostate cancer cells, but the selenium effect was modest.   In contrast to what have been observed in other cell lines, selenium treatment had little or no effect on survivin expression in several androgen-independent prostate cancer cell lines.   Survivin knockdown sensitized these cells to selenium growth inhibition and apoptosis induction. In nude mice bearing PC-3M xenografts, survivin knockdown synergizes with selenium in inhibiting tumor growth.

**Conclusions**

Selenium could inhibit the growth of hormone-refractory prostate cancer cells both *in vitro* and *in vivo*, but the effects were modest.   The growth inhibition was not mediated by downregulating survivin expression.   Survivin silencing greatly enhanced the growth inhibitory effects of selenium.

**Background**

Prostate cancer (PCa) is the second most commonly diagnosed cancer and the second leading cause of cancer deaths in men in the United States [1]. Surgery and radiation therapy are effective for localized disease [2], but there is no effective treatment strategy for recurrent or metastatic PCa that has failed surgery, radiation or hormonal therapy. Chemotherapeutic drugs could only extend the lives of men with advanced prostate cancer by months, and they are also associated with dose-limiting toxicity. With the recent advances in the understanding of molecular pathways involved in prostate cancer progression, targeted therapies that are designed to interfere with the way cancer cells grow and survive offer new hope in prostate cancer therapeutics.

Survivin, a structurally unique member of the inhibitor of apoptosis (IAP) protein family, is involved in the control of mitotic progression and inhibition of apoptosis [3]. It is abundantly expressed in cancer tissues but is undetectable in normal, differentiated adult tissues. Survivin expression is associated with poor prognosis in many cancer types [4]. The expression of survivin increases gradually from normal prostate tissue, to primary low-grade prostate carcinoma, and to primary high-grade carcinoma, with the highest expression observed in foci of prostate cancer metastasized to the lymph nodes [5]. This pattern of gene expression suggests that survivin is associated with disease progression, and therefore making survivin an attractive target for prostate cancer therapeutics.

Selenium is an essential trace element. Several case control studies have demonstrated an inverse correlation between serum selenium level and the risk of developing prostate cancer [6-8]. The Nutritional Prevention of Cancer (NPC) trial demonstrated that supplementation of selenium, in the form of selenized yeast, could reduce the incidence of prostate cancer by ~50% % [9,10]. Although the interim analysis of the Selenium and Vitamin E Chemoprevention Trial (SELECT) indicated no reduction in prostate cancer risk associated with selenium supplementation [11], the finding should not be simply interpreted as selenium is ineffective against prostate cancer. In the Discussion, we provide several potential explanations for the discrepancy of the findings in SELECT and the NPC trial. The negative SELECT finding makes it more important and imperative to study the efficacy of new selenium compounds [12-14], including the compounds used in the study, methylseleninic acid (MSA) and methylselenocysteine (MSC), for prostate cancer intervention.

One of mechanisms proposed for the anticancer activity of selenium is apoptosis induction. Due to the role of survivin in conferring cytoprotection against apoptosis in cancer cells, we set out to examine the effect of selenium on the expression of survivin in several hormone refractory prostate cancer cell lines. To our surprise, selenium treatment did not affect survivin expression in these cells. Based on this finding, we

hypothesize that selenium treatment and survivin knockdown would complement the action of each other, leading to a greater anticancer effect.   The present study was designed to test this hypothesis, and to explore the underlying mechanisms.

**Methods**

**Reagents**

MSA and MSC were obtained from PharmaSe (Lubbock, TX).   The rabbit anti-human polyclonal antibodies against survivin and caspase 3 were from Santa Cruz Biotech (Santa Cruz, CA).   The rabbit anti-human Ki-67 polyclonal antibody was obtained from Neomarker (Fremont, CA).   Trizol, RPMI 1640, fetal bovine serum (FBS), and lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA).   Acridine orange (AO) and ethidium bromide (EB) were from Sigma (St. Louis, MO).

**Prostate tissue acquisition and immunohistochemical (IHC) analysis**

Prostate cancer specimens were obtained from patients undergoing prostatectomy in the Second and the Third Affiliated Hospitals of Jilin University.   Normal prostate tissues were obtained from patients undergoing surgery for benign prostatic hyperplasia (BPH) in these hospitals.   This study was approved by the Research Ethics Committee of Norman Bethune College of Medicine, Jilin University and was in compliance with the Helsinki

Declaration.    The tissues were examined by the pathologists to confirm the diagnosis before IHC analyses.   All specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4 µm-thick slides. The slides were dewaxed, and the endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution in methanol for 20 min.   Epitope retrieval was performed by treating the slides with 10 mM sodium citrate buffer (pH 6.0) and heating in a microwave oven for two times at the high power for 6 min each.   Non-specific binding was prevented by blocking with normal goat serum (1:10) for 10 min.   Immunostaining of survivin was performed using a rabbit anti-human survivin polyclonal antibody.   Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody.   The staining procedure was carried out manually at room temperature, using an avidin-biotin-peroxidase complex method. The presence of survivin and Ki-67 was evaluated by staining with rabbit anti-survivin and anti-Ki-67 antibody, respectively.   After incubation with the primary antibody for 60 min, the slides were incubated with the biotinylated goat anti-rabbit IgG (H+L) (DAKO, Carpinteria, CA) at 37°C for 30 min, followed by incubation with a 1:200 streptavidin-biotin-peroxidase complex (Sigma, St. Louis) for 30 min.   Reactive products were visualized with 3,3'-diaminobenzidene (DAB) as the chromogen, and the slides were counterstained with hematoxylin and coverslipped.   Sections previously known to express survivin and Ki-67 were included in each run, receiving either the primary antibody as the positive control, or a mouse IgG as the negative control.   The stained slides were analyzed with a

microscope at 200× magnification.   The slides were examined by two independent

pathologists who had no knowledge of the clinical outcomes and of the results obtained by

the other.   Differences in interpretation were reconciled by reviewing the slides jointly.

Cellular brownish staining was scored as positive and the threshold was set at 10%.   The

proliferation index was defined as percent of tumor cells stained positively for Ki-67.


**Construction of the short hairpin RNA (shRNA) vectors for survivin silencing**

Oligonucleotides for anti-survivin shRNA expression were designed, synthesized, and

cloned into the pGCsilencer-U6/Neo/GFP plasmid (GeneChem Co, Shanghai, China).

The sequences are: sense, 5'-gatcccGCAGTTTGAAGAATTAACCttcaagaga

GGTTAATTCTTCAAACTGCtttttggat-3'; and antisense, 5'-agctatccaaaaaGCAGTTTG

AAGAATTAACCtctcttgaaGGTTAATTCTTCAAATGCgg-3'.   Sequences in uppercase

letters indicate nucleotides 394-412 of the survivin cDNA.   BLAST searches of the

human genome database were carried out to ensure that the sequences would not target

any other transcripts.   The recombinant plasmid was named pGCsh-survivin (referred to

as sh-survivin hereafter).    Recombinant pGCsh-scrambled plasmid (referred to as

sh-scrambled hereafter), which used a scrambled sequence that has no significant

homology to any mouse or human gene sequences, was constructed as the negative

control.

**Cell culture and transfection**

The PC-3M, C4-2B, and 22Rv1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 unit/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM of L-glutamine and incubated at 37℃ in 5% $CO_2$. For transfection, $5 \times 10^5$ cells were plated onto 6-well plates 24 hours before transfection. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions. Transfection media were removed 6 hours after transfection and replaced with fresh complete medium containing 10% FBS. Controls included lipofectamine 2000 treated cells and sh-scrambled transfected cells.

**Gene expression analysis by semi-quantitative reverse-transcription PCR (RT-PCR) and Western analysis**

The effects of survivin shRNA and MSA, alone or in combination, on cellular expression of survivin were measured by semi-quantitative RT-PCR and Western blot analysis. PC-3M cells were divided into 5 groups: (a) control (mock-transfected); (b) scrambled (transfected with sh-scrambled); (c) sh-survivin (transfected with sh-survivin); (d) MSA (treated with 5 µM MSA); (e) combination (transfected with sh-survivin and treated with 5 µM MSA), and cells were harvested after 66 hours following transfection and treatment. Total RNA was isolated using the Trizol reagent according to the manufacturer's instructions. Survivin gene expression was determined by

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semi-quantitative RT-PCR, using the primer pairs 5'-GAATTCATGGGTGCCCCGA

CGTTGCC-3' and 5'-AGATCTTTCTTCTTATTGTTGGTTTCC-3'. The housekeeping

gene β-actin was amplified as an internal standard.


For Western blot analysis, cells were lysed with HEPES lysis buffer (30 mM HEPES,

1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl).

Fifty micrograms of protein were electrophoresed on a 10% SDS-PAGE gel, transferred

onto a PVDF membrane (Millipore, Bedford, MA), and probed with the anti-survivin or the

anti-caspase-3 antibody. A goat anti-rabbit antibody labeled with horseradish peroxidase

(Amersham) was added as the secondary antibody. Immunoblots were developed using

a chemiluminescence detection system (Amersham Pharmacia Biotech).


**Cell proliferation and apoptosis assays**

Cell numbers were determined at 66 hours after treatment using the 3-(4,5-

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and quantified by

using a microplate reader (Bio-Rad). For apoptosis, $10^6$ cells were fixed in 70% (v/v)

ethanol, stained with PBS containing 50 μg/mL propidium iodide, 10 mg/mL RNase, and

0.1% (v/v) Tween-20 for 30 minutes at room temperature, and analyzed for cell apoptosis

with a FACScan flow cytometer (Becton Dickinson). For AP/EB staining, PC-3M cells

were collected by trypsinization, washed with PBS (pH 7.4), and fixed in 4%

paraformaldehyde. Fixed cells were washed twice with PBS and resuspended in PBS containing 16 µg/ml AO and 16 µg/ml EB, then mounted on a glass slide and observed under a fluorescent microscope.

**Antitumor effect of survivin siRNA and selenium *in vivo***

Five-week-old pathogen-free athymic nude mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). PC-3M cells in exponential growth phase were harvested and single-cell suspensions ($2 \times 10^6$ cells in 100 µl PBS) were injected subcutaneously (s.c.) into the right dorsal flank of nude mice. Tumor size was measured every 2 to 3 days, and tumor volume calculated as $0.5236 \times width^2 \times length$. After palpable tumors had developed, the mice were divided randomly into five groups (six mice per group): (a) control (mock-transfected); (b) scrambled (transfected with sh-scrambled); (c) sh-survivin (transfected with sh-survivin); (d) MSC (given MSC at 100 µg/mouse/day); (e) combination (transfected with sh-survivin and given MSC). Transfection of the siRNAs was performed by an electroporation method. Twenty micrograms of plasmid suspended in 50 µl PBS was injected percutaneously into the tumors by using a syringe with a 27-gauge needle. Immediately after injection, tumors were pulsed with an electroporation generator (ECM830, BTX, Holliston, MA). Pulses were delivered at a frequency of 1 pulse/sec, 150V/cm, for 50 milliseconds. This process was performed twice, first on day 15 and then on day 25. For selenium treatment, MSC was given by

daily intragastric administration starting at day 15.  Mice were sacrificed on day 35 when

tumor burden in the control group approaching 10% of body weight. Tumors were excised, fixed

in 4% buffered formalin, and embedded in paraffin.   The animal study was conducted following

internationally recognized guidelines and was approved by the Animal Research Committee of

Norman Bethune College of Medicine, Jilin University.

**Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay**

Apoptotic cells in tissues were determined by using the TUNEL assay (Roche

Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.   Briefly, after

deparaffinization and rehydration, sections were incubated with proteinase K (16.2 µg/mL

in 10 mM Tris·HCL, pH 7.4) for 20 min at 37℃. Slides were rinsed with PBS and

incubated with 3% $H_2O_2$ in methanol for 10 min at room temperature to block endogenous

peroxidase activity, followed by PBS washing and incubation in 0.1% Triton X-100 in 0.1%

sodium citrate for 2 min on ice.   Sections were then incubated with a mixture of terminal

deoxynucleotidyl transferase (TdT) solution and biotin-dUTP solution in a humidified

chamber at 37℃ for 60 min, followed by washing with PBS and incubation with

peroxidase-conjugated streptavidin in a humidified chamber for 30 min at room

temperature.   After an additional wash with PBS, the slides were incubated in DAB and

counterstained with hematoxylin.   Paraffin-embedded sections of normal tonsils were

used as positive control.   Negative control was obtained by replacing the TdT solution

with distilled water.  The presence of clear nuclear staining was indicative of apoptotic cells.  At least 1,000 tumor cell nuclei were randomly selected and examined.  The number of TUNEL-positive tumor cell nuclei was counted and the apoptotic index was calculated as follows: apoptotic index = (number of apoptotic cells / total cell number) ×100%.

**Statistical analysis**

Chi-square test was used to analyze the difference in survivin expression between normal and cancerous prostate tissues. Pearson correlation analysis was performed to test the correlation of Gleason Score and survivin expression in the prostate cancer cases. Data were presented as mean ± standard deviation (SD) when appropriate. Two-tailed *Student's t*-test was used to evaluate the differences between treatment and control values, and $P<0.05$ was considered statistically significant.

**Results**

**Survivin is overexpressed in prostate cancer tissues**

We first analyzed the expression of survivin by IHC staining in a collection of prostate cancer and normal tissues in a Chinese cohort.  As shown in Fig.1, the survivin protein was virtually undetectable in normal prostatic tissue but highly expressed in prostate

cancer.   The cellular localization of survivin is mostly cytoplasmic, but some weak staining can be found in the nucleus.   Overall, survivin was expressed in 24 of 28 (85.7%) prostate cancer specimens, and no survivin expression could be detected in 22 normal prostate tissues (Table 1).   The difference is statistically significant (*P*<0.001).   Pearson correlation analysis identified a strong correlation between Gleason score and survivin expression among the cases (R=0.7446, *P*<0.0001). Please see additional file-1 for the original data used to perform this analysis.   These results confirmed previous findings that survivin is expressed in clinical prostate cancers [5], suggesting survivin is a promising target for prostate cancer therapy.

**MSA induces apoptosis in PC-3M cells but has little effect on survivin expression**

Since MSA has been shown to induce apoptotic cell death in a number of prostate cancer cell lines [15,16], we set out to examine the effect of MSA on apoptosis in PC-3M cells.   PC-3M is a metastatic subline derived from PC-3.   We chose PC-3M because it has the highest level of survivin expression among the cell lines we have tested (data not shown).   Cells were treated with 5 µM MSA for 48 and 66 hr and the extent of apoptosis was quantified by flow cytometric analysis of cells labeled with propidium iodide (PI). This dose of selenium has been shown to be achievable in human subjects taking selenium supplementation [17].   As shown in Fig 2, only 0.9% of the control cells were positively labeled with PI.   In cells treated with MSA, the proportion of apoptotic cells increased to 3.6% by 48 hr, and to 14.7% by 72 hr.   These results showed that MSA was

capable of inducing apoptosis in PC-3M cells, but the effect was modest and a long treatment (>48 hr) was needed to achieve a significant induction of apoptosis.

As a member of the IAP family, the best known function of survivin is to protect the cells from undergoing apoptosis.   It has been shown that selenium decreased survivin expression in several prostate cancer cell lines, including LNCaP, PC-3, and C4-2 [18]. We decided to examine survivin expression in PC-3M cells following MSA treatment.   In contrast to what have been observed in other cell lines, both semi-quantitative RT-PCR and Western blotting analysis showed that treatment with 5 µM MSA for 66 hr did not influence survivin expression in PC-3M cells (Fig. 3A & 3B), indicating the induction of apoptosis by MSA in these cells is not mediated by decreasing survivin expression. In addition to PC-3M cells, we analyzed survivin expression in C4-2B and 22Rv1 prostate cancer cells following MSA treatment and the results were similar (Fig. 3B).

**Survivin gene silencing sensitizes prostate cancer cells to growth inhibition by MSA**

Based on the data from the previous experiments, we hypothesized that survivin knockdown will sensitize prostate cancer cells to growth inhibition by MSA.   To test this hypothesis, we designed a plasmid-based shRNA vector for survivin gene silencing and tested its efficacy.   As shown in Fig. 3A, sh-survivin effectively reduced the level of the

survivin transcript, whereas the scrambled shRNA had no effect.   Western blotting

analysis confirmed the down-regulation of survivin expression by sh-survivin in all 3 cell

lines (Fig. 3B). Quantitation by volume densitometry showed the knockdown efficiency at

the protein level is in the range of 60-70% in these cell lines.


We next quantitated changes in cell viability after cells were transfected with the

shRNAs and treated with various concentrations of MSA for 66 hr.   The results are shown

in Fig. 4.   Cells transfected with sh-scrambled have similar viability as the

mock-transfected control cells under all circumstances. Silencing of survivin reduced cell

viability by 50-60% ($P$<0.01).   In the absence of sh-survivin, MSA reduced the cell

viability in a dose-dependent manner in all three cell lines.   However, cells transfected

with sh-survivin are significantly more sensitive to MSA growth inhibition, manifested by

the marked downward shift of the dose-response curves.    In addition, cells transfected

with sh-survivin and treated with MSA, the decrease in cell viability was significantly

greater than sh-survivin alone ($P$<0.01).


**Survivin gene silencing enhances selenium's efficacy on apoptosis induction.**

Next, we analyzed the effect of apoptosis induction by sh-survivin and MSA in PC-3M

cells by flow cytometry. Transfection and MSA treatment were carried out as described in

the previous section.   Very few PI-positive cells were found in untreated cells or in cells

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transfected with sh-scrambled (Fig. 5A). Silencing of survivin resulted in a robust increase of apoptotic cells, confirming the antiapoptotic role of survivin. The combination of survivin knockdown and MSA induced apoptosis more effectively than either alone ($P$ <0.01). In addition, AO/EB staining was performed to visualize the apoptotic cells and similar results were obtained (see additional file-2 for detail).

Caspases are important mediators of apoptosis. Activation of caspase-3 by enzymatic cleavage is a hallmark event in caspase-dependent apoptosis. We investigated caspase-3 activation by Western blotting and the results are shown in Fig. 5B. Cleaved caspase-3 (19 KDa) was induced similarly by sh-survivin and MSA, whereas their combination led to a modestly but significantly stronger activation ($P$ <0.05). The changes in caspase-3 activation did not involve changes in gene expression, as the band intensity for procaspase-3 (32 KDa) was not changed by either sh-survivin, or MSA, or their combination. The modest increase in caspase-3 activation in the combined group suggest that the synergistic effect on apoptosis induction could also be mediated by caspase-independent mechanisms, since survivin has also been shown to inhibit both caspase–independent apoptosis [19]. In general, the apoptosis data are in agreement with the MTT results, showing that survivin gene knockdown greatly enhances the anticancer effect of selenium in prostate cancer cells.

**Inhibition of tumor growth *in vivo* by survivin knockdown and selenium**

A xenograft nude mouse tumor model with subcutaneously implanted PC-3M cells was used to investigate the effects of survivin knockdown and selenium *in vivo*. Fifteen days after injection, the tumors reached an average volume of 122.5±15.36 mm$^3$. The animals were then randomly divided into 5 groups receiving PBS, sh-scrambled, sh-survivin, MSC, or the combination of MSC and sh-survivin, respectively. MSC was administrated at a dose of 100 µg/mouse/day through an oral route and the shRNA plasmids were delivered by electroporation twice during the experiment, first at day 15 and then at day 25. As shown in Fig. 6A, tumor xenografts in the control and the scrambled groups grew at similar rates, whereas xenografts in all 3 treatment groups demonstrated reduced growth by day 30, when compared to their respective controls ($P$ <0.05). Consistent with the *in vitro* results, selenium by itself has a modest inhibitory effect on the growth of PC-3M xenografts, but the combination with survivin knockdown achieved a robust growth inhibition. In fact, tumors in the combination group maintained their original sizes after treatment, and were significantly smaller than those in the single treatment groups by day 35 ($P$ <0.05). Similar conclusions can be reached by examining the final tumor weight (Fig. 6B). These results demonstrate a strong inhibitory effect on tumor growth *in vivo* by the combination of survivin knockdown and selenium treatment.

To explore the mechanisms of tumor inhibition *in vivo*, tumors were collected after the animals were sacrificed. The expression of survivin was first analyzed by IHC staining.

As shown in Fig. 7A, the staining results showed that the electroporation-based delivery method was effective as survivin was down-regulated by sh-survivin. MSC has no effect on the expression or the cellular localization of survivin. Cell proliferation was quantitated by IHC staining of Ki-76 (Fig. 7B) and apoptotic cells were identified by the TUNEL assay (Fig. 7C). Tumors from the control or the sh-scrambled groups have proliferating cells (Ki-67 positive) and less apoptotic cells (TUNEL positive) than those in the sh-survivin or MSC. The lowest number of proliferating cells and highest number of apoptotic cells were found in tumors from the combination group. Quantitative analyses of the stained slides were performed and the results are summarized in Table 2. In tumors receiving sh-survivin or MSC, the proliferation index was significantly lower ($P$ <0.01), and the apoptotic index was significantly higher ($P$ <0.01), when compared with the control tumors. Once again, the combination group was more effective in blocking tumor growth and inducing cell death than either of the single treatment groups ($P$ <0.01). These results confirmed our *in vitro* observations and showed survivin knockdown greatly enhanced the efficacy of selenium in inhibiting the growth of prostate cancer cells *in vivo*.

**Discussion**

Soon after the development of the RNA interference (RNAi) technology a few years ago, the potential of using this highly specific and powerful gene silencing approach in

treating cancer has generated a great deal of interest. Obviously, the key to the success of this approach is to identify a gene which is expressed universally in cancer cells, but not in normal cells. Survivin appears to be such a candidate. As a member of the IAP family, survivin has been reported to be up-regulated in human cancers of various origins. Consistent with previous reports [5,20], we found in the present study that survivin is highly expressed in the majority of cancerous prostate tissues, but not in normal prostatic tissues. Silencing of survivin expression in PC-3M cells by a specific shRNA led to decreased cell growth and increased cell death. Elevated survivin expression has been associated with resistance to chemotherapeutic agents [21-24]. Therefore, targeted suppression of survivin expression represents a potential therapeutic strategy for prostate cancer, either being used alone or in a neoadjuvant setting.

Several potential reasons have been discussed to explain the discrepancy of the findings in SELECT and the NPC 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 2 tertiles [25]. 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) [11]. Another important consideration is how selenium exerts its anticancer activity. By using a

nested case-control design within a prospective study (the Physicians' Health Study) among 586 men diagnosed with prostate cancer during 13 years of follow-up and 577 control subjects, Li et al. showed a ~50% reduction in risk of advanced prostate cancer in individuals within the highest quintile of selenium level compared to those within the lowest quintile [26]. Such association was not observed for localized prostate cancer, suggesting that selenium might function by slowing down tumor progression [26]. 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, it raises the hypothesis of whether selenium might only be effective only in selected subsets of men with lower selenium levels at baseline.

The formulation and dose of selenium used in the SELECT study have also been hot topics of debate. The compounds used in the current study, MSA and MSC, are monomethylated forms of selenium and are considered second-generation selenium compounds. Metabolically, they are very different from selenomethionine, the formulation used in the SELECT. MSA and MSC can be easily converted to methylselenol, which is considered to be the critical metabolite for the anticancer activity of selenium [14,27]. Selenomethionine, on the other hand, can be incorporated nonspecifically into proteins in place of methionine [14]. Due to its compartmentation into tissue proteins, selenomethionine is not as readily available as MSA or MSC for further

metabolism.   The metabolism of selenomethionine to methylselenol requires at least 5 transsulfuration steps and the action of thiol methyltransferase [12-14].   Studies published prior to and after the start of the SELECT study have showed that MSA and MSC have stronger anticancer activities than selenomethionine [28-30].

Several studies have shown that selenium induces growth arrest and cell death in prostate cancer cells and inhibits the growth of prostate cancer xenografts [15,16,31,32], leading support to the idea that selenium could be used in the treatment of prostate cancer. In the present study, we confirmed the anticancer activity of selenium in the androgen-independent human prostate cancer cells. In contrast to a report that selenium suppresses survivin expression in a number of PCa cell lines, including the parental PC-3 and LNCaP lines [18], our *in vitro* and *in vivo* data both showed that MSA treatment had little or no effect on survivin expression in PC-3M, C4-2B, and 22Rv1 cells.   This is consistent with results published by Azrak et al showing at similar doses, selenium treatment did not affect survivin expression in mouse prostate cancer cells or ovarian cancer cells [33,34].   When used alone, MSA had a modest effect on growth inhibition and apoptosis induction in PC-3M cells cultured *in vitro*.   Similarly, MSC inhibited the growth of PC-3M xenografts but the efficacy appears to be inferior to its inhibitory effect against PC-3 xenografts [29].   The insensitivity of PC-3M cells to selenium could be related to its inability to suppress survivin expression in these cells.   In support of the

notion, we found that the effect of selenium was greatly enhanced when survivin expression was silenced.   Indeed, in nude mice bearing PC-3M xenografts, survivin knockdown in combination with selenium treatment stopped tumor growth completely.   In the study by Azrak et al which showed MSA did not inhibit survivin expression in skov3 ovarian cancer cells, survivin knockdown greatly enhanced the efficacy of combination therapy by MSA and paclitaxel, converting the interaction between these two agents from antagonistic to synergistic [34].

**Conclusions**

Survivin was highly expressed in clinical prostate cancers but not in normal prostates.   In prostate cancer cells where selenium had no effect on survivin expression, growth inhibition and apoptosis induction effects of selenium were modest.   Both the *in vitro* and *in vivo* efficacies of selenium were greatly enhanced when survivin expression was silenced.   The current study suffers from the limitation that the results are obtained mainly from cell culture and animal experiments.   Nonetheless, the results suggest a potential mechanism of cancer cell resistance to the cytotoxic effect of selenium compounds and a strategy to improve the efficacy of selenium.   This is important, especially in the context of the negative findings of the SELECT trial with regard to the anticancer activity of selenium.   Further preclinical studies are needed to confirm the findings before these results can be incorporated into clinical practice.

**Competing interests**

The authors have no competing interests to disclose.

**Authors' contributions**

This study was designed by XL, YD, XZ, and HZ.  XL carried out the majority of the experiments and performed the data analysis.  RG helped with the animal experiments, data analysis, and manuscript preparation.  YD provided assistance with data analysis, result interpretation, and manuscript preparation.  LG helped with shRNA design and YZ with flow cytometry analysis.  LZ and XZ were responsible for patient sample collection and coordination of the study.  The manuscript was written by XL and HZ.  HZ also contributed to data analysis and interpretation.  All authors have read and approved the final manuscript.

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**Table 1. Expression of survivin in normal and cancerous prostatic tissues.**

| Tissue Type | survivin staining status | | P |
|---|---|---|---|
| | positive N (%) | negative N (%) | |
| Normal | 0 (0%) | 22 (100%) | |
| | | | <0.001 |
| Prostate cancer | 24 (85.71%) | 4 (15.29%) | |

**Table 2. Cell proliferation and apoptosis indices from PC-3M xenografts.**

| Group (n =6) | Proliferation index | Apoptotic index |
|---|---|---|
| control | 86.75±9.43 | 3.64±1.66 |
| sh-scrambled | 81.64±9.25 | 4.45±1.86 |
| sh-survivin | 16.82±5.32* | 31.3±3.64* |
| MSC | 56.2±8.65* | 14.7±2.23* |
| combination | 6.8±1.64*# | 48.6±2.67*# |

*, P<0.01 vs sh-scrambled;   #, P<0.01 vs sh-survivin or MSC group.

**Figure Legends**

**Fig. 1. Representative images from immunohistochemical analysis of survivin expression in normal and cancerous prostatic tissue.** A, normal prostate. B, prostate cancer. The images were obtained at 200× magnification.

**Fig. 2. MSA induces apoptosis in PC-3M cells.** Untreated cells (A) or cells treated with 5 µM MSA for 48 hr (B) or 72 hr (C) were labeled with propidium iodide (PI) and the histograms of DNA content were obtained by flow cytometry analysis. The x-axis indicates PI fluorescence intensity, and y-axis indicates cell number. The numbers above the histogram are percentage of cells in the sub-G1 (apoptotic) phase.

**Fig. 3. Survivin expression in various prostate cancer cells treated with MSA, sh-survivin, or the combination.** A, RT-PCR analysis in PC-3M cells. B, Western blot analysis in PC-3M, C4-2B, and 22Rv1 cells. The band intensity was quantified by volume densitometry and normalized to that of β-actin. The results are expressed as fold induction over untreated.

**Fig. 4. Growth inhibition by survivin knockdown and MSA treatment.** PC-3M, C4-2B, and 22Rv1 cells were transfected with the shRNAs and treated with various concentrations of MSA for 66 hr. Cell numbers were quantitated by the MTT assay. The

viability of the mock-transfected control cells were set arbitrarily to 100% and the viabilities

of other samples were expressed as percentage of the control.   The data presented are

mean ± SD (n=18). Control, mock-tranfected cells.   Sh-scrambled, cells transfected with

sh-scrambled.   Sh-survivin, cells transfected with sh-survivin.    *, $P$<0.01 vs MSA alone;

#, $P$<0.01 vs sh-survivin alone.

**Fig. 5. Apoptosis induction by survivin knockdown and MSA treatment in PC-3M**

**cells.**   Cells were transfected with the shRNAs and treated with 5μM MSA for 66 hr. A,

Quantitation of apoptotic cells by PI staining and flow cytometry analysis.   Apoptosis

index was calculated as percentage of cells in the sub-G1 population on the DNA

histogram. The data presented are mean ± SD (n=3). The values are 0.9%±0.65%,

2.8%±0.71%, 23.1±2.36%, 12.7±1.45%, and 38.6±2.87%, respectively. B, Western blot

analyses of caspase-3.   β-actin was used as the loading control. B. Quantitation of

cleaved caspase-3 by volume densitometry. The intensity of the cleaved caspase-3 band

was quantified by volume densitometry and normalized to that of β-actin. The result was

expressed as fold to control.

**Fig. 6. Inhibition the growth of PC-3M xenografts by survivin knockdown and MSA.**

The shRNA plasmids were transfected by electroporation twice at day 15 and 25, as

indicated by the arrows. A, tumor volume. B, final tumor weight. The data presented are

mean ± SD (n=6).*, P<0.05 vs control; **, P<0.05 vs sh-scrambled; #, P<0.05 vs

sh-survivin; &, P<0.05 vs MSC group.


**Fig. 7.   Immunohistochemical staining in PC-3M tumor xenografts**. (A) survivin

expression, (B) Ki-67 expression , and (C) TUNEL assay. The images were taken at

200×magnification.



**Additional files**

Additional file-1: Table_Survivin_Gleason_PSA.pdf

This file contains the original data used for the Pearson correlation analysis for survivin

expression and Gleason score.   It also contains the PSA measurements.


Additional file-2: AO_EB_apoptosis.pdf

This file contains additional figure for apoptosis detection using AO/EB staining as
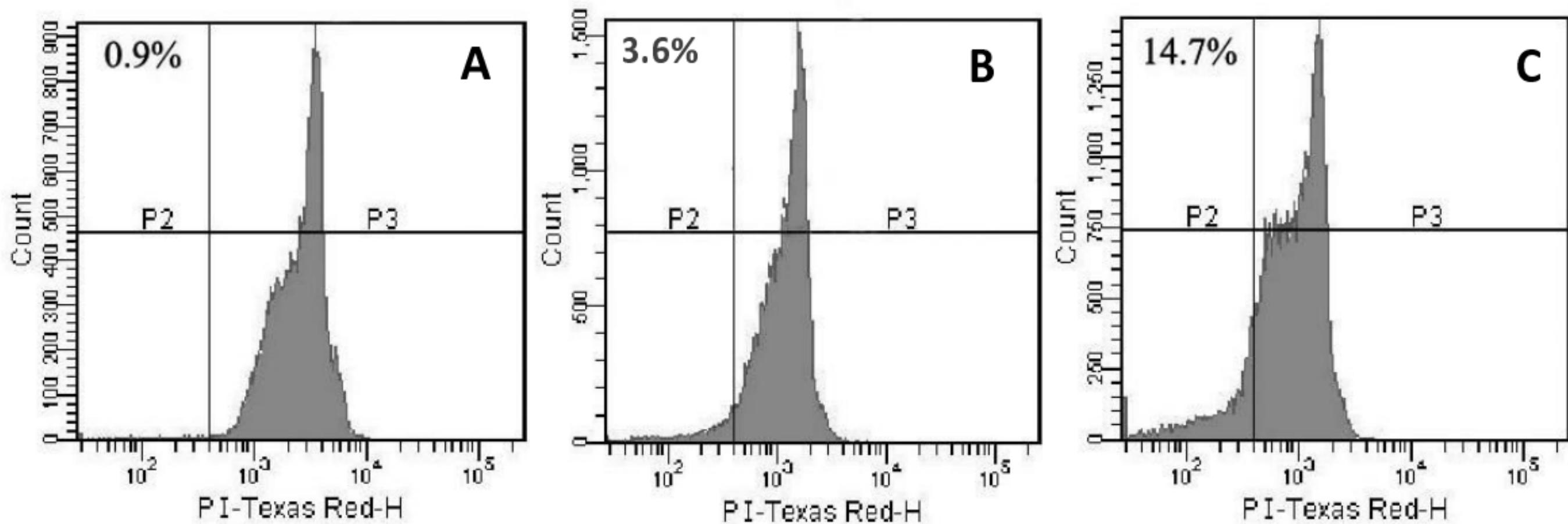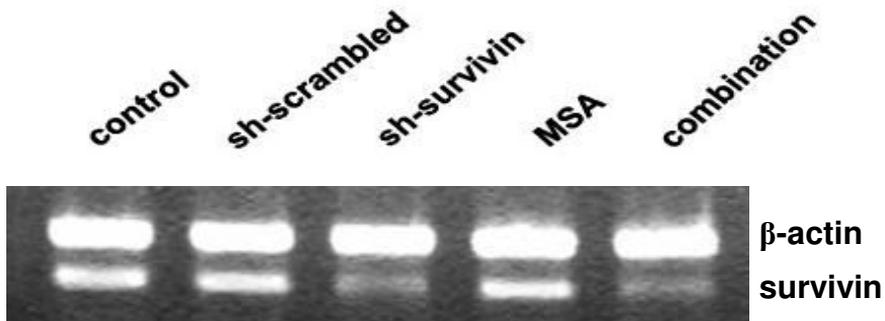
described in the text.

Figure 1

Figure 2

A

| | control | sh-scrambled | sh-survivin | MSA | combination |
|---|---|---|---|---|---|

β-actin
survivin

B

| | control | sh-scrambled | sh-survivin | MSA | combination |
|---|---|---|---|---|---|

survivin

β-actin                                                                          PC-3M

survivin/actin   1.00   1.03   0.40   0.86   0.32

survivin

β-actin                                                                          C4-2B

survivin/actin   1.00   0.98   0.43   0.85   0.29

survivin

β-actin                                                                          22Rv1

survivin/actin   1.00   1.00   0.29   0.78   0.18
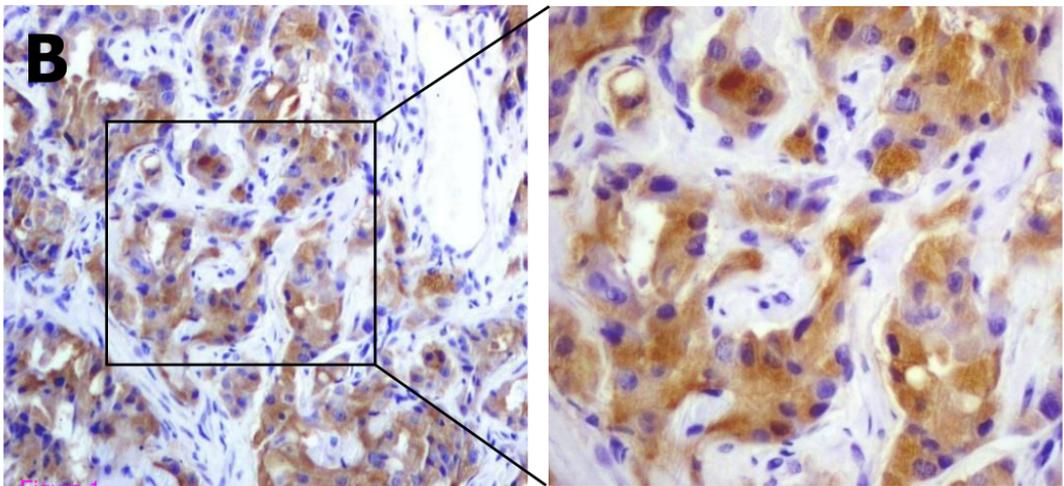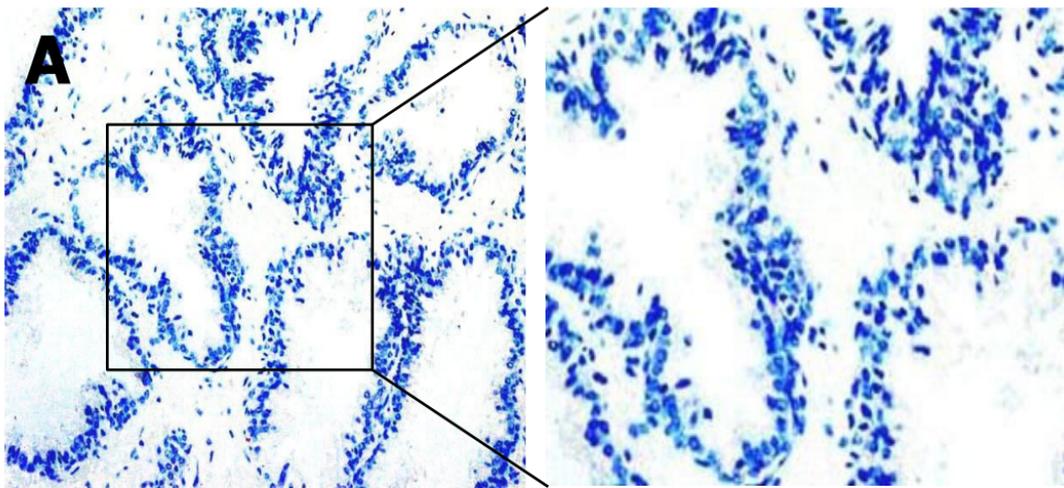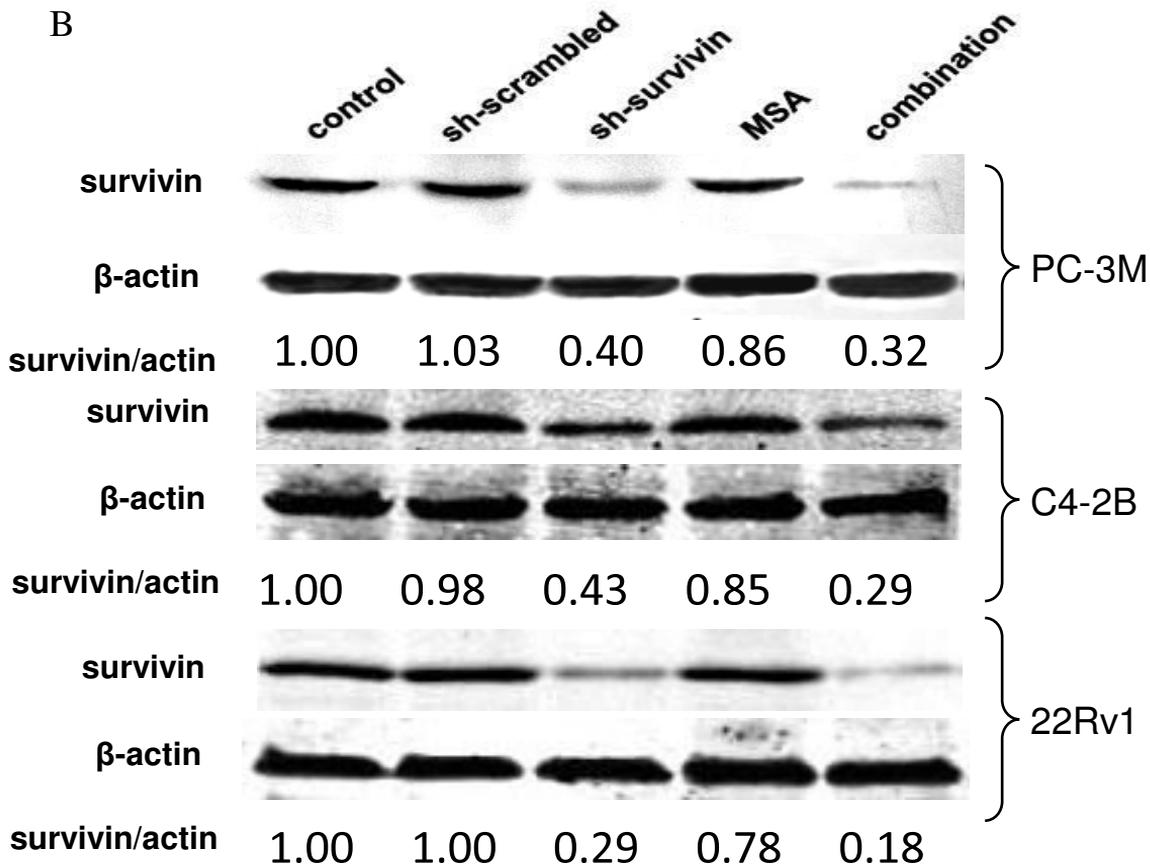
Figure 3

**A. PC-3M**

**B. C4-2B**

**C. 22Rv1**

Figure 4

**A**

Apoptotic Index

*P*<0.01

*P*<0.01

*P*<0.01

control  sh-scrambled  sh-survivin  MSA  combination

**B**

control  sh-scrambled  sh-survivin  MSA  combination

Procaspase-3                                                          32KDa

Cleaved caspase-3                                                     19KDa

β - actin                                                            42KDa

fold of change
(Normalized)        1.00    0.90    5.07    4.59    6.29

Figure 5

Figure 6

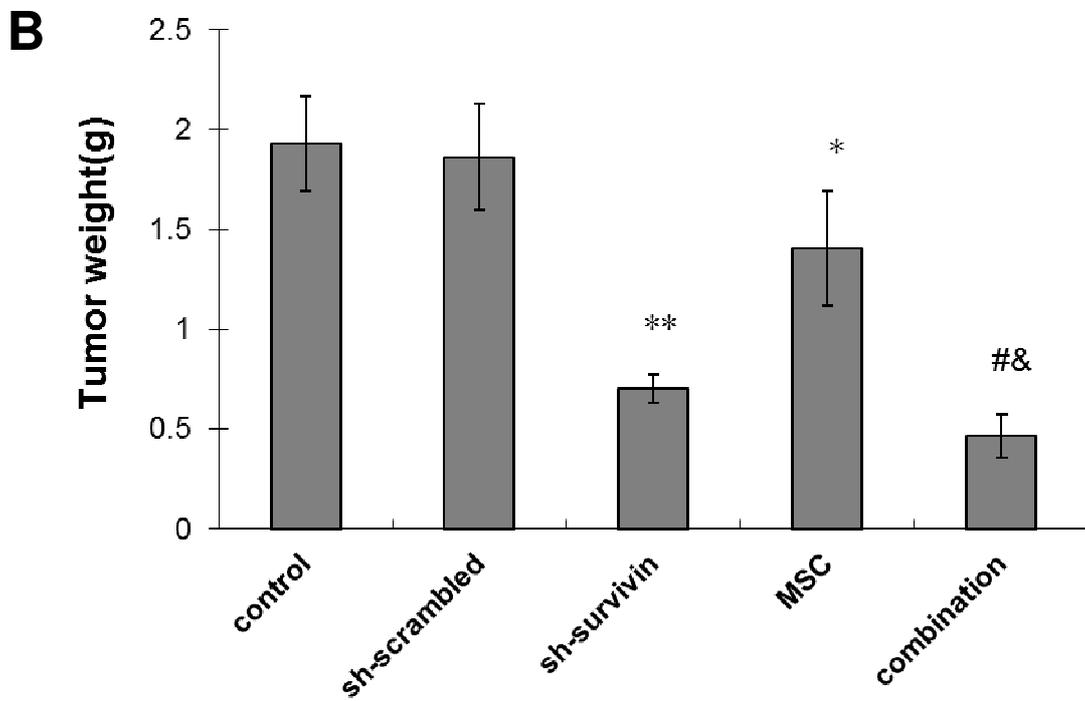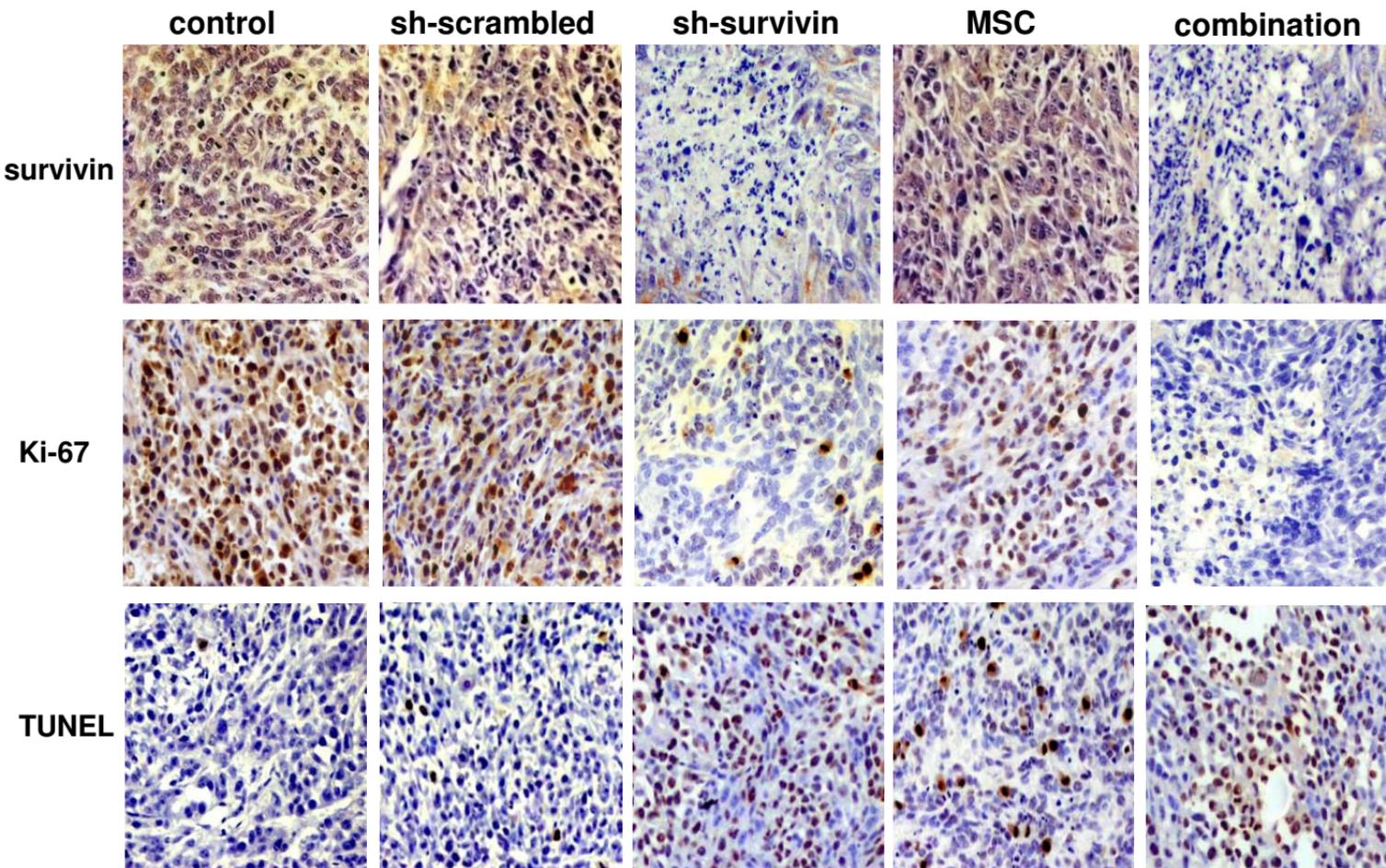|  | control | sh-scrambled | sh-survivin | MSC | combination |
|---|---|---|---|---|---|
| survivin | | | | | |
| Ki-67 | | | | | |
| TUNEL | | | | | |
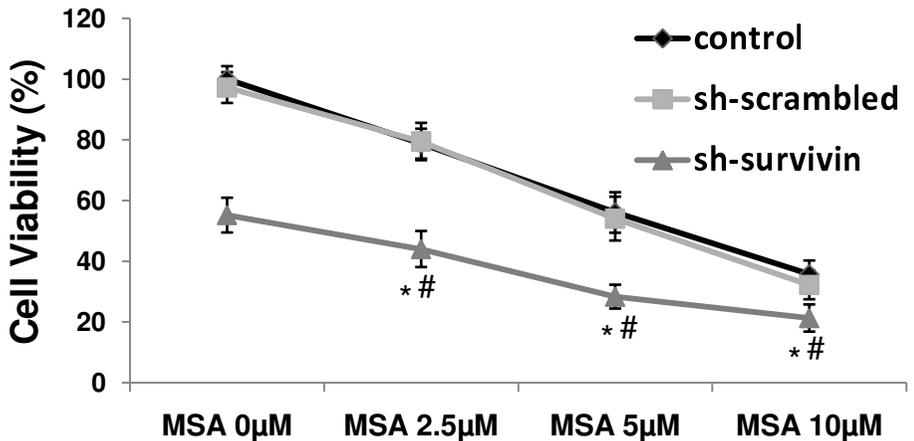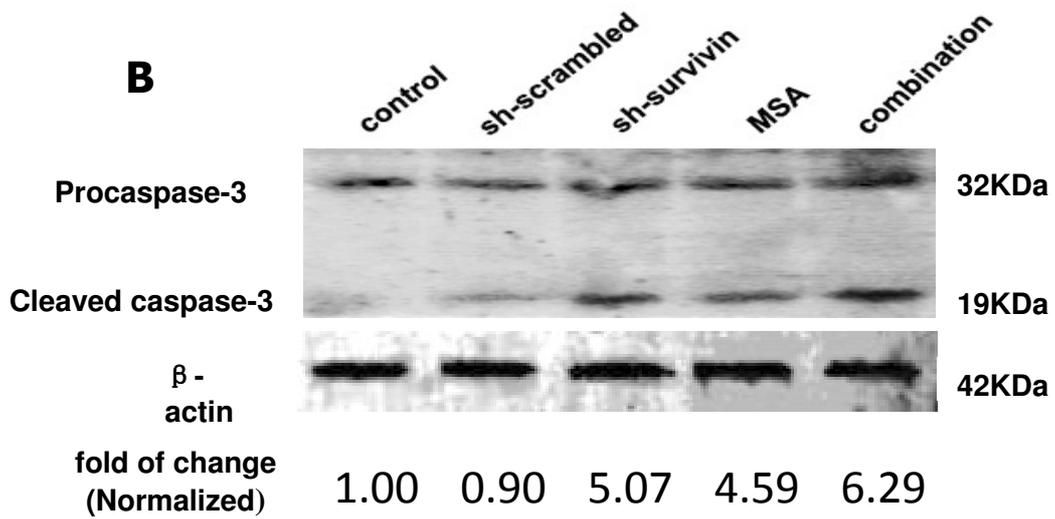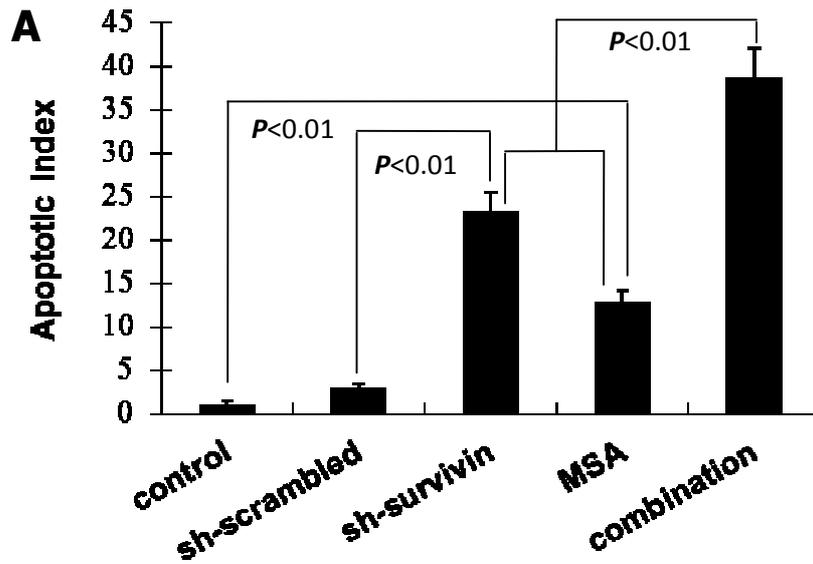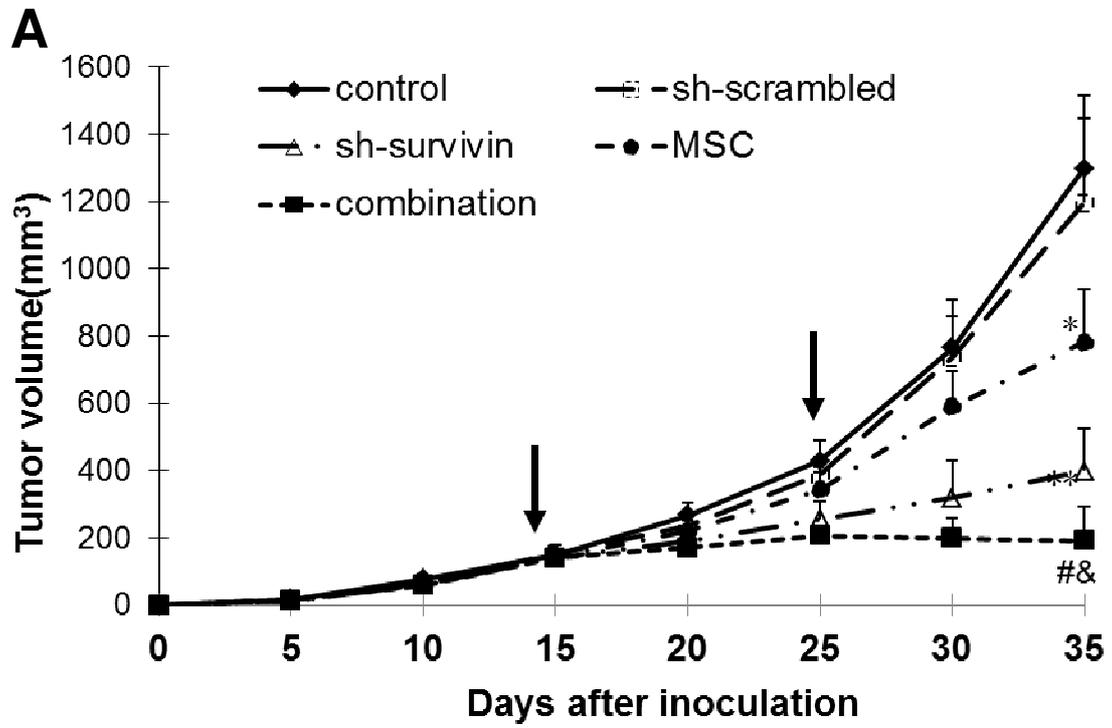
Figure 7

**Additional files provided with this submission:**

Additional file 1: Table_Survivin_Gleason_PSA.pdf, 44K
http://www.biomedcentral.com/imedia/1613166535336981/supp1.pdf
Additional file 2: AO_EB_apoptosis.pdf, 120K
http://www.biomedcentral.com/imedia/1729637093369817/supp2.pdf