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Original Article

Androgen deprivation and stem cell markers in prostate cancers

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Abstract: In our previous studies using human LNCaP xenografts and TRAMP (transgenic adenocarcinoma of mouse prostate) mice, androgen deprivation therapy (ADT) resulted in a temporary cessation of prostate cancer (PCa) growth, but then tumors grew faster with more malignant behaviour. To understand whether cancer stem cells might play a role in PCa progression in these animal models, we investigated the expressions of stem cell-related markers in tumors at different time points after ADT. In both animal models, enhanced expressions of stem cell markers were observed in tumors of castrated mice, as compared to non-castrated controls. This increased cell population that expressed stem cell markers is designated as stem-like cells (SLC) in this article. We also observed that the SLC peaked at relatively early time points after ADT, before tumors resumed their growth. These results suggest that the SLC population may play a role in tumor re-growth and disease progression, and that targeting the SLC at their peak-expression time point may prevent tumor recurrence following ADT.

Key words: Prostate cancer, stem-like cells, androgen deprivation, xenograft tumor, TRAMP mice

Introduction

A very small population of cells in cancers has been recognized as having self-renewal and differentiation capabilities and, hence, is referred to as cancer stem cells (CSC) [1, 2]. Many scientists believe that cancers arise from CSC, although this is currently under debate. According to this hypothesis, CSCs are responsible for the occurrence of distant metastases and for tumor recurrence after initial effective treatment (reviewed in [3]).

Targeted therapies against CSC are currently being developed. However, there is a major difficulty in determining how to effectively attack such a small population within a tumor. A recent study demonstrated that the proportion of tumor cells expressing CD44, a marker for identifying CSC, increased in human breast cancers 12 weeks after chemotherapy, and these cells had a great propensity for self-renewal as measured by their ability to form mammospheres in culture and tumors in mice [4]. An increase of CSC was also observed in xenograft models of human colorectal cancer after chemotherapy [5] and in human gliomas after radiation [6]. Thus, time-dependent changes in CSC may have important implications for therapeutic response. In this report, we designate cells that express stem cell markers in tumor tissues as stem-like cells (SLC).

In our previous studies, castration resulted in cessation of human prostate LNCaP tumor growth [7]. However, this anti-tumor effect only lasted two to three weeks and was followed by accelerated tumor growth and chemotherapy-resistance [7]. In TRAMP mice, most PCa were initially very sensitive to androgen deprivation therapy (ADT). Significant atrophy of prostates and tumors was observed by 10 wks after castration, with increased expressions of bcl-2 and Grp78/Bip in tumors at this time point [8]. However, tumors eventually re-grew in all castrated mice, and significantly increased distant metastases were observed in castrates (78%)

relative to intact animals (30%) by the end of the experiment (36-wks of age). In addition, a high incidence of neuroendocrine carcinoma (NEC) was only associated with castrated TRAMP mice [9]. In an effort to determine whether SLC might have played a role in tumor re-growth and increased malignancy, we used immunohistochemistry, western blots and real time PCR to study the expression patterns of stem cell markers in LNCaP xenograft tumors and TRAMP prostate cancers at different time points after castration.

Materials and methods

Animal models and androgen ablation

Two animal models were used in this study: the LNCaP human prostate cancer xenograft model and the TRAMP (transgenic adenocarcinoma of mouse prostate) mouse model. Male SCID (severe combined immunodeficiency) mice, six to eight weeks old, were purchased from Harlan Laboratory, (Indianapolis, Indiana). TRAMP mice were bred in-house under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine. The breeding and genome typing procedures, and the nature of the prostate cancers that develop in these mice have been reported previously [8, 9]. Surgical castration was used for androgen ablation in both mouse models. SCID mice were castrated when tumors reached approximately 400 mm^3 [7]. TRAMP mice were castrated at the age of twelve weeks; mice without castration were used as controls.

Tumor growth and sample collection

LNCaP cells grown in 10% FBS RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 1% penicillin and streptomycin (Invitrogen) were harvested from culture flasks at 80-90% confluence. 2 x 10^6 cells were inoculated subcutaneously into the flanks of SCID mice (two flanks per mouse). Xenograft tumors were collected 10, 15, 20, 25, and 30 days after castration (n=10 per time-point). Tumors from non-castrated mice were removed on the same days for controls (n=5 per time-point). Endogenous PCa develops in TRAMP mice by the age of 12 weeks with 100% incidence [8, 10]. Castrated TRAMP mice were euthanized 10 wks (Cas-10) or 20 wks (Cas-20) after the surgery. The DLP (dorsal-lateral prostate) lobes of mouse prostates, which have the highest incidence of PCa development in the TRAMP model (10), were dissected under an anatomic microscope for protein analysis, gene expression, and IHC staining. All prostates and main organs were stained with H&E (hematoxylin/eosin) for histopathology.

Immunohistochemistry (IHC)

LNCaP xenograft tumors and DLP lobes from TRAMP mice were either embedded in OCT and frozen in liquid nitrogen for cryo-sectioning (7-μm thickness) or fixed immediately in 4% formalin for 24 hours followed by routine paraffin embedding (FFPE) and sectioning (10-μm thickness). The detailed procedures for IHC are described elsewhere [7, 8]. Briefly, antibodies for Ki67 (DAKO, CA), CD44 (eBioscience, San Diego, CA), CD133, CD117 (c-Kit) (Santa Cruz, Santa Cruz, CA), and Sca-1 (for mouse tissue only) (BD Bioscience, San Jose, CA) were diluted in blocking buffer (10% BSA in PBS) to appropriate concentrations, and incubated with cryo-tissues (CD133 and CD44) and/or FFPE-tissues (Ki67, c-Kit, and Sca-1) on glass slides. Isotype-matched immunoglobulins were used as negative controls. HRP-conjugated EnVision+Dual Link system reagent (DAKO, Carpinteria, CA) was then added to the tissue sections, and the positive signals were visualized with DAB substrate (DAKO). After hematoxylin counter-staining, IHC images were photographed (5-10 pictures/slide) and saved as TIFF files. These image files were then digitized with MICD 7.0 software under the auto-scan function to measure the total grain area of positive signals in each file. The significance of the differences among various time-points was analyzed using the student t-test.

Real time PCR

Total RNA was extracted from tumor tissue samples using QIAGEN RNeasy Mini Kit (Cat. 74104) according to the manufacturer's instructions. Briefly, homogenized tumor tissues were passed through 21G needles several times, then subjected to RNA binding column followed by DNase digestion. After washing, total RNA was eluted from the column with RNase-free water. First strand complementary DNA (cDNA) was synthesized from less than one microgram of total RNA using iSCRIPT

cDNA synthesis Kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was added into duplicate wells for each tumor sample, and real-time PCR was performed using Fast Real-Time PCR System (Applied Biosystems). Primers for amplifying human CD44 were 5'TCCACCCCA ACTCCATCTGT-3' (sense) and 5'- GGAGCTGAA GCATTGAAG-3' (antisense), and for hGAPDH they were 5'-GGTGGTC TCCTCTGACTTCAACA-3' (sense) and 5'-GTTGCTGTAGCCAAATTCGTTGT-3' (antisense). The amplification procedures included enzyme activation at 95^{0}C for 20 sec followed by 40 cycles of denaturing at 95^{0}C for 1 sec, and annealing/extension at 60^{0}C for 20 sec. cDNA from PC3 cells was used as the positive control, and PCR reaction without template served as the negative control. CD44 expressions were normalized with respect to GAPDH and analyzed by the ΔΔCt method [11]. The student t-test was used to evaluate the differences between groups of samples.

Western blots

Detailed procedures have been described previously [7-9]. Briefly, anti-human antibodies against ALDH (BD PharMingen, CA), Shh, Notch1 and p63 (Millipore, Billerica, MA), and antibodies that cross-react with both human and mouse BCRP, bcl-2 (Santa Cruz, CA) and Bip/Grp78 (Millipore), as well as β-actin (Sigma-Aldrich, St Louis, MO) were diluted in blocking buffer (5% BSA in TBST) to appropriate concentrations and incubated with tissue blots (on PVDF membrane) individually. Following reaction with HRP-conjugated secondary antibodies, the signals were developed with enhanced chemiluminescence (ECL) substrate (GE Healthcare, Piscataway, NJ). The band densities were digitized using Bio-Spectrum AC Imaging System (UVP LLC, Cambridge, CA); the readouts were then normalized to β-actin and the differences among samples were analyzed with the student t-test.

Magnetic isolation of cancer stem cells

DLP lobes (including tumors, if present) were dissected from mouse prostates and cut into small pieces (1-2 mm^3). Following the company's instructions (StemCell Technologies, Vancouver, BC), the tissues were digested in collagenase/hyaluronidase solution for three hours at 37^{0}C while being gently shaken. When necessary, DNase I was used to hydrolyze DNA released from dead cells. After a brief wash in culture medium (5% PrEGM; Lonza, MD), cells were passed through a 40 μM cell strainer to obtain a single cell suspension; the isolated cells were counted with a hematocytometer. Single cell suspensions were then incubated with rat anti-mouse Sca-1 antibody-coated magnetic beads (StemCell Technologies) at the suggested ratio, and separated magnetically. Sca-1$^+$ cells were counted and the proportions of these cells in the total single cell population of DLPs were calculated.

Results

SLC in LNCaP xenograft tumors after castration

Castration effect on LNCaP tumors: Consistent with our previous study [7], castration resulted in cessation of LNCaP tumor growth. As shown in **Figure 1A**, this cessation only lasted about three weeks; tumors resumed growth after day-20 and doubled in size within the next 10 days. IHC showed a reduction in proliferating cells (Ki67$^+$) relative to intact mice (controls), but only during the first two weeks after castration, and a sharp increase was observed between day-15 and day-20 (**Figure 1B**). This indicates that active cellular events occur in LNCaP tumors during the day-15 to day-20 period, even though tumor volumes do not change during this time (**Figure 1A**).

Stem cell markers in xenograft tumors after castration: To determine whether SLC play a role in the re-initiation and acceleration of tumor growth in this animal model, tumors were investigated at different time points after castration (days 10, 15, 20, 25, and 30) for the expressions of stem cell markers. Tumors from intact mice on the same days were used as controls.

The common stem cell markers CD44, CD133, and c-Kit (CD117) were evaluated by IHC (n=5 per time point). The expressions of c-Kit and CD44, but not CD133, were significantly increased in tumors 15 days post castration (day-15); no differences were observed between tumors at other time-points post castration, or in control tumors at any time point (data not shown). Selected images of tumors at day-15 and day-30 are shown in **Figure 2A**. Although both CD44 and c-Kit were over-expressed in day-15 tumors, their distribution

patterns were different. Most of the CD44+ cells in the xenograft tumors were distributed in the periphery of tumor islands, while the c-Kit+ cells were within the tumor mass (**Figure 2A**). This suggests that the CD44+ cells and c-Kit+ cells represent different types of cells in LNCaP tumors; each might play a role in tumor progression.

An increase in CD44 expression was also detected by real-time PCR at day-15 after castration, but not at day-30 (**Figure 2B**). Similar to the IHC results, real-time PCR data did not show significant differences in CD44 mRNA levels among tumors from other time points after ADT and among the controls (data not shown).

The expression of SLC markers in tumor tissues at different time points (four or six tumors per time point) was also analyzed by western blots. Antibodies against human ALDH (aldehyde dehydrogenase), Shh (sonic hedgehog), p63, BCRP (breast cancer resistant protein), Notch1, bcl-2, and Grp78/Bip were employed. After normalization to β-actin, bands that were at least double the mean of the non-treated control samples were considered to be overexpressed (**Table 1**). Among the seven molecules tested, ALDH, Shh, p63, and BCRP were overexpressed at day-15 (4/4 or 6/6) after castration. Three of six tumors also expressed higher levels of ALDH at day-20. Bcl-2 and Notch1 were overexpressed in 2/4 (50%) and 4/6 (67%) tumor samples, respectively, at day-15. In contrast, similar expression levels of the above proteins were found at other time points and in controls. Grp78/Bip, a cell stress-related molecule, was highly expressed only in day-30 tumors (4/4). Representative western blots demonstrating the differences among tumors at day-15 and day-30 post-castration are shown in **Figure 3**. The overall results for protein expressions are listed in Table 1.

Thus, except for Grp78/Bip, all proteins tested by western blot analyses were highly expressed in most day-15 tumors, compared to the other time points and the non-treated con-

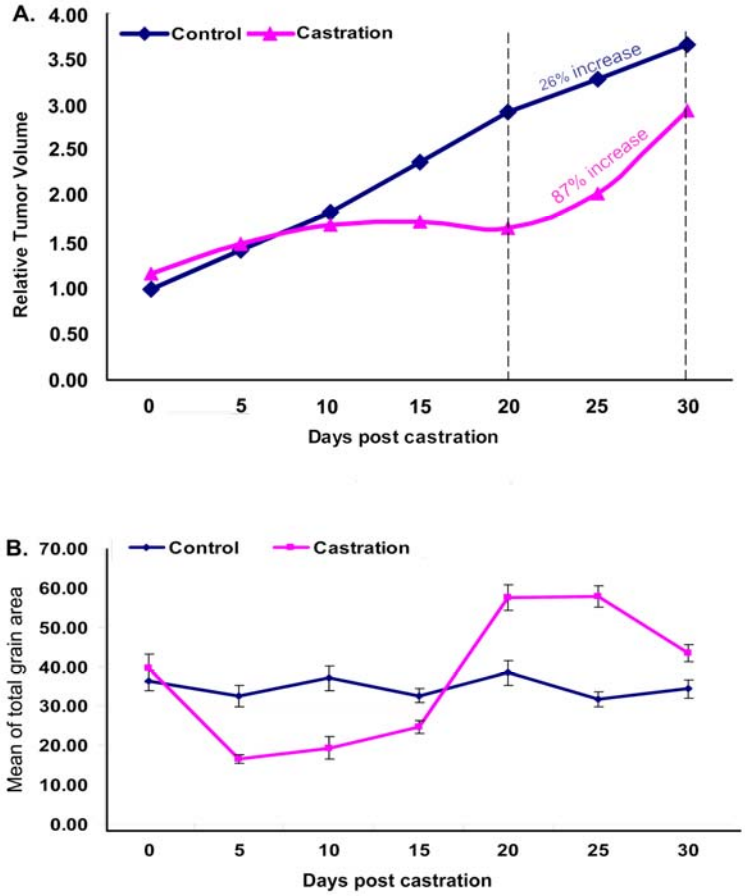


Figure 1. Response of LNCaP tumors to castration. **A.** Tumor volumes at different time points (5 mice for control and 10 mice for castrated groups at each time point). Mean tumor volume at each time point was normalized to the initial volume at day-0 (castration day). **B.** Ki67+ cells at different time points post castration. Three mice were evaluated per time point for both control and castration groups. Images (5-10/sample) were digitized using MCID7.0 software. A sharp increase in the number of proliferating cells was observed between day-15 and day-20 in castrated mice.

trols. Taken together with the tumor volume measurements and IHC results, our data suggest that SLC may play a role in tumor recovery from the initial treatment of ADT. Furthermore, around day-15, there seems to be a turning point for tumor re-growth and disease progression in the LNCaP xenograft model.

Table 1. Proportion of tumors with at least 2-fold increase in protein expression

	Day-5	Day-10	Day-15	Day-20	Day-25	Day-30
ALDH	0/6	0/6	6/6	3/6	0/6	0/6
Shh	0/6	0/6	6/6	0/6	0/6	0/6
Notch	0/6	0/6	4/6	0/6	0/6	0/6
p63	0/4	nd	4/4	nd	nd	0/4
BCRP	0/4	nd	4/4	nd	nd	0/4
bcl-2	0/4	nd	2/4	nd	nd	0/4
Grp78	0/4	nd	0/4	nd	nd	4/4

After normalization to β-actin, a band density at least double the mean of the non-castrated control samples in each reaction was considered to be an overexpression. nd: not done.

SLC in TRAMP model after castration

To further analyze the role of SLC in tumor recurrence, tumors from castrated TRAMP mice were also investigated.

Due to *PB-SV40 T(t)-Ag* expression in prostatic epithelial cells, 100% of untreated TRAMP male mice develop prostate cancers by the age of 12-wks [8, 10, 12]. In this study, none of the TRAMP mice at the age of 4-wks (n=4)

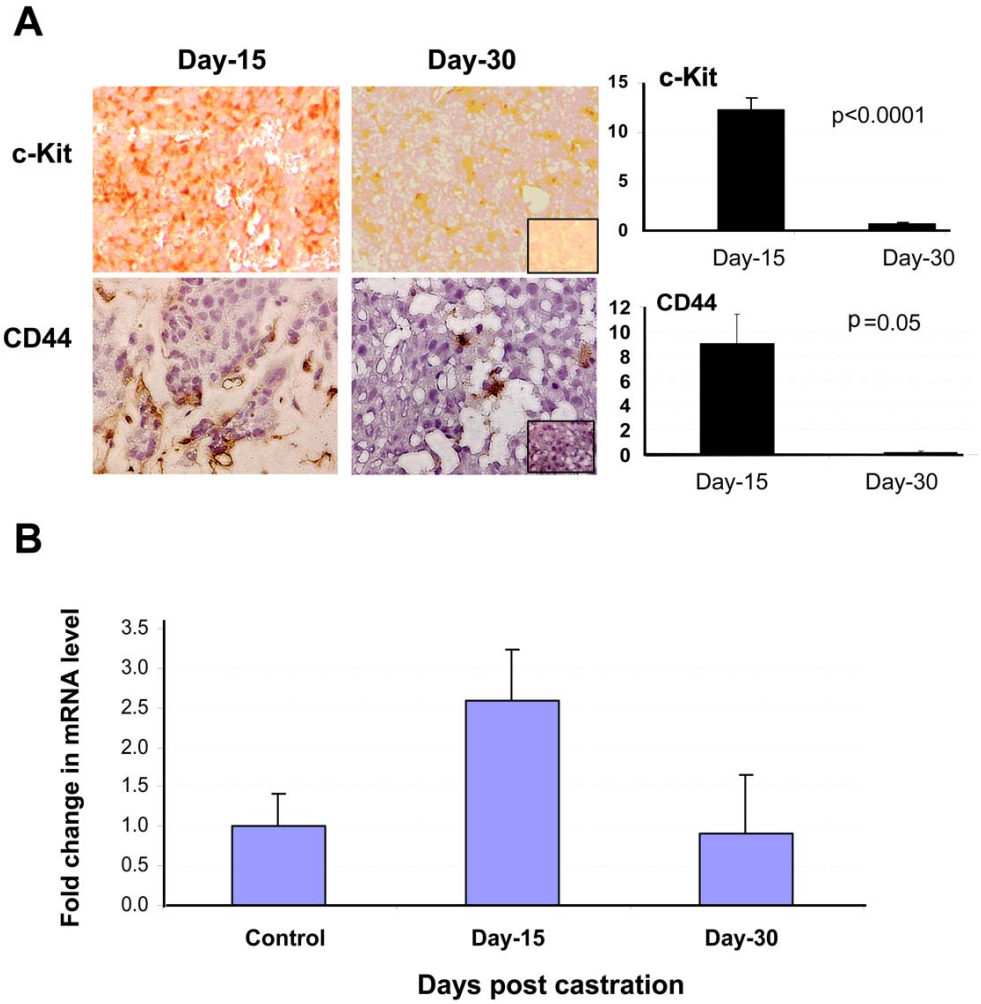


Figure 2. Expression of CD44 and c-Kit in LNCaP xenografts after castration. **A**. IHC. Cryosections of tumors from day-15 (n=5) and day-30 (n=5) time points were reacted individually with CD44 (200x) and c-Kit (100x) antibodies. Inserts represent negative controls. Images were digitized using MCID7.0 software and data analyzed with student t-test (right panel). **B**. Real-time PCR. CD44 gene expression in tumors at day-15 (n=3) and day-30 (n=3) relative to controls (n=3).

developed PCa by the age of 36-wks (end of the experiment). In contrast, all mice that were castrated at the age of 12-wks (n=10) developed PCa before reaching 36-wks of age (**Figure 4A**). Histopathology demonstrated that there were no neoplastic lesions in the prostates of 4-wk-old mice (n=4), but well- to poorly-differentiated tumors were observed in all prostates of 12-wk-old mice (n=4) (Figure 2B, H&E stain). The stem cell antigen-1 (Sca-1) was detected by IHC only in neoplastic lesions of the 12-wk-old prostate glands (Figure 4B). This suggests that Sca-1^{+} cells may play an important role in tumor initiation.

Stem cell marker expressions in TRAMP PCa after castration: We previously reported that TRAMP tumors respond to castration in two different ways [8]; for the SLC study, only those that responded positively to castration (tumor shrinkage) were used. The DLP lobes, which have the highest incidence of PCa de-

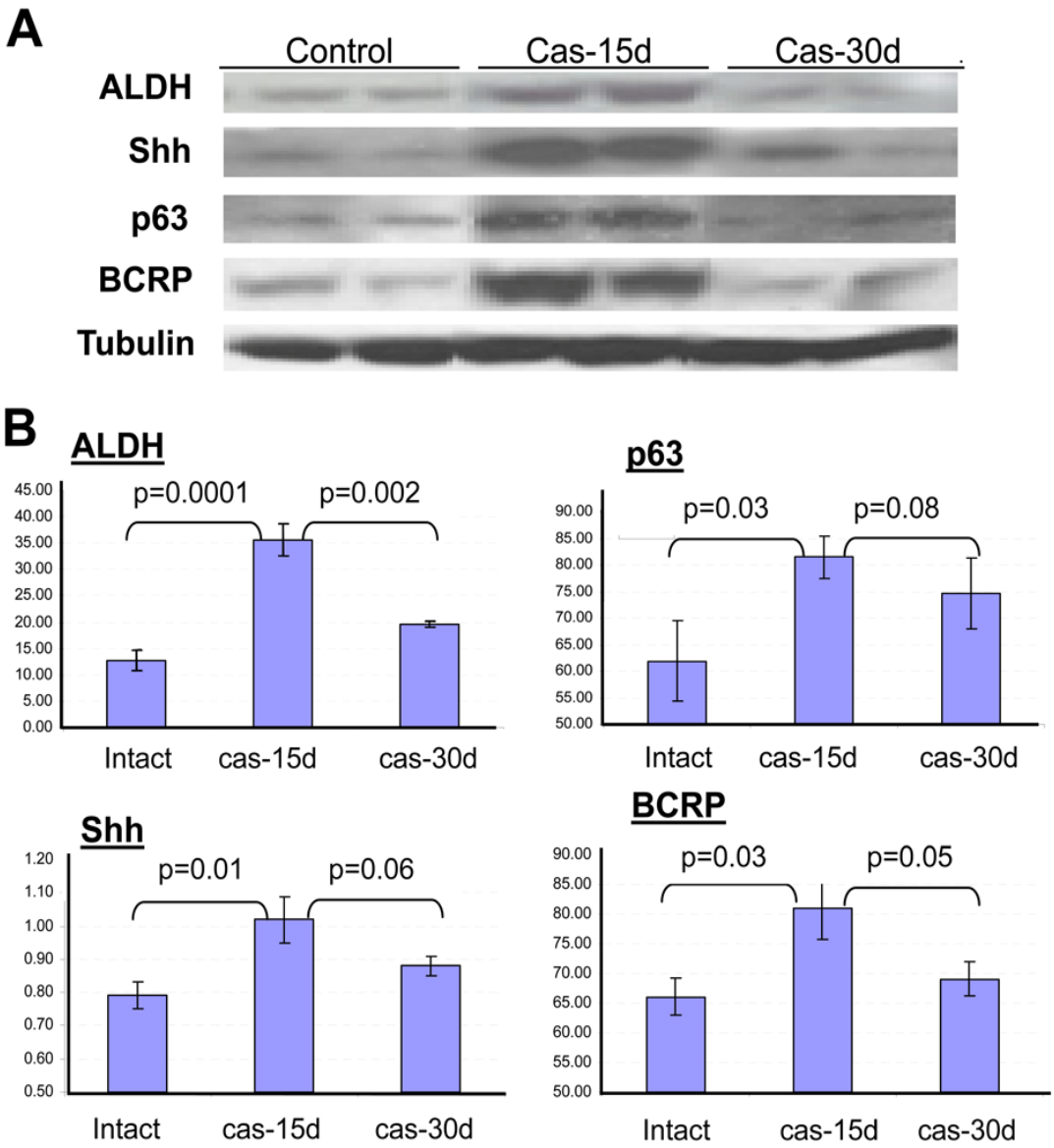


Figure 3. Stem cell related proteins in LNCaP xenografts after castration. **A**. Representative western blots of stem cell-related proteins in tumors at day-15 and day-30 after castration and in non-castrated controls. 30 μg of protein extracts were loaded per lane. **B**. Densitometry evaluation of western blot results. Bars represent results of 3 experiments ±S.D.

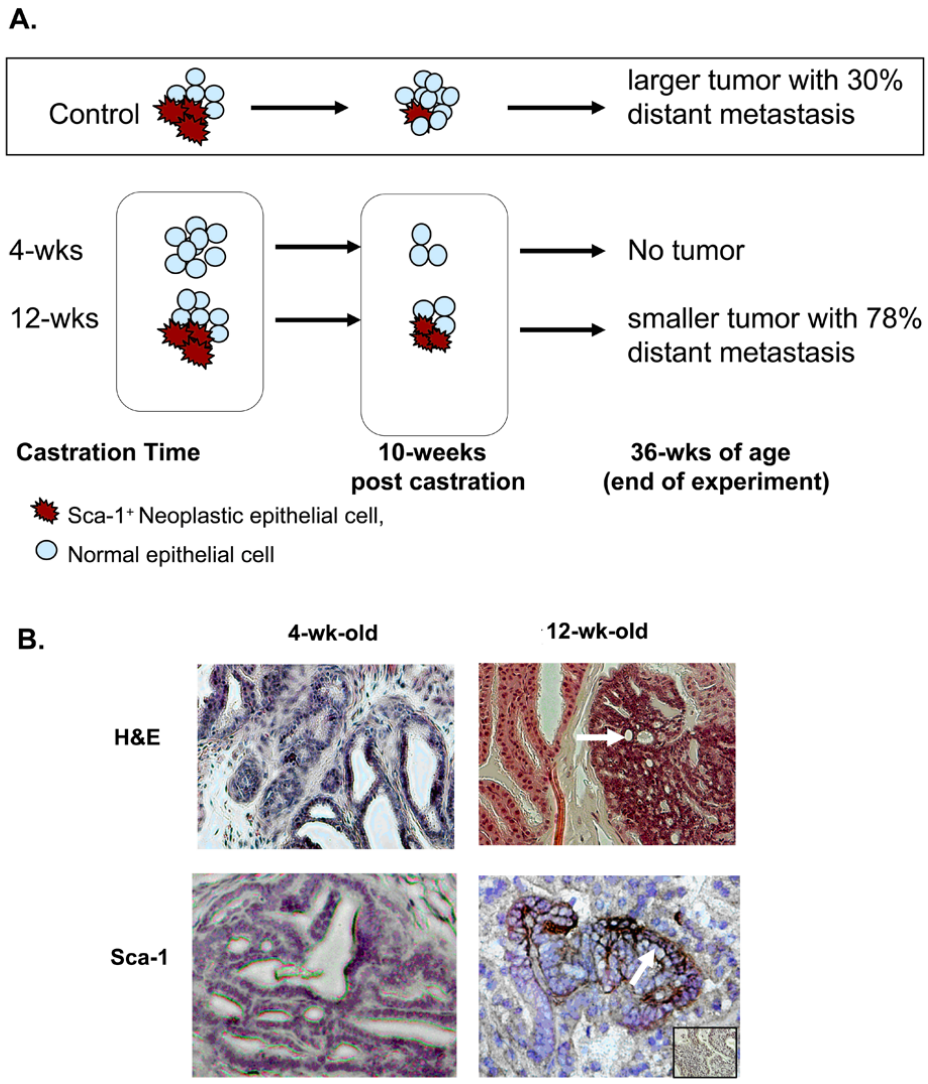
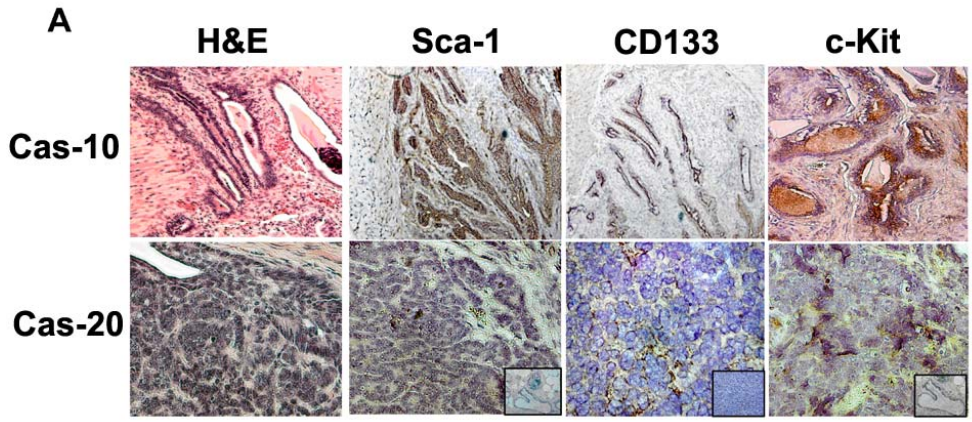


Figure 4. Comparison of TRAMP prostates at 4 wks and 12 wks of age. **A**. Representation of the impact of time at castration on tumor development in TRAMP mice. **B**. Upper panel. Histopathology (H&E stain; 100x) shows neoplastic proliferation in the prostate glands of 12-wk-old non-castrated mice (n=7) but not in non-castrated 4-wk-old mice (n=4). Lower panel. Immunostaining for Sca-1 (200x) shows that only tumor cells are Sca-1$^+$ in 12-wk-old prostates, while Sca-1 is not detected in 4-wk-old prostates. Insert box is negative control.

velopment in the TRAMP model [10], were dissected from mice castrated at 12-wks of age at two subsequent time-points: 10 wks (Cas-10; n=12) and 20 wks (Cas-20; n=9) after the surgery. Consistent with previous studies, most of the DLPs of Cas-10 mice (11/12) had regressed glands with no infiltrative neoplastic lesions, and the mice had no distant metastases by histopathology. In contrast, 7/9 (75%) Cas-20 mice developed poorly-differentiated PCa, and six (67%) had distant metastases to the lungs and/or livers. This result suggests that transition of tumors from repression by ADT to re-growth and progression occurred after 10 wks post castration.

To determine whether SLC have any impact on this transition, IHC staining for Sca-1, CD133, CD44, and c-Kit was performed on DLP tissues of Cas-10 and Cas-20 mice. Sca-1, CD133, and c-Kit were overexpressed in the luminal cells of prostate glands in Cas-10 DLP, but not



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	WT	Cas-10	Cas-20
Sca-1^{+} cells	1.3 x 10^{-2}	4.3 x 10^{-2}	0.06 x 10^{-2}

Figure 5. Expression of stem cell markers in TRAMP mice after castration.**A**.IHC. Representative prostates from mice 10 wks (Cas-10; n=5) and 20 wks (Cas-20; n=5) post castration immunostained for Sca-1, CD133, and c-Kit (100x). Insert boxes are negative controls.**B**. Western blots. 30 μg of protein extracts from prostate DLP lobes of Cas-10 (n=5) and Cas-20 mice (n=5) were analyzed for bcl-2 and Grp78 expression.**C**. The proportion of Sca-1+ cells in wild-type (wk 12; n=2), Cas-10 (n=2) and Cas-20 (n=2) mouse prostates. Sca-1+ cells were isolated with anti-Sca-1 antibody conjugated magnetic beads. The proportion of Sca-1+ cells was determined relative to the total number of single cells in suspension.

in Cas-20 DLP (**Figure 5A**). No differences were found in CD44 expressions (data not shown).

For western blot analyses, antibodies against mouse bcl-2, Grp78/Bip, and BCRP were used. The expressions of bcl-2 and Grp78 were significantly higher in Cas-10 compared to Cas-20 prostates (**Figure 5B**), while no differences were observed in the expressions of BCRP (data not shown).

Using anti-Sca-1 antibody-bound magnetic beads (StemCell Inc), Sca-1^{+} cells were isolated from the DLP lobes of prostates of TRAMP mice and age-matched wild type mice. The average proportion of Sca-1^{+} cells in Cas-10 prostates (n=2) was 4.3 x 10^{-2} (4.3%) of the total isolated cells, while the average in Cas-20 prostates (n=2) was only 0.06 x 10^{-2} (0.06%), which was 72-fold lower than the number in Cas-10 prostates and 21-fold lower than the wild-type controls (Figure 5C).

Discussion

Although SLC seem to be a special subset of tumor cells, newer studies suggest that they are a heterogeneous population with diverse biological properties, and that multiple populations with stem cell characteristics can co-exist in the same tumor [13-15]. In addition, there is currently no standard marker for identifying cancer stem cells (CSC) in tumor tissues. Based on these considerations, to de-

termine whether CSC play a role in tumor recurrence, we evaluated several stem cell-related cell surface antigens and intracellular proteins in LNCaP xenograft tumors and TRAMP PCa after ADT.

The markers evaluated in this study have been frequently used in stem cell and CSC studies.The cell surface antigens CD44, c-Kit, CD133, and Sca-1 (mouse-specific) have been reported to be overexpressed in prostate cancers [16-21]. The cellular proteins that were investigated in the tumor tissues after ADT were all related to stem cells or the "stemness" condition. It has been reported that ALDH plays an important role in the early differentiation of stem cells [22]. Higher ALDH activity has been shown in murine and human hematopoietic and neural stem and progenitor cells [23-26], as well as in normal and malignant human mammary stem cells [27]. Strong expression of ALDH is associated with poor prognosis in patients [28]. Shh and Notch1 are key signal regulatory factors in embryonic development. They are both involved in early prostate development and duct differentiation [29-31]. Bcl-2 and p63 are two well-known proteins that are highly expressed in prostate basal cells [32, 33]; whether the basal cells are stem cells is currently not clear. The drug-resistant protein BCRP and the cell stress-related protein Grp78/Bip have also been reported to be overexpressed in stem cells and embryo cells [34, 35].

Varied results have been reported in the literature regarding the proportion of CSC in tumors. In our study, many stem cell markers increased as a function of time after ADT. In both human xenograft and transgenic TRAMP models, we observed significantly increased expression of stem cell markers mainly at relatively early time points post castration. In the LNCaP xenograft model, peak expression of most stem cell markers occurs at approximately two weeks after ADT. Around this time point, there continues to cessation in tumor growth, as evidenced by the stationary tumor volumes and minimal number of proliferating cells within tumors (**Figures 1A** and **1B**). After three weeks, when tumor growth started to accelerate, the number of SLC declined significantly (**Table 1** and **Figure 1A, 2**). A similar phenomenon was observed in TRAMP tumors, although tumor growth dynamics were more protracted in these mice (**Figure 5**).

Recent studies on other types of cancer have reported increased CSCs following chemotherapy [4, 5] or radiation [6], suggesting that cellular stress caused by anti-cancer therapies may be one of the inducers for the "stem cell phenomenon". That ADT can result in enhanced expression of SLC markers in two independent *in vivo* models of PCa extends these observations to include hormone ablative therapy as another potential inducer of "stemness". With regard to the origin of these SLC, there are two possibilities. Chemical or physiological alterations in the microenvironment due to anti-cancer therapies could induce some tumor cells back to the "stemness" condition to avoid cellular damage and death. Consistent with this notion, reversion of cancer cells to SLC *in vitro* has been reported [36, 37]. Alternatively, because anti-cancer therapies kill the bulk of the tumor cells, this might cause a change in the relative proportion of SLC in a tumor. With adaptation to a changed microenvironment, surviving SLC may then engage in self-renewal as well as "differentiation" towards the cancer phenotype, eventually resulting in tumor progression. At the early stages after treatment, as the cell population is enriched for SLC [37-39], peak expression of SLC markers would be observed. As more and more tumor cells arise from progenitor cells, the relative expression of stem cell markers would decrease and return to basal levels, consistent with our observations in both the LNCaP and TRAMP models.

In summary, our results demonstrate that ADT can induce a "stemness" condition in PCa, which might contribute to the survival of tumor cells in response to ADT, and provide a nidus for subsequent tumor growth and progression. These data also raise the possibility that a window for therapeutic targeting of SLC may exist for PCa after hormone ablation.

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