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Methylselenocysteine preventing castration-resistant progression of prostate cancer

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Abstract

Background—Castration-resistant progression of prostate cancer after androgen deprivation therapy remains a critical challenge in the clinical management of prostate cancer. Resurgent androgen receptor activity is an established driver of castration-resistant progression, and upregulation of androgen receptor expression has been implicated to contribute to the resurgent androgen receptor activity. We reported previously that methylselenocysteine can decrease the expression and activity of androgen receptor. Here we investigated the ability of methylselenocysteine to inhibit castration-resistant progression of prostate cancer.

Methods—The regrowth of LNCaP prostate cancer xenografts after castration was monitored. The levels of prostate-specific antigen in mouse serum were measured by ELISA. Tumor cell proliferation and apoptosis were analyzed via Ki-67 immunohistochemistry and TUNEL assay, respectively. Intratumoral angiogenesis was assessed by immunohistochemistry staining of vascular endothelial growth factor and CD31.

Results—We showed that methylselenocysteine delayed castration-resistant regrowth of LNCaP xenograft tumors after androgen deprivation. This was accompanied by decreased serum levels of prostate-specific antigen, inhibition of prostate cancer cell proliferation and tumor angiogenesis, as well as downregulation of androgen receptor and induction of apoptosis in the relapsed tumors.

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Conclusions—The present study represents the first to show the preclinical efficacy of methylselenocysteine in delaying castration-resistant progression of prostate cancer. The findings provide a rationale for evaluating the clinical application of combining methylselenocysteine with androgen deprivation therapy for the treatment of advanced prostate cancer.

Keywords

methylselenocysteine; prostate cancer; castration resistance

INTRODUCTION

As the first-line treatment for disseminated prostate cancer, androgen deprivation therapy disrupts androgen receptor (AR) signaling through androgen ablation or AR antagonists. While this regimen is effective initially, progression to castration-resistant prostate cancer (CRPC) is almost inevitable [1]. Despite recent advances in prostate cancer therapy, metastatic CRPC remains incurable. Resurgent AR activity is an established driver of castration-resistant progression of prostate cancer [2, 3].

Prostate cancer can adapt to androgen deprivation therapy by mutating AR, amplifying AR, upregulating constitutively-active AR splice variants, activating AR by androgen-independent mechanisms, and/or increasing intra-tumoral androgen levels through *de novo* androgen synthesis [4–17]. Importantly, the expression of AR was shown to be prevalently upregulated in prostate tumors that relapsed after androgen deprivation [10]. Moreover, elevated expression of AR was shown to sensitize the receptor to low levels of androgen [18] and to convert prostate cancer growth from a castration-sensitive to a castration-resistant stage [10]. Knocking down AR by shRNA in xenograft models can delay the progression of prostate cancer to castration resistance [19]. Therefore, therapeutic approaches that can diminish the availability of AR should offer considerable benefit in preventing or delaying prostate cancer recurrence after androgen deprivation therapy.

Previously, we showed that methylselenocysteine (CH_3-Se-CH_2-CH(NH_2)-COOH, MSC), a methyl-selenium compound, is effective in downregulating the expression of AR and AR target, such as the prostate-specific antigen (PSA), in both the castration-sensitive LNCaP prostate tumor xenografts and the castration-resistant 22Rv1 prostate tumor xenografts, leading to inhibited growth of these tumors [20, 21]. In the present study, we tested the hypothesis that MSC prevents or delays prostate cancer recurrence after androgen deprivation. It is important to point out that MSC has a very different biological and pharmacological activity than selenomethionine, the form of selenium used in the selenium and vitamin E chemoprevention trial [22–27], and that MSC has shown no evidence of toxicity in a single-dose Phase I trial [28].

MATERIALS AND METHODS

Cell Lines and Reagents

LNCaP cells were obtained from American Type Culture Collection at Passage 4. The cells used in this study were within 20 passages (~3 months of non-continuous culturing) and

cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. MSC was obtained from PharmaSe.

Tumor Xenograft Models

Male nude mice were obtained from the Vital River Laboratories Co. Ltd. at 5–6 weeks of age. After one week of adaptation, mice were inoculated subcutaneously with 4×10^6 LNCaP cells suspended in 50% Matrigel on the right flank. When the tumor size reached ~65 mm^3, castration was performed *via* a scrotal approach. The day following castration, the mice were randomly assigned to two groups, *viz.*, vehicle control (saline) and 5 mg/kg/day MSC through oral gavage [21, 29, 30]. The tumor dimensions and body weights were measured weekly. Tumor volume was calculated as $0.524 \times width^2 \times length$ [31]. At the termination of the experiment, mice were anesthetized with Ketamine/Xylazine, blood collected for serum PSA determination, and euthanized by CO_2. Tumors were removed, weighed, and fixed in 10% formalin for paraffin embedding and histological analyses. All animal procedures were approved by the Jilin University Institutional Animal Care and Use Committee.

Determination of Serum PSA Levels

Serum PSA levels were quantified with the use of the quantitative ELISA kit (United Biotech) per instruction of the manufacturer.

Immunohistochemical Analyses

Immunohistochemistry (IHC) was conducted as described previously [32] with a Ki-67 (NovoCastra), vascular endothelial growth factor (VEGF; abcam), or CD31 (NovoCastra) primary antibody at a 1:200, 1:500, or 1:500 dilution, respectively. As the negative control, the primary antibody was replaced with a non-immune IgG at the same concentration, and no reactivity was detected. Apoptosis was detected by the TUNEL assay using the DeadEnd™ Colorimetric TUNEL System (Promega) per manufacturer's protocol. Five random microscopic fields were captured for each tumor section at 10x magnification, and the images were analyzed by using the ImmunoRatio online analysis tools [33]. For Ki-67 and VEGF staining as well as the TUNEL results, the percentage of positively stained cells was calculated for each image. The data are presented as average % of positively stained cells in each group. For CD31 staining, the ratio of vessel area to total analyzed area was calculated by using the Microvessel Analysis V1 software.

Statistical Analysis

The Student's two-tailed *t* test was used to determine the mean differences between treatment and control, and $P < 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

RESULTS

MSC Inhibition of Castration-Resistant Progression of LNCaP Tumors

We evaluated the effect of MSC on prostate cancer recurrence after androgen deprivation therapy in the LNCaP xenograft model. LNCaP is an androgen-dependent human prostate cancer cell line [34]. The development of LNCaP xenograft tumors requires androgens, and castration causes an initial decelerated growth of the xenografts [35, 36]. However, similar to human prostate cancers, the xenografts eventually become castration resistant and resume growth [35–39]. Male nude mice were implanted with LNCaP cells on the right dorsal flank. Castration was performed when the tumors reached ~65 mm^3. Castrated mice were then divided into two groups receiving either saline as control or 5 mg/kg/day MSC through oral gavage. The dose of MSC was the same as we previously used in the study with castration-sensitive LNCaP tumor xenografts [20]. Treatment was initiated the day following castration.

The tumors in the control group resumed growth starting from day 21 after castration as a result of acquiring castration resistance, whereas the tumors in the MSC group remained small (Figure 1A). From day 35, the difference in the average tumor size between the two groups became statistically significant. At the termination of the experiment, the average tumor size was 956 mm^3 in the control group, but 323 mm^3 in the MSC group, indicating ~66% inhibition of tumor regrowth by MSC (Figure 1A). In line with this result, the average tumor weights were 1.2 g and 0.2 g in control and MSC groups, respectively (Figure 1B). During the 58-day period of treatment, MSC did not appear to cause toxicity in the mice as indicated by no decrease in body weight (Figure 2A) or weights of various organs, including the prostate, compared to the control group (Figure 2B). Collectively, the data showed the potential of MSC to inhibit prostate cancer relapse after androgen deprivation therapy without causing overt toxicity.

MSC Reduction of Serum PSA Levels

Prostate tumor relapse after castration is associated with elevated PSA levels in the serum [40] and sustained or increased AR levels in the tumors [2, 3]. We measured the levels of PSA in mouse serum using ELISA. As shown in Figure 2, MSC supplementation led to an almost 50% drop in mean serum PSA level at the termination of the experiment, from 32 ng/ml in the control group to 16 ng/ml in the MSC group. We also measured AR protein expression by Western blotting. As shown in Figure 3, MSC treatment decreased the mean level of AR protein in the tumors by ~30%. The data thus support the ability of MSC to suppress rise in serum PSA levels after androgen deprivation therapy and to reduce AR protein levels in castration-resistant prostate tumors.

MSC Inhibition of Cell Proliferation and Induction of Apoptosis

We further evaluated the effect of MSC on proliferation and apoptosis in the tumors via immunohistochemistry using an antibody against Ki-67, a marker of cell proliferation, and TUNEL assay, respectively. As shown in Figure 4, MSC supplementation caused an approximately 30% reduction of the percentage of Ki-67-positive cells and a 2.5-fold induction of the percentage of apoptotic cells in the tumors. The data thus indicate that

inhibition of cell proliferation and induction of apoptosis may underlie MSC inhibition of prostate cancer relapse after androgen deprivation therapy.

MSC Inhibition of Angiogenesis

Angiogenesis plays an important role for supporting the growth of prostate tumors, and a recent study reported that castration-resistant prostate cancer is associated with increased angiogenesis [41]. We therefore evaluated the effect of MSC on intratumoral angiogenesis *via* IHC using VEGF and CD31 as markers. As shown in Figure 5, MSC treatment caused a ~50% reduction of the percentage of VEGF-positive cells and microvessel density, suggesting that inhibition of tumor angiogenesis may represent an additional mechanism by which MSC prevents prostate cancer relapse after androgen deprivation therapy.

Discussion

We demonstrated previously that MSC inhibits the growth of the castration-sensitive LNCaP prostate tumor xenografts in gonad-intact mice and castration-resistant 22Rv1 prostate tumor xenografts and downregulates AR expression and activity in these tumors [20, 21]. In the present study, we extended the observations to a castration-resistant progression model, showing, for the first time, the potential of using MSC to prevent or delay the development of castration-resistant prostate cancer and to suppress rise in serum PSA levels after androgen deprivation therapy. Mechanistically, MSC downregulates AR expression, inhibits prostate cancer cell proliferation and tumor angiogenesis, as well as induces apoptosis in prostate tumors. The findings provide a strong rationale for a combination therapy using MSC to improve the therapeutic outcome of androgen deprivation therapy.

Selenium has been shown to be an effective anticancer agent in cancers of various sites, including the prostate, by numerous preclinical studies, epidemiological observations, and clinical trials [23, 42]. Although the interim analysis of the Selenium and Vitamin E Chemoprevention Trial (SELECT) indicated that supplementation of healthy individuals with nutritional dose of selenium did not reduce prostate cancer risk [25], the finding should not have been entirely unexpected. The anticancer efficacy of selenium depends on the form and dosage of selenium administered and the stage of the disease progression [22–24, 26, 43]. In fact, animal studies using xenograft models have shown that selenomethionine, the form of selenium used in the SELECT, is ineffective against prostate cancer growth, whereas “second-generation” selenium compounds, including MSC, are very effective in inhibiting tumor growth [24, 26]. This is in concordance with the fact that selenomethionine is converted to the active metabolite, methylselenol, much less efficiently than the much more potent new selenium compounds [22, 23, 27]. Additionally, in most of the preclinical studies that showed a positive association between selenium administration and tumor inhibition, the experiments were conducted by using pharmacological doses of selenium, not the nutritional dose that was used in the SELECT. Therefore, the negative SELECT finding underscores the urgency of studying the efficacy of the new selenium compounds, particularly at pharmacological doses, for prostate cancer intervention. Selenium has a low toxicity profile. While the tolerable upper intake level for selenium is set at 400 μg a day for adults [44], prostate cancer patients supplemented with 3,200 μg selenium per day for an

average of one year did not develop any serious selenium-related toxicities [45]. Therefore, selenium supplementation at pharmacological doses will be well tolerated.

Our previous studies showed that MSC, at pharmacological doses, can effectively reduce the abundance of both the full-length and/or the constitutively-active splice variants of AR in LNCaP and 22Rv1 prostate tumor xenografts [20, 21]. Increased expression of the full-length and splice variants of AR has been indicated to be one mechanism of resistance to traditional androgen deprivation therapy and the new androgen deprivation drugs abiraterone and enzalutamide [10, 15, 46–50]. To date, none of the anti-androgens currently used in clinics can target AR directly to reduce its availability. In addition to MSC, several other compounds have been shown pre-clinically to reduce the levels of AR and to inhibit the growth of castration-resistant prostate cancer cells [21, 51–56]. These compounds may serve as an effective antidote to overcoming resistance to androgen deprivation therapies. Determining the combinatory efficacies and the best sequences of treatments is an area of our ongoing research.

Conclusions

In summary, the present study represents the first to show the preclinical efficacy of methylselenocysteine in delaying castration-resistant progression of prostate cancer. The findings not only provide a rationale for further developing MSC or its analogue for intervention of CRPC, but also substantiate reducing AR availability as a viable approach to improve therapeutic outcome of androgen deprivation therapy.

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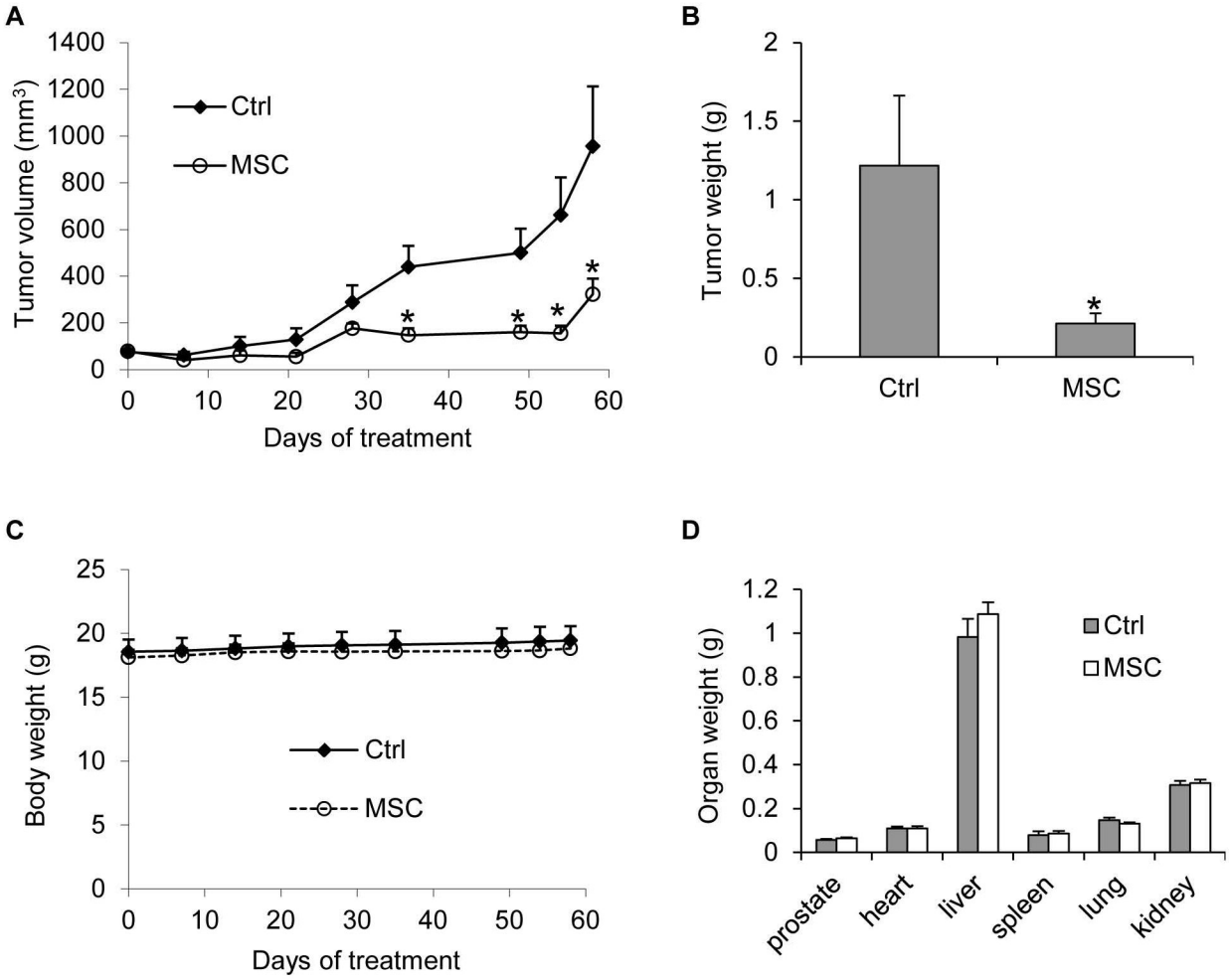

Figure 1. MSC inhibition of castration-resistant progression of LNCaP xenograft tumors

LNCaP cells were inoculated into gonad-intact nude mice, and surgical castration was performed when the tumors reached ~ 65 mm^3. Treatment with 5 mg/kg/day MSC through oral gavage was initiated the day following castration (n = 5). **A.** Tumor volumes. **B.** Tumor weights at the conclusion of the experiment. **C.** Mouse body weights. **D.** Weights of various organs at the conclusion of the experiment. *, $P < 0.05$ from the control group. Error bars, SEM.

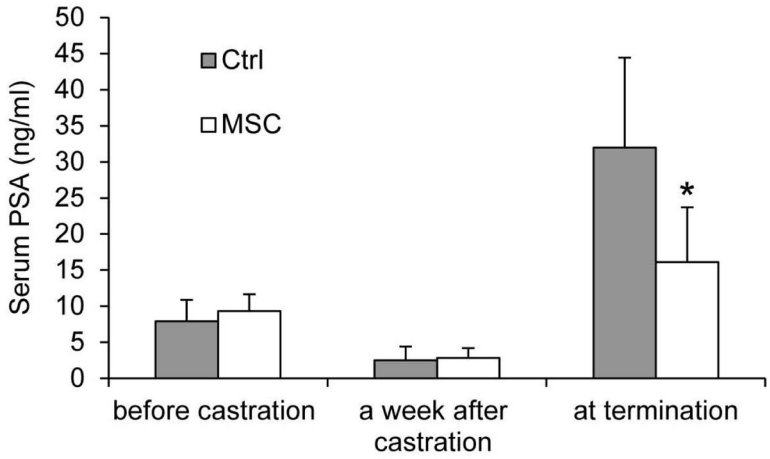

Figure 2. MSC suppression of rise in serum PSA levels during castration-resistant progression of LNCaP xenograft tumors

Serum PSA levels were determined by ELISA before castration, a week after castration, and at the conclusion of the study. *, $P < 0.05$ from the control group. n = 5. Error bars, SEM.

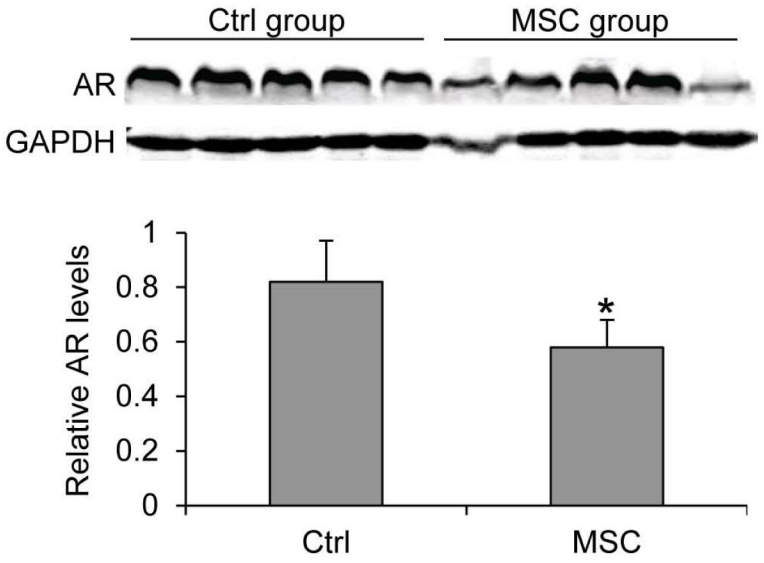

Figure 3. MSC downregulation of AR expression in castration-resistant LNCaP xenograft tumors

AR protein levels in the tumors were determined by Western blotting. Densitometric quantitation of the Western data is shown below the blots. *, $P < 0.05$ from the control group. Error bars, SEM.

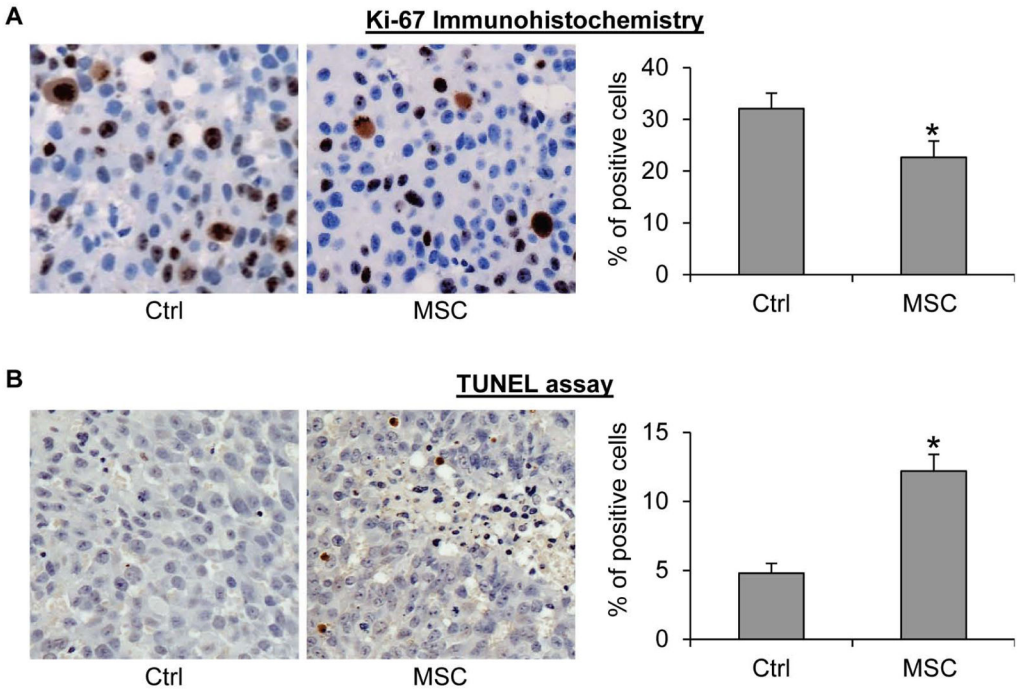

Figure 4. MSC inhibition of cell proliferation and induction of apoptosis in castration-resistant LNCaP xenograft tumors

A. Ki-67 immunohistochemical staining of the tumor sections. **B.** TUNEL assay of the tumor sections. Left panels, representative images. Right panels, quantitation of the data. *, $P < 0.05$ from the control group. Error bars, SEM.

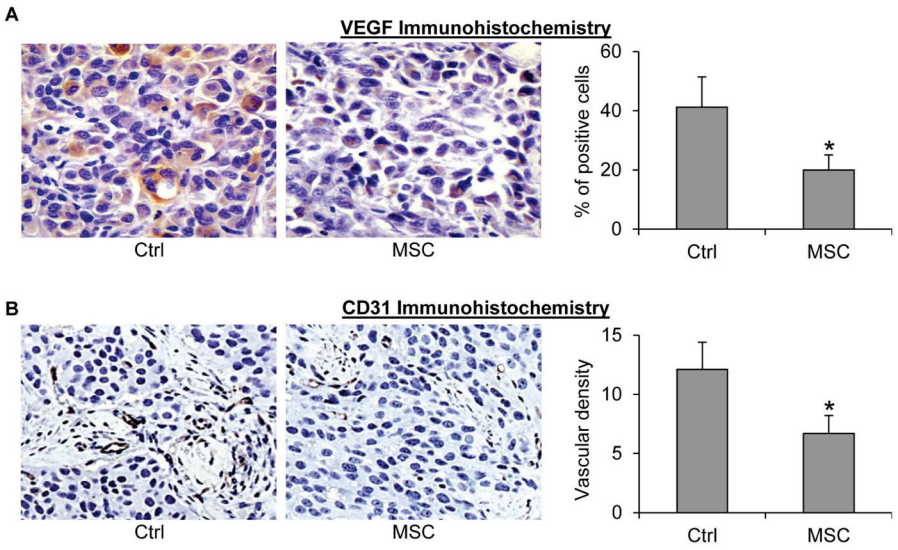

Figure 5. MSC inhibition of angiogenesis in castration-resistant LNCaP xenograft tumors

A. VEGF immunohistochemical staining of the tumor sections. **B.** CD31 immunohistochemical staining of the tumor sections. Left panels, representative images. Right panels, quantitation of the data. *, $P < 0.05$ from the control group. Error bars, SEM.