Published in final edited form as:

J Cell Physiol. 2014 September ; 229(9): 1170–1176. doi:10.1002/jcp.24546.

Dependence of Castration-Resistant Prostate Cancer (CRPC) Stem Cells on CRPC-Associated Fibroblasts

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Abstract

We previously established a role for cancer-associated fibroblasts (CAF) in enhancing the self-renewal and differentiation potentials of putative prostate cancer stem cells (CSC). Our published work focused on androgen-dependent prostate cancer (ADPC) using the conditional *Pten* deletion mouse model. Employing the same model, we now describe the interaction of CAF and CSC in castration-resistant prostate cancer (CRPC). CAF isolated from ADPC (ADPCAF) and from CRPC (CRPCAF) were compared in terms of their ability to support organoid formation and tumor initiation by CSC from CRPC (CRPCSC) in vitro and in vivo. CRPCSC formed spheroids in vitro and well-differentiated glandular structures under the renal capsules of recipient mice in vivo more effectively in the presence of CRPCAF compared to ADPCAF. Furthermore, whereas CSC with CAF from ADPC formed mostly well-differentiated tumors in our previous study, we now show that CRPCSC, when combined with CRPCAF (but not ADPCAF), can form aggressive, poorly-differentiated tumors. The potential of CRPCAF to support organoid/tumor formation by CRPCSC remained greater even when compared to 10-fold more ADPCAF, suggesting that paracrine factors produced specifically by CRPCAF preferentially potentiate the stemness and tumorigenic properties of the corresponding CSC. This apparently unique property of CRPCAF was notable when the CAF and CSC were grafted in either intact or castrated recipient mice. In both environments, CRPCAF induced in the epithelial compartment higher proliferative activity compared to ADPCAF, indicated by a higher Ki67 index. Factors released by CRPCAF to regulate CRPCSC may be targeted to develop novel therapeutic approaches to manage advanced prostate cancer.

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Androgen deprivation therapy through chemical or surgical castration is the standard of care for advanced prostate cancer. Despite initial tumor regression in response to androgen withdrawal, most tumors eventually relapse in a low-androgen environment that is normally insufficient for prostate epithelial cell growth. The recurrent cancer is known as androgen depletion-independent cancer (Roy-Burman et al., 2005; Collins and Maitland, 2006; Sharifi et al., 2006; Miki and Rhim, 2008) or more recently, castration-resistant prostate cancer (CRPC) (Dayyani et al., 2011; Ryan and Tindall, 2011; Grimm et al., 2012). It has been proposed that CRPC develops from cancer stem cells (CSC). These are rare cells that express low levels of the androgen receptor and do not depend on androgen for survival (Nikitin and Matoso, 2007; Oldridge et al., 2012). Dormant CSC are thought to escape hormonal therapy and, having self-renewal capability, they ultimately break loose and give rise to CRPC (Pienta and Bradley, 2006; Taylor et al., 2010). CSC reside in stem cell niches, where signals are provided by fibroblasts, vascular, and other cell types to facilitate survival and the self-renewing capability of the CSC (Calabrese et al., 2007; Yang and Wechsler-Reya, 2007). Cancer-associated fibroblasts (CAF) are known to play a role in CRPC (Clark et al., 2013; Sluka and Davis, 2013), and here we specifically assess the extent of dependence of CSC isolated from CRPC on CAF from homologous origin versus CAF from the primary tumor, that is, the androgen-dependent prostate cancer (ADPC).

The conditional *Pten* deletion mouse model recapitulates several aspects of human prostate cancer, including the development of primary adenocarcinoma, progression to micrometastases, tumor regression in response to castration, as well as recurrence of castration resistant disease (Wang et al., 2003; Liao et al., 2007). In the model in which luciferase expression is coupled to prostate-specific *Pten* deletion, tumor growth can be monitored non-invasively over time by bioluminescence imaging (Liao et al., 2007). Using this *cPten*^{-/-}*L* mouse model, we recently isolated CAF and CSC from ADPC and demonstrated the critical role that the fibroblasts played in supporting the abilities of the CSC to form spheroids in vitro and initiate glandular structures and tumors in vivo (Liao et al., 2010). In the present study, we investigated the influence of CRPCAF on CSC found in recurrent tumors of castrated *cPten*^{-/-}*L* mice. We also compared the influence of the CRPCAF to that of ADPCAF and found that those isolated from CRPC were more potent than those from ADPC in supporting CSC of homologous origin.

Materials and Methods

Animals

Cells employed in this study were isolated from a prostate cancer mouse model, *cPten*^{-/-}*L*, in which conditional *Pten* deletion in the prostate is associated with simultaneous activation of the luciferase reporter (Liao et al., 2007). For renal capsule transplantation assays, we employed either intact or castrated NOD/SCID mice purchased from NCI-Frederick, MD. Animals were maintained and handled in accordance with federal, state, and local guidelines mandated by the University of Southern California Institution Animal Care and Use Committee (Assurance Number: A-3518-01).

Isolation of prostate CSC

Cancer stem-like cells (CSC) were isolated from 12 to 14-month-old castrated *cPten*^{-/-}*L* mice that developed recurrent prostate cancer (Liao et al., 2007). Two to three prostate tumors were pooled, minced and digested into single cell suspensions overnight at 37°C in DMEM/F12 medium (Life Technologies, Grand Island, NY) containing 5% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), 0.5 mg/ml collagenase (Sigma, St. Louis, MO), 0.5 mg/ml hyaluronidase (Sigma) and 0.5 µg/ml DNase I (Sigma). The cells were filtered using a 40 µm strainer and stained using biotinylated Lin antibodies against CD31, CD45 and Ter119 (BD Biosciences, San Jose, CA), PE/Cy5-conjugated streptavidin (Biolegend, San Diego, CA), PE-conjugated Sca-1 antibody (Biolegend), and Alexa647-conjugated CD49f antibody (Biolegend). Lin⁻Sca-1^{high}CD49f^{high} (LSC^{hi}) cells were then isolated by fluorescence-activated cell sorting (FACS) using BD FACS Aria™ Cell Sorting System with BD FACSDiva™ software.

Isolation of cancer-associated fibroblasts

CAF were isolated as described (Yang et al., 2008) from 12 to 14 month-old *cPten*^{-/-}*L* mice that developed androgen-dependent (ADPCAF) or castration-resistant prostate cancer (CRPCAF), cultured in BFS medium (Yang et al., 2005) with or without testosterone, and used within 5–9 passages. Multiple sources of ADPCAF and CRPCAF were included in the in vitro and in vivo experiments.

In vitro spheroid formation assay

Culture conditions were adapted from published protocols (Lang et al., 2001; Xin et al., 2005, 2007). 10⁴ LSC^{hi} cells isolated from *cPten*^{-/-}*L* mice that developed CRPC were suspended in Prostate Epithelial Growth Media–PrEGM (Lonza, Walkersville, MD) and mixed with Matrigel (BD Biosciences) at 1:1 ratio in a total volume of 250 µl. The cells were then seeded in 24-well plates and co-cultured with 10⁴ CAF in PrEGM with 1% Charcoal-Stripped Serum (Gemini Bio Products). The CAF, derived from either androgen-dependent (ADPCAF) or castration-resistant prostate cancer (CRPCAF), were placed in cell-culture inserts (8.0 µm pore size) above the CSC in a manner that prevents direct contact between the two cell types. Half of the growth medium was replaced every 3 days. Spheroid number and size were determined after 14 days of culture at 37°C.

In vivo renal transplantation assay

10⁴ CRPC LSC^{hi} cells were mixed with either 10⁴ or 10⁵ ADPCAF or CRPCAF in 80 µl neutralized rat tail collagen type I (BD Biosciences) and cultured in BFS medium (Yang et al., 2005) with or without testosterone in a 12-well plate overnight at 37°C prior to engrafting under the renal capsules of either intact or castrated 8–12 week-old NOD/SCID male mice. Grafts were grown for 10–12 weeks before collected for analysis.

Histopathology

Kidney capsule grafts were collected from the NOD/SCID mice after a 10–12 week period and incubated in 4% paraformaldehyde overnight at 4°C and then washed twice in PBS for 30 min before storing in 70% ethanol. Fixed tissues were embedded in paraffin, and 5 µm

sections were mounted onto glass microscope slides, and stained with hematoxylin and eosin after removal of paraffin and rehydration (Zhou et al., 2006, 2007).

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as previously described (Adisetiyo et al., 2013). Androgen receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:200 dilution. Anti Ki67 antibody was purchased from Vector Laboratories (Burlingame, CA) and used at 1:400 dilution. Anti Vimentin antibody (1:200) was from Cell Signaling Technology (Boston, MA), and Cytokeratin 8 (TROMA-1) antibody (1:100) was from the Development Studies Hybridoma Bank (Iowa City, IA). Affinity purified biotinylated goat anti-rabbit IgG (H + L) and affinity purified biotinylated rabbit anti-rat IgG (H + L) were purchased from Vector Laboratories and used as secondary antibodies.

Proliferation index

Grafts recovered from renal capsules were sectioned and immunostained using Ki67 antibody. Areas containing glandular structures and three random areas of sections with poorly-differentiated tumor morphology were photographed at 400× magnification. Ki67 positive cells and total number of cells in each image were counted using Image J software and the proliferation index was calculated as the fraction of cells positive for Ki67.

Statistical analysis

We used an unpaired, two-tailed *t* test to make statistical comparisons between various samples. Each parameter was measured at least three times independently. *P*-value <0.05 was considered statistically significant.

Results

In this study, we compared CAF from ADPC versus those from CRPC in terms of their ability to support organoid formation from CRPC stem-like cells (CRPCSC) in vitro and in vivo. ADPCAF were isolated from prostate tumors that spontaneously developed in 12–14 month-old *cPten*^{-/-} *L* mice in the presence of androgen and CRPCAF were isolated from recurrent tumors that developed in *cPten*^{-/-} *L* mice 6–9 months post-castration (Liao et al., 2007). The CSC were isolated from the CRPC tumors based on absence of Lineage markers (CD31⁻, CD45⁻, and Ter119⁻, collectively known as Lin⁻) and high expression levels of stem cell antigen-1 (Sca-1) and the prostate basal cell marker CD49f (Fig. 1A).

First, we compared abilities of CRPCAF and ADPCAF to support spheroid formation by CRPC CSC in vitro. The Lin⁻Sca-1^{high}CD49f^{high} (heretofore LSC^{hi}) cells were suspended in Matrigel and placed at the bottom of 16 mm culture wells. Inserts containing CAF were placed above the LSC^{hi} cells and spheroid formation was assessed after 14 days. As shown in Figure 1B, the CSC formed 10-fold more spheroids in co-cultures containing CRPCAF as compared to ADPCAF (*P* < 0.05). Counted separately, the number of large spheroids (100 μm in diameter) was sixfold greater in the presence of CRPCAF compared to ADPCAF. LSC^{hi} cells cultured without CAF infrequently produced spheroids, and those that formed

were very small (Fig. 1B and C). These data suggest that CAF derived from both androgen-dependent and castration-resistant prostate cancer secrete signaling molecule(s) that enhance the ability of CSC from CRPC to form spheroids in vitro, with the CRPCAF-derived signal being remarkably stronger.

We next compared the in vivo effect of CAF from ADPC versus CRPC on the “stemness” of the CSC isolated from CRPC. The LSC^{hi} (10⁴) cells were mixed with an equal number of either ADPCAF or CRPCAF and engrafted under the renal capsules of intact male NOD/SCID host mice (Table IA). After ten to twelve weeks, the grafts were harvested, sectioned, stained with H&E and analyzed microscopically. Glandular structures were found in all four grafts where LSC^{hi} cells were combined with CRPCAF, with their number ranging from 9 to 21 per graft (Table IA). When grafted along with ADPCAF, the LSC^{hi} cells formed glandular structures (N = 11) in only 1 of 4 recipient mice.

To better model CRPC, we compared glandular structure formation in LSC^{hi}/CRPCAF versus LSC^{hi}/ADPCAF grafts in castrated NOD/SCID mice. As depicted in Table IB, glandular structures developed in 9 of 12 LSC^{hi}/CRPCAF grafts (2 < N < 16), but only in 1 of 11 LSC^{hi}/ADPCAF grafts (N = 1). Thus, compared to ADPCAF, CRPCAF possess a greater capability to stimulate CRPC LSC^{hi} cells in forming glandular structures in vivo regardless of the androgen level in the host environment.

The greater potential of CRPCAF compared to ADPCAF to support glandular structure formation may be due to the more abundant supply of paracrine factors that elicit this potential in the LSC^{hi} cells. We therefore asked whether 10-fold more ADPCAF would stimulate glandular structure formation by LSC^{hi} cells to levels similar to those seen with CRPCAF. As shown in Table IB, however, none of five grafts with 10⁴ LSC^{hi} cells and 10⁵ ADPCAF formed any glandular structures. These results suggest that the CRPCAF acquire distinctive properties that are absent or hardly present in ADPCAF. Thus, the potential of CRPCAF to support glandular structure formation by CRPC LSC^{hi} cells in vivo is far greater than that of ADPCAF.

We next examined the microscopic morphology of the glandular structures to assess whether LSC^{hi}/CRPCAF grafts were histologically different from LSC^{hi}/ADPCAF grafts. Whereas all glandular structures found in LSC^{hi}/ADPCAF grafts appeared well-differentiated (Fig. 2A,a,c), we observed in several LSC^{hi}/CRPCAF grafts a more aggressive, poorly-differentiated tumor morphology (Fig. 2A,e). In addition to the incidence of 20–25% LSC^{hi}/CRPCAF grafts with poorly-differentiated tumor morphology (Table I), the glandular structures in LSC^{hi}/CRPCAF grafts with well-differentiated morphology were generally larger (Fig. 2A,d) than those found in the LSC^{hi}/ADPCAF grafts. Furthermore, whereas the glandular structures in LSC^{hi}/ADPCAF grafts ranged from 24 to 160 μm in diameter, those found in LSC^{hi}/CRPCAF grafts ranged from 13 to 670 μm.

Poorly-differentiated, aggressive prostate cancer is characterized by co-expression of AR, epithelial (e.g., CK8) and mesenchymal cell markers (e.g., Vimentin). Indeed, IHC analysis of the LSC^{hi}/CRPCAF grafts with poorly-differentiated morphology demonstrated positive staining for AR (Fig. 2B,e), CK8 (Fig. 2C,e), and Vimentin (Fig. 2D,e). In contrast, the well-

differentiated glandular structures found in both LSC^{hi}/ADPCAF and LSC^{hi}/CRPCAF grafts displayed benign to prostatic intraepithelial neoplasia (PIN) morphology and expressed AR and CK8, with Vimentin expression primarily confined to the stromal compartment surrounding the glandular structures (Fig. 2B–D).

The higher incidence and number of glandular structures induced by CRPCAF compared to ADPCAF could be mediated by mitogen(s) secreted specifically by CRPCAF. We functionally tested the presence of such mitogen(s) by determining the Ki67 proliferation index in the glandular structures. Indeed, Ki67-positive cells were markedly more abundant in glandular structures within LSC^{hi}/CRPCAF compared to LSC^{hi}/ADPCAF grafts (Fig. 3A, b vs. a), and this was more apparent in the castrated hosts (Fig. 3A, d vs. c). The average Ki67 index in LSC^{hi}/CRPCAF glandular structures was four and twofold higher than the Ki67 index in the LSC^{hi}/ADPCAF glandular structures in castrated and intact hosts, respectively (Fig. 3B). There was no significant difference between the Ki67 indices of the well-differentiated glandular structures and the poorly-differentiated tumors found in LSC^{hi}/CRPCAF grafts recovered from castrated hosts (Fig. 3B).

Discussion

While the transition from ADPC to CRPC has been extensively investigated in terms of changes that occur in the epithelial compartment, less is known about alterations to the stromal compartment. In the present study, we show that CAF from CRPC are far more potent than their ADPC counterparts in inducing CRPC CSC to form spheroids in vitro (Fig. 1) and glandular structures in NOD/SCID mice (Table I). The high potency of the CRPCAF to induce organoid formation is attributable to their higher mitogenic activity upon the epithelial compartment as compared to ADPCAF (Fig. 3). Furthermore, whereas well-differentiated glandular structures were formed in the presence of both ADPCAF and CRPCAF in vivo, only CRPCAF supported the development of aggressive poorly-differentiated tumors (Table I, Fig. 2).

The enhanced ability of CRPCAF to support formation of glandular structures and poorly-differentiated tumors was evident in both intact and castrated recipient NOD/SCID mice. The experiments using castrated mice best model CRPC, which develops under low androgen conditions. Similar observations made in intact mice suggest that the stronger influence of CRPCAF compared to ADPCAF on CRPC LSC^{hi} cells is independent of androgen deprivation. It remains to be determined whether CRPCAF would remain more potent than ADPCAF when tested for their influence on LSC^{hi} stem-like cells isolated from ADPC. Although we did not directly compare LSC^{hi} cells from ADPC versus those from CRPC, it is important to note that in our previous work with LSC^{hi} cells and CAF from ADPC (Liao et al., 2010), we routinely observed well-differentiated glandular structures but did not encounter the more aggressive, poorly-differentiated tumors. It appears, therefore, that CRPCSC have the special potential to form aggressive, poorly-differentiated tumors, and that CRPCAF are required to manifest this potential. Possibly, co-evolution of CAF and CSC during the development of CRPC results in optimal cooperativity that gives rise to aggressive tumors when both compartments are derived from CRPC. Detection of both well-differentiated and poorly-differentiated lesions in grafts containing CSC and CAF from

CRPC (this paper) but only well-differentiated lesions in grafts consisting of the respective cells from ADPC (Liao et al., 2010) may suggest that the CRPCSC population is inherently more heterogeneous than the CSC from ADPC. These observations are novel, at least with respect to the tests conducted with the cells from the *Pten*-deletion model of prostate cancer, although their validity and generality remain to be determined in other model systems of prostate cancer and, more importantly, in human prostate cancer.

In conclusion, while changes in the epithelial compartment during the transition from ADPC to CRPC are well established, our results clearly demonstrate that parallel changes occur within the CAF. Co-evolution of the epithelial and stromal compartments during prostate cancer progression may be associated with the establishment of new cues that CSC received from CAF and possibly other cells within the tumor microenvironment. Next generation therapeutic approaches for the treatment of prostate cancer will likely include targeting of the interaction between CSC and CAF. The present work suggests that such approaches may have to target different mechanisms in ADPC and CRPC.

Acknowledgments

We would like to thank Lora Barsky and David Stanley from the USC Flow Cytometry Core for their assistance with FACS, Ryan Park and Lindsey Hughes from the USC Small Animal Imaging Core for bioluminescence imaging, the USC Department of Animal Resources for maintenance of the mice, as well as Gohar Saribekyan from the USC Stem Cell Core Facility and members of the Merrill, Roy-Burman, and Frenkel laboratories (especially Gillian Little) at USC for helpful discussions and for sharing reagents and expertise.

Contract grant sponsor: California Institute for Regenerative Medicine;

Contract grant numbers: TG2-01161.

Contract grant sponsor: National Institutes of Health;

Contract grant numbers: R01CA059705, R01CA113392.

Contract grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases;

Contract grant numbers: R01DK71122.

Abbreviations

ADPC	androgen-dependent prostate cancer
ADPCAF	androgen-dependent prostate cancer-associated fibroblasts
CAF	cancer-associated fibroblasts
CRPC	castration-resistant prostate cancer
CRPCAF	castration-resistant prostate cancer-associated fibroblasts
CRPCSC	cancer stem cells isolated from castration-resistant prostate cancer
FACS	fluorescence-activated cell sorting
LSC^{hi}	Lin ⁻ Sca-1 ^{high} CD49f ^{high}
PIN	prostatic intraepithelial neoplasia

Sca-1 stem cell antigen-1

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Fig. 1. CRPCAF are more effective than ADPCAF in supporting spheroid formation by CRPC LSC^{hi} cells in vitro. (A) Representative FACS data showing the isolation of LSC^{hi} population from castration-resistant murine prostate tumor. Lin⁻ cells were first isolated by removing Lin⁺ cells (CD31⁺, CD45⁺, and Ter119⁺), and were then further sorted to yield Lin⁻/Sca-1^{high}/CD49f^{high} (LSC^{hi}) cells based on high expression of Sca-1 and CD49f. (B) LSC^{hi} cells were seeded in matrigel and co-cultured with CRPCAF or ADPCAF on inserts placed above the wells for 14 days. Spheroids were enumerated as a whole or as those larger than 100 μm in diameter. * $P < 0.05$ for the difference between LSC^{hi}/CRPCAF and LSC^{hi}/ADPCAF co-cultures for both the total number of spheroids and those >100 μm. (C) Representative images of spheroids that were formed by LSC^{hi} cells co-cultured with CRPCAF as compared to LSC^{hi} cells cultured in the presence of ADPCAF or without any fibroblasts. Microscopic images were taken at 100× magnification. Bar, 100 μm.

Fig. 2. Histological analysis of LSC^{hi}/ADPCAF versus LSC^{hi}/CRPCAF grafts grown in vivo. LSC^{hi} cells isolated from CRPC tumors of *cPten*^{-/-} *L* mice were implanted along with a similar number of CAF from either ADPC or CRPC in the renal capsules of either castrated (c–e) or intact NOD/SCID mice (a–b). Grafts were analyzed after 10–12 weeks by H&E staining (A) and immunostaining for AR (B), CK8 (C) and Vimentin (D). Shown are representative images of well-differentiated glandular structures formed in both LSC^{hi}/ADPCAF grafts (*top row*) and LSC^{hi}/CRPCAF grafts (*middle row*) as well as poorly-

differentiated tumors that developed only in LSC^{hi}/CRPCAF grafts (*bottom row*). Microscopic images were taken at 400× magnification. Bar, 50 μm.

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Fig. 3.

Higher proliferative rates in LSC^{hi}/CRPCAF compared to LSC^{hi}/ADPCAF grafts. (A) Grafts isolated from intact and castrated NOD/SCID mice were stained for Ki67 to assess proliferation rate. Microscopic images were taken at 400× magnification. Bar, 50 μm. (B) Ki67 indices in well-differentiated glandular structures in LSC^{hi}/CRPCAF and LSC^{hi}/ADPCAF grafts, as well as in poorly-differentiated tumors in LSC^{hi}/CRPCAF grafts. For glandular structures, N = 11 except for the LSC^{hi}/ADPCAF grafts in castrated recipients, where N = 1 (because only one glandular structure was detected). For poorly-differentiated tumors, N = 3 for castrated recipients. Mean ± S.D.