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Calcitonin induces stem cell-like phenotype in prostate cancer cells

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Abstract

Stem cell-like-cancer cells are key drivers of tumor growth, metastasis, and relapse of cancer following remission. Prostate stem cell-like cancer cells isolated from human prostate cancer (PC) biopsies express CD44⁺/α₂β₁^{hi}/CD133⁺ cell surface markers and can self-renew *in vitro*. Expression of calcitonin (CT) and its receptor (CTR) is frequently elevated in PCs and activation of CT-CTR axis in non-invasive PC cells induces an invasive phenotype. We investigated whether CT-CTR autocrine axis induces stem cell-like phenotype in two PC cell lines. CT-CTR axis in these cell lines was activated by enforced expression of CTR. The cells were then examined for the changes in the expression of CD44 and CD133, collagen adherence, tumorigenic, metastatic and repopulating characteristics. The activation of CT-CTR axis led to a large increase in adherence to collagen and a remarkable increase of CD44 and CD133 in PC-3 and LNCaP cells. This was accompanied by a strong increase in tumorigenic, metastatic and repopulation properties of PC cells. However, the mutation of CTR-C PDZ-binding site in CTR almost abolished CTR-mediated increases in stem cell-like characteristics of PC cells. These results support an important role for CT-CTR axis in the progression of PC from localized cancer to an aggressive form, and a majority of proinvasive CTR actions may be mediated through its interaction with its partner protein at the PDZ-binding site. These results suggest that CT/CTR can serve as a valuable target to prevent the generation of stem-like PC cells.

Key Words

- ▶ Calcitonin receptor
- ▶ stem cell antigens
- ▶ tumor growth
- ▶ metastasis
- ▶ prostate cancer

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Introduction

Prostate cancer (PC) is the most frequently diagnosed cancer in men, exceeded only by lung cancer as the leading cause of cancer-related mortality among men (Ferland *et al.* 2010). Despite recent advances in the field, effective therapy for patients with locally advanced and/or metastatic disease is not effective (Shibata *et al.* 2013). Although androgen and its receptors are important for the maintenance and function of the prostate, other factors such as neuroendocrine secretions are known to contribute to the progression of PC to its androgen-independent form (Beltran *et al.* 2016). Recent evidence suggests the presence of a biologically distinct

subpopulation of prostate tumor cells, which possess stem cell-like properties such as the expression of stem cell antigens CD44 and CD133 and are described as cancer stem cells or cancer repopulating cells (CRCs) (Oktem *et al.* 2014a, b). These cells have proliferative potential to maintain tumor bulk and resist chemotherapy in order to repopulate the tumor and cause metastasis after cancer treatment (Reya *et al.* 2001). Moreover, these cells also display other phenotypes such as high levels of the α₂β₁ integrins, a potential to establish and maintain a prostate epithelium with secretory activity, higher invasive and self-renewal properties. Moreover, these cells can be

enriched by their strong affinity to specific ECMs such as collagen I, collagen IV or laminin.

Calcitonin (CT), a neuroendocrine peptide, and its receptor are frequently elevated in prostate cancer and the activation of CT-CTR autocrine axis in non-invasive PC cells has been shown to induce an invasive phenotype in multiple PC cell lines (Shah *et al.* 1992, 1994, 2008, Thomas & Shah 2005, Thomas *et al.* 2006, 2007b). CT activates multiple cellular events such as disassembly of tight junctions and adherence junctions, activation of Wnt- β -catenin signaling, cadherin switching, induction of epithelial-to-mesenchymal transition (EMT), and major changes in cell adhesion properties of PC cells to extracellular matrix (ECM) (Thomas *et al.* 2007a, Shah *et al.* 2009a). CTR expressed in the prostate gland is type II receptor, which is dually coupled to stimulatory GTP binding proteins G α s and G α q and stimulates cyclic-AMP- and phospholipase C-activated pathways (Shah *et al.* 1994, Chien & Shah 2001). Recent studies from this laboratory have shown that CTR contains a canonical PDZ-binding site in its cytoplasmic tail, and the site plays a critical role in CTR-mediated increase in invasiveness of PC cells and in the formation of distant metastases by orthotopically implanted PC cells in immunodeficient mice (Aljameeli *et al.* 2016). Our recent studies have shown that CTR-ZO1 interaction is critical for proinvasive and metastatic actions of CT in primary prostate cancers as well as multiple prostate cancer cell lines including LNCaP and PC-3 cells (Aljameeli *et al.*, 2016). Ability of CTR to interact with ZO-1 can be abolished by the removal of PDZ-binding site ESSA on CTR-C-tail by Δ ESS mutation. PC cell lines expressing mutant CTR (CTR- Δ ESS) do not display proinvasive characteristics and fail to grow to form tumors and distant metastases when implanted orthotopically in the prostates of nude mice (Aljameeli *et al.*, 2016). Since PC stem-like cells exhibit characteristics similar to those exhibited by PC cells with activated CT-CTR axis, the primary objective of the present study was to examine whether CT-CTR axis induces or increases the stem cell-like characteristics of PC cells and identify the potential mechanism associated with this CTR action. We fractionated PC cells with collagen I because the activation of CT-CTR axis in these cells induces the surface expression of the α 2-integrin subunit and rapid adherence to type I collagen (Thomas *et al.* 2007a, Sariisik *et al.* 2013). We then examined stem cell-like and oncogenic characteristics of PC-3 and LNCaP cell subfractions. The expression of stem cell antigens CD44, CD133 as well as their oncogenic characteristics

such as proliferation, migration, spheroid-forming ability and metastasis-forming abilities were examined.

Materials and methods

Materials

Antibodies for flow cytometry CD44, CD133 and α 2 β 1) experiments were purchased from Abcam, BD Transduction Laboratories (San Jose, CA, USA), Proteintech (Rosemont, IL, USA) and Bio-Rad respectively. Collagen I was purchased from Sigma-Aldrich. The working collagen solution was prepared by diluting the stock collagen solution 1:5 with tissue culture grade water. Culture dishes were coated with the diluted collagen solution under UV light inside the culture hood for at least 1 h.

Methods

Animals

Male balb/c nu/nu mice (6–8 weeks old) were purchased from Envigo and housed one per cage in micro-isolator units in a barrier facility on a high-efficiency particulate air (HEPA)-filtered rack under standard conditions of 12-h light/dark cycles, fed *ad lib* on a standard autoclaved laboratory diet, and quarantined for 1 week prior to their use in the study.

Surgical orthotopic implantation

The surgical orthotopic implantation was performed under ketamine/xylazine anesthesia as previously described (Chien *et al.* 1999, Shah *et al.* 2008) (Shah *et al.* 2009b). In brief, fast adherent and slow adherent cell suspensions of three, PC-3v, PC-3CTRwt and PC-3CTR Δ ESS cell lines (1×10^5 cells/20 μ L) were injected into the dorsal prostate, the mice were maintained on the laboratory diet *ad libitum* for up to 60 days, and were regularly monitored for tumor growth/metastasis with fluorography using Kodak 4000 MM imaging station (Eastman Kodak Company, Rochester) and killed within 60 days (Shah *et al.* 2008). Six animals per group were used in this group of experiments. This number was derived by using the power calculations for $P < 0.05$ as previously described (Dell *et al.* 2002, Charan & Kantharia 2013). The sample size was determined to test the hypothesis that fast adherent PC3-CTRwt cells will form significantly larger prostate xenografts along with distant metastases as compared to those produced by PC-3v or PC3-CTR Δ ESS cells. The animals were observed on a daily basis and killed by CO₂ inhalation when they met the following humane endpoint criteria: prostration,

skin lesions, significant body weight loss, difficulty breathing, epistaxis, rotational motion and body temperature drop. Several organs were collected, fixed and examined for tumor metastasis. At necropsy, primary tumor and other organs were harvested and weighed. The tissues were fixed in neutral buffered formalin and processed for H&E staining. The use of animals was necessary to evaluate tumorigenicity and metastasizing ability of cell lines expressing wild type or mutant CTR. Killing was performed by trained investigators. All animal procedures were conducted strictly under the protocol approved by the Institutional Animal Care and Use Committee at University of Louisiana at Monroe. These procedures are in accordance with the principles and procedures outlined by the NIH.

Cell culture

PC-3 and LNCaP cells were obtained from ATCC and maintained as recommended by the provider (Shah *et al.* 2009b). PC-3 cells express CT but lack CTR. We activated CT-CTRaxis in PC-3 and LNCaP cells by enforced expression of either CTR-wt or CTR-ΔESS, a mutant CTR that lacks the PDZ-binding site as described recently (Aljameeli *et al.* 2016). PC-3 cells were stably transfected with the carrier plasmid without CTR insert. The cell lines and modified sublines were used only up to passage 12. Primary cell lines were used within 6 months of their purchase. Therefore, PC-3 and PC-3CTR cell lines were authenticated by STR profiling at the John Hopkins University Medical Center (Thakkar *et al.* 2016). The sublines were used immediately after the stable transfection.

Western blotting

The lysate fractions of PC-3 and LNCaP sublines expressing either control plasmid or expressing CTR-wt or CTR-ΔESS were boiled for 5 min in 2 Laemmli solution containing 20 mM dithiothreitol, and 50 g of protein was loaded per lane of 10% SDS-polyacrylamide gel, and fractionated proteins were electrically transferred to a nitrocellulose membrane. The blots were incubated with appropriate antisera as described under 'Results' for 18 h at 4°C, followed by appropriate horseradish peroxidase-conjugated secondary antisera. The immune complexes were visualized on chemiluminescence radiography film using Western blot ECL detection system. The blots were then washed and reprobed for β-actin. The same experiment was repeated two more times. The density of the bands was semiquantitatively estimated by densitometry.

Fractionation of PC-3 and LNCaP cells on the basis of collagen adherence capacity

PC cells were fractionated into fast adherent, slow adherent and non-adherent cells based on their ability to adhere to collagen within 5 min, 20 min or longer (Fig. 1A). The cells were seeded on collagen I-coated plates for 5 min. The adhered cells were used as fast adherents. The cells that did not adhere to collagen after 5 min were harvested and reseeded on a new collagen-coated plate for 20 min. This fraction of adhered cells was used as slow adherent cells. The cells that did not adhere to collagen after 20 min were considered as non-adherent cells (Fig. 1A). All cell lines used for the fractionation were from passages six or earlier.

Flow cytometry

After fractionation, the cells were washed twice in PBS, and labeled with CD44, CD133 and α2β1 antibodies for 45 min on ice in dark. The cells were then washed and stained with FITC-conjugated secondary antibodies for 45 min and analyzed using FACSCalibur flow cytometer. Data acquisition and analysis were done using CellQuest2™ software (BD Biosciences).

Immunocytochemistry

To check whether both CD44 and CD133 are present on the surface of same cells, we immunolabeled collagen-fractionated PC-3, PC3-CTRwt and PC3-CTRΔESS cells with primary antibodies (mouse Anti-CD44, BD Transduction Laboratories; rabbit Anti-CD133, Proteintech) for overnight at 4°C. The appropriate secondary antibodies conjugated with fluorophores FITC or TRITC were applied. After washes, the slides were observed under Nikon Optiphot-2 microscope equipped for epifluorescence. The images were captured with Retiga 1300 camera connected to an iMac computer loaded with the iVision image analysis program (Biovision Technologies, Exton, PA, USA). All antibodies were characterized for specificity and cross-reactivity as described earlier.

Image analysis and interpretation

Approximately ten different microscope fields of each ICC chambers were photographed, and the images were quantitatively analyzed using iVision Image Analysis program. The images were then analyzed for co-localization of CD44 and CD133, as well as for quantification of immunostaining.

Co-localization of CD44 and CD133 in labeled cell population was checked using co-localization module of

Figure 1

Fractionation of PC-3 cells. (A) Experimental protocol for fractionation of PC-3 cells on the basis of collagen adherence. (B) Percentage of PC-3 cells separated from total cells separated in each stage of fractionation. The results are expressed as mean $\pm$ s.e.m. of percent of cells fractionated at each stage of eight separate experiments. (C) Percentage of LNCaP cells separated from total cells separated in each stage of fractionation. The results are expressed as mean $\pm$ s.e.m. of percent of cells fractionated at each stage of eight separate experiments. (* $P < 0.001$ (one-way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)). (D1) CTR immunoreactivity in plasma membranes of PC-3 and LNCaP cell lines expressing either CTR-wt or CTRESS (50 μ g protein/lane) was determined by Western blot analysis. The blot was reprobed for β -actin for normalization. Representative of three separate experiments. (D2) Optical density of CTR and actin bands on each blot was obtained by image analysis on Kodak 4000 MM Image station. The figure presents the group data (ratio of CTR OD/actin OD $\pm$ s.e.m. of three blots).

iVision program. The program evaluated co-localization of both fluorescent dyes and calculated Pearson's co-efficient (maximum being 1.000).

For quantification of CD44 and CD133 immunostaining, the images were (positive or negative) scored by two individuals independently using established methods and the mean reading was taken (Thakkar *et al.* 2013). The staining intensity was assigned an arbitrary value, on a scale of 0–3 as follows: (–), 0; (+/–), 0.5; (+), 1; (++) 2; and (+++), 3. An ICC index for each sample was calculated by multiplying staining intensity with the percentage of positive cells. The results were graded from 0 (negative) to 300 (all cells display strong staining intensity). Reproducibility of the analysis was verified by rescoring of randomly chosen slides. Duplicate readings gave similar results.

Statistical analysis

Statistical calculations were performed using Prism 5 computer program (GraphPad Software). Results are generally expressed as mean IHC index $\pm$ standard error of the mean (s.e.m.) unless otherwise stated. $P < 0.05$ was

considered to indicate a statistically significant result. One-way ANOVA and t -tests were used to compare CD44/CD133 ICC index across the cell fractions.

Immunohistochemistry

Paraffin-embedded specimens were deparaffinized, hydrated, subjected to antigen retrieval by heating the slides for 5 min in 5 mM sodium citrate. The sections were then stained for prostate stem cell antigen (PSCA) as previously described (14). Incubations with primary antibodies were followed by FITC-conjugated secondary antibodies. The slides were then counterstained with DAPI.

Controls

Tissue sections were incubated either in the presence of no primary antibody, or no secondary antibody.

MTT assay

The proliferation of PC cells was assessed by MTT assay using kit purchased from American Type Culture Collection.

Exponentially growing cells were plated in a 96-well plate at a density of 8×10^3 cells per well and cultured for 24 h. The complete growth medium was then replaced with 100 μ L of basal incubation medium (RPMI 1640 containing 0.1% BSA, 10 mM HEPES, 4 mM L-glutamine, 100 IU/mL penicillin G, and 100 g/mL streptomycin), and the culture was continued at 37°C under 5% CO₂ for 24 h. The cells were then treated with MTT solution at 37°C for 4 h, and the color reaction was stopped with the stop solution (100 L/well). The incubation was continued until the formazan product was completely dissolved. Absorbance of the samples was read at 595 nm with an ELISA plate reader (Bio-Rad Laboratories, Inc.).

Wound-healing assay

The cells were plated in eight-chamber cell culture slides until they were 80% confluent. Wound was formed by a single scratching of 20 μ L pipette tip on a cell layer. The culture was continued overnight in starvation medium (basal incubation medium). Next day, the migrated cells were counted under the microscope and photographed.

In vitro invasion assay

PC cells (40,000 is optimum) were plated in serum-free media on Matrigel-coated transwell inserts (Corning, Bedford, MA, USA) with the lower chambers containing growth media with 10% FBS. After 24 h, the cells were washed with PBS. The invaded cells (on the outside bottom of transwell insert) were stained using Diff Quick stain kit. The stained cells were counted under the microscope.

Soft Agar clonogenic assays

Soft agar assays were performed as described previously (Thomas *et al.* 2006). An underlay of 0.5% agar in RPMI 1640 containing 5% fetal calf serum was prepared by mixing equal volumes of 1% agarose and 2 RPMI 1640 plus 10% fetal calf serum. Two milliliters of this mixture were pipetted into the wells of six-well plates and allowed to set. Cells (5×10^3) were seeded in each well of a six-well culture dish containing 0.3% top low-melt agarose. The agarose was allowed to set, and the plates were incubated in a humidified chamber at 37°C for 14 days. Colonies were counted in a blinded manner using a 10 objective on a Zeiss inverted microscope (Carl Zeiss). Colonies with a diameter larger than 50 μ m were scored. Data are expressed as average number of colonies formed as well as the average diameter of colonies.

Spheroid formation assay

Spheroids were prepared from single cell suspension of prostate cell lines as described before (Thomas *et al.* 2007a). In brief, 5×10^4 per mL cells in RPMI 1640 serum-free medium were placed on 96-well low-attachment tissue culture plates. The plates were rocked on gyrotory shaker in a CO₂ incubator at 37°C for 2 days, at the end of which the spheroids measuring 150–300 μ m in diameter ($\sim 4 \times 10^4$ cells/spheroid) were formed. Spheroids greater than 50 μ m were counted.

Results

Fractionation of PC cell lines on the basis of collagen adherence

Collagen adherence of PC-3 cells was remarkably increased following the expression of CTR-wt, and to a smaller extent with CTR- Δ ESS (Fig. 1B). Among different fractions, only 20% of PC3-v cells were fast adherents. This increased to almost 80% for PC3-CTRwt, but was about 50% for PC3-CTR Δ ESS. A significant decline in non-adherent PC3 cell subpopulation was observed. This fraction consisted of almost 35% of PC3-v cells, but only 5 and 15% of cells expressing CTRwt and CTR Δ ESS respectively. LNCaP cell subfractions also exhibited very similar characteristics (Fig. 1C). To ensure that the differences in adhesion of cells expressing CTR-wt or CTR- Δ ESS were not due to the differences in relative levels of CTR in these sublines, we analyzed CTR immunoreactivity in all PC-3 and LNCaP cell sublines by Western blot analysis. The results of Fig. 1D show that CTRwt and CTR- Δ ESS levels in respective PC-3 and LNCaP cell lines were relatively very similar, which suggests that the differences in adhesive properties of PC-3 and LNCaP sublines were entirely due to the differences in the functional properties of CTR-wt and CTR- Δ ESS.

Effect of CT-CTR axis activation on the expression of stem cell antigens

Recent studies have shown that stem cell antigens CD44, CD133 and $\alpha 2\beta 1$ are associated with tumorigenic/adherent properties of PC cells. It has been shown that PC-3 cells express CT, but not CTR (Chigurupati *et al.* 2005). Therefore, the expression of CTR will activate CT-CTR autocrine axis. We examined the influence of CTR-wt and CTR- Δ ESS on the expression of stem cell antigens CD44 and CD133. We fractionated the cells on

the basis of their adherence to collagen 1. As expected, $\alpha 2\beta 1$ antigen expression of all fractions was high in both cell lines (Table 1). However, both cell lines displayed significant differences in the expression of CD44 and CD133 among these fractions.

The fast adherent fraction of PC-3 cells exhibited very low percentage of cells expressing CD44 expression (1.62%) and CD133 (0.8%). In contrast, the percentage of cells expressing CD44 (34.54%) and CD133 (38.23%) was very high in fast adherent PC3-CTRwt subfraction. Corresponding percentages of CD44- and CD133-positivity for PC3-CTRAESS subfraction was relatively moderate at about 14 and 17.15 respectively (Table 1; Supplementary Figs 1, 2, 3, 4, 5, 6, 7, 8 and 9, see section on [supplementary data](#) given at the end of this article). Similarly, fast adherent subfractions of LNCaP cells also displayed similar characteristics (Table 1, Supplementary Figs 10, 11, 12, 13, 14, 15, 16, 17 and 18). For example, CD44-positive cell population was only 1.88%, but increased to 38.12% when CTR-wt was over-expressed. The increase was very moderate (9.12%) when CTR- Δ ESS was expressed. The corresponding increases of CD133 expression in fast adherent LNCaP subfractions were 24.87% (CTRwt) and 0.81% (CTRAESS) as compared to 0.42% for the vector-control LNCaP cells (Table 1).

Among slow adherents, the profile of both cell lines was similar to that of fast adherents qualitatively but more moderate in extent. For example, only 3% of low adherent PC-3 cells expressed either CD44 and CD133. However, the expression of CTRwt or CTR- Δ ESS induced a subtle shift in the percentage of cell expressing these antigens. For example, CD44-positive cells increased from 3 to 12.94% when CTRwt was expressed. Corresponding increase in CD133 expression was slightly higher at 16.9%. Similarly, the corresponding numbers for CTR- Δ ESS cells were 14.9 and 8.9% respectively. These results again suggest that the expression of CTR induces the expression of stem cell antigens CD44 and CD133. However, the increase is remarkably smaller than that seen in fast adherents. In case of LNCaP subfractions, CD44 was expressed by 2.97% of LNCaP-vector control cells. This increased to 17.37% with CTR-wt expression, but decreased to 8.37% when CTR- Δ ESS was expressed. Similar changes were observed in CD133 as well (Table 1).

Interestingly, CTR Δ ESS, but not CTR-wt, caused a remarkable increase in CD44 and CD133 expression in non-adherent cell population of both cell lines.

Next, we examined whether same cells exhibit both stem cell antigens CD44 and CD133. We verified this by double ICC of collagen-fractionated PC cell lines.

Table 1 Expression of stem cell antigens

Percent CD44-positive cells	Fast adherents	Slow adherents	Non-adherents	Antibody controls
PC3v	1.62	3.18 ^a	4.61 ^{ab}	0.51 ^{abc}
PC3-CTRwt	34.54	12.9 ^a	1.72 ^{ab}	0.88 ^{abc}
PC-3-CTRAESS	14.11	14.94	24.93 ^{ab}	1.1 ^{abc}
Percent CD133-positive cells				
PC3v	0.8	3.0 ^a	4.82 ^{ab}	0.51 ^{abc}
PC3-CTRwt	38.23	16.09 ^a	2.26 ^{ab}	0.68 ^{abc}
PC-3-CTRAESS	17.15	8.90 ^a	23.50 ^{ab}	0.97 ^{abc}
Percent $\alpha 2\beta 1$ -positive cells				
PC3v	91.49	94.62 ^a	90.7 ^b	0.35 ^{abc}
PC3-CTRwt	97.21	86.18 ^a	28.4 ^{ab}	2.71 ^{abc}
PC-3-CTRAESS	86.87	87.07	81.64 ^{ab}	0.36 ^{abc}
Percent CD44-positive cells				
LNCaPv	1.88	2.97 ^a	4.79 ^{ab}	0.63 ^{abc}
LNCaP-CTRwt	38.12	17.37 ^a	5.49 ^{ab}	0.65 ^{abc}
LNCaP-CTRAESS	9.12	8.71	11.37 ^{ab}	0.37 ^{abc}
Percent CD133-positive cells				
LNCaPv	0.42	2.77 ^a	5.01 ^{ab}	0.46 ^{bc}
LNCaP-CTRwt	24.87	18.49 ^a	2.68 ^{ab}	0.29 ^{abc}
LNCaP-CTRAESS	0.81	4.13 ^a	10.15 ^{ab}	0.65 ^{bc}
Percent $\alpha 2\beta 1$ -positive cells				
LNCaPv	95.81	92.13 ^a	92.15 ^a	0.25 ^{abc}
LNCaP-CTRwt	94.03	92.21 ^a	57.98 ^{ab}	0.88 ^{abc}
LNCaP-CTRAESS	83.81	89.13 ^a	93.25 ^{ab}	0.25 ^{abc}

The percentile proportions depicting flow cytometry measurements over ten thousand events of all the four groups (control, fast adherents, slow adherents and non-adherents) from both the PC3 and LNCaP cell lines were individually compared with each other using Chi-squared test analysis recommended by Campbell and Richardson. The confidence interval was set at 95% and the statistical differences were denoted by ^acompared to control group, ^bcompared to fast adherent group, and ^ccompared to slow adherent group as significant for *P* values less than 0.05.

Figure 2

Co-expression of CD44/CD133 on surface of PC-3 cells. (A) Percentage of cells double-stained with CD44/CD133 antibodies. The results are mean $\pm$ S.E.M. of four separate experiments. (B) CD44/CD133 immunocytochemistry of PC-3 cell subfractions. Fast-, slow- and non-adherent fractions of PC-3, PC-3-CTRwt and PC-3-CTRAESS cells were double-labeled for CD44 and CD133 as described in 'Methods' section. The slides were photographed and their IHC index was calculated. The results are mean $\pm$ S.E.M. of three separate experiments. **Significantly different from its corresponding PC-3 subfraction. Level of significance: $P < 0.001$ (one-way ANOVA and Newman-Keuls test). For example, PC-3-CTRwt fast vs PC-3 fast; PC-3CTRwt slow vs PC-slow; PC-3CTRwt-non vs PC-3-non. ^Significantly different from PC-3 CTRwt fast or PC-3-CTRwt-slow. Levels of significance: $P < 0.01$ (one-way ANOVA and Newman-Keuls test).

Analysis of co-localization suggested that almost all cells co-localized CD44 and CD133, with Pearson's co-efficient of 0.93. The results of Fig. 2A suggest that the percentage double-labeled cells in collagen fractions of PC cells were comparable to those in Table 1, and raise a possibility that the expression of CTRwt enhance the adherence of PC-3 cells on collagen, also increase the expression of CD44 and CD133 in same cells. Moreover, we observed a similar increase in the IHC index suggesting the increase in the amounts of CD44/CD133 antigens per cell (Fig. 2B). CTR-ΔESS moderately increased collagen adherence as well as CD44, CD133 expression.

Slow adherent CTRwt cells and CTRΔESS cells also expressed higher CD44/CD133 expression as compared to non-adherents, but this increase was remarkably lower as compared to that observed in fast adherent fractions.

Effect of CTR-CTR axis activation on rate of cell proliferation

Earlier studies from this and other laboratories reported that the activation of CTR-CTR axis led to a moderate increase in the rate of cell proliferation (Thomas *et al.* 2006, Shah *et al.* 2009a). We reexamined this phenomenon in fractionated cells on the basis of their collagen adherence. The results of Fig. 3 show that all three subfractions of PC3 cells displayed a similar rate of cell proliferation. However, the expression of CTRwt caused a significant, two-fold increase in the rate of proliferation among fast adherent cells. No significant change was observed in other two cell fractions. CTRΔESS caused a smaller increase in cell proliferation of fast adherent cells. These results suggest that a subpopulation of PC3 cells displaying high collagen adherence are much more responsive to CT-CTR activation than the remaining subpopulations.

CT-CTR axis and tumorigenic potential, self-renewal and invasiveness

In a wound-healing assay, we observed that the fast adherent fraction of PC-3v cells showed much greater migration than the slow adherent fraction. CT-CTR activation by the CTR-ΔESS did not have any significant impact on overall migratory characteristics. In contrast, the activation of CT-CTR axis by CTRwt led to over a seven-fold increase in the migration of fast adherent fraction. The other two fractions also displayed remarkable increase in their migratory capacity (Fig. 3), demonstrating the importance CTR-C PDZ-binding site for this CTR action (Aljameeli *et al.* 2016).

Figure 3

Rate of cell proliferation of PC-3 cell subfractions: Effect of CTR. Fast-, slow- and non-adherent fractions of PC-3, PC-3CTR and PC3-CTRAESS cells were analyzed for cell proliferation by MTT assay as described in 'Methods' section. The results are expressed as mean $\pm$ S.E.M. of four separate experiments. * $P < 0.001$ (one-Way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)).

Figure 4

Migration of PC-3 cell subfractions: Effect of CTR. Fast-, slow- and non-adherent fractions of PC-3, PC-3CTR and PC-3CTRΔESS cells were analyzed for cell migration in a Matrigel™ invasion assay as described in 'Methods' section. The results are presented as (A) representative micrographs as well as (B) pooled data of four separate experiments (mean ± s.e.m.). * $P < 0.001$ (one-way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

Matrigel™ invasion assays also displayed very similar results (Fig. 4). CTRwt remarkably increased invasion of all subfractions, but fast adherents were most invasive. Once again, CTRΔESS did not have any stimulatory effect on invasion of all subfractions.

Stem cell-like cancer cells display the ability to form floating spheroids (known as colonospheres) in a serum-free defined medium. It has been suggested that this ability to aggregate in spheroids indicates the self-renewal capacity of stem cell-like cancer cells (Shaheen *et al.* 2016). We examined the capacity of PC-3 subfractions to form spheroids, and the influence of CTRwt and CTRΔESS in this process. Both, CTRwt and CTRΔESS increased spheroid formation of PC-3 cells. But CTRwt was significantly more potent than CTRΔESS (Fig. 5). Once again, fast adherent fractions displayed most self-renewal capacity as assessed by spheroid formation ability.

Cancer cells, but not normal cells, can grow in the absence of substratum or anchorage-independent growth (Thomas *et al.* 2006). Growth in soft agar in the form of colonies is also reflective of their tumorigenicity, that is, their ability to invade and grow in unnatural environment and form metastases. We examined this property or clonogenicity of PC-3 subfractions. The ability of PC-3 cell subfractions to form colonies over agar was robustly increased only with the expression of CTRwt, but not CTRΔESS (Fig. 6).

Orthotopic tumor growth and formation of distant metastases in nude mice

Next, we examined whether fast adherent cells are more tumorigenic and aggressive than slow adherents and whether CTRwt and CTRΔESS have an influence on their tumorigenicity and metastatic capacity. Fast adherent cells expressing CTRwt developed tumor within

15 days compared to 30 days for fast adherent PC-3 cells without CTR. Moreover, fast adherent PC-3 CTRwt cells showed significantly larger mass of prostate tumor as compared to slow adherent PC-3CTRwt cells as assessed by wet weight of the prostate at necropsy (Fig. 7A).

Figure 5

Invasion of PC-3 cell subfractions: Effect of CTR. Fast-, slow- and non-adherent fractions of PC-3, PC-3CTR and PC-3CTRΔESS cells were analyzed for cell invasion in a wound healing assay. The results are presented as (A) representative micrographs as well as (B) pooled data of four separate experiments (mean ± s.e.m.). * $P < 0.001$ (one-way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

Figure 6

Spheroid formation by PC-3 cell subfractions: Effect of CTR. Fast-, slow- and non-adherent fractions of PC-3, PC-3CTR and PC3-CTRAESS cells were analyzed for their ability to form spheroid in defined conditions as described in 'Methods' section. The results are presented as (A) representative micrographs as well as (B) pooled data of four separate experiments (mean $\pm$ s.e.m.). * $P < 0.001$ (one-way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

Interestingly, the fast adherent PC-3-CTRAESS cells formed even smaller tumors than the corresponding fraction of PC3-v cells, further conforming our previous report that the CTR-C PDZ-binding site is critical for tumorigenic activity of CTR (Aljameeli *et al.* 2016). These results are further confirmed by H&E histology and PSCA immunofluorescence (Fig. 7B). The results demonstrate that prostates of mice implanted with PC3-CTRwt cells completely lost normal morphology as the tissue was completely taken over by tumor cells. This was evident in both, fast adherent and slow adherent, fractions of PC3-CTRwt-cells. In contrast, the prostates receiving either fractions, fast or slow adherents of PC3-CTRAESS cells, displayed normal morphology and occasional presence of dividing cells in a few pockets. PSCA expression was consistent with H&E histology.

Distant metastases

The presence of micrometastases in distant organs was examined by visualization as well as by histology and

PSCA immunofluorescence. The frequency and extent of micrometastases formed by different cell fractions in distant organs are presented in Tables 2 and 3. The results demonstrate that fast adherent cells expressing CTRwt were most aggressive in forming distant metastases, and their most favored target organs were kidneys, lungs and femur (Fig. 8). Fast adherents expressing CTR Δ ESS were much less aggressive and formed much smaller and much less frequent metastases in lungs, femur and kidneys. These results were further corroborated by H&E histology and PSCA staining of those organs (Fig. 9A and B). Tables 2 and 3 describe the frequency and extent of micrometastases in host organs.

Discussion

Present results have shown that fast adherent PC cell lines expressing CTRwt displayed several characteristics attributed to stem-like prostate cancer cells such as high expression of stem cell antigens CD44 and CD133,

Figure 7

Clonogenicity of PC-3 cell subfractions: Effect of CTR. Fast-, slow- and non-adherent fractions of PC-3, PC-3CTR and PC3-CTRAESS cells were analyzed for clonogenic activity. The results are presented as (A) representative micrographs as well as (B) pooled data of four separate experiments (mean $\pm$ s.e.m.). * $P < 0.001$ (one-way ANOVA and Newman-Keuls test (PC-3CTRwt-fast vs all other PC-3CTRwt subfractions)). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

Table 2 Frequency of micrometastases in host organs.

Organs	PC3-CTRwt	Frequency of micrometastases		PC3-CTRΔESS	Number of animals
	Fast adherents	Slow adherents		Fast adherents	
Femur	4	1		1	6
Kidneys	6	2		0	6
Brain	3	0		0	6
Lungs	5	2		3	6

high proliferative, invasive, tumor-forming, metastatic and self-renewal activities. These results are consistent with our earlier results that the activation of CT-CTR axis induces an increase in tumorigenic and metastatic properties of prostate cancer cells, but further demonstrate that CT-CTR axis can also induce the expression of stem cell antigens in PC-3 cells (Sabbisetti *et al.* 2005, Shah *et al.* 2009a). Moreover, rapid adherence to collagen can be used to enrich a population of stem-like PC cells from androgen-independent PC cell lines.

Although the effect of CTR on the expression of stem cell antigens is direct or indirect remains to be investigated, present results show that CTRwt was much more potent than CTR-ΔESS for inducing the expression CD44 and CD133 in same cells. Our recent studies have shown that CTR contains a canonical PDZ-binding site-ESSA in its cytoplasmic C-tail (Aljameeli *et al.* 2016). We have also shown that when CTR is activated, its PDZ-binding site binds to the PDZ3 domain of tight junction protein zonula occludens-1 (Aljameeli *et al.* 2016). The binding of CTR with ZO-1 facilitates the phosphorylation of ZO-1 by PKA, which consequently leads to the disassembly of tight junctions (Thakkar *et al.* 2016). Deletion of the PDZ-binding site with ESS-to-AAA mutation in CTR-C tail does not affect CTR-Gαs interaction. The mutant receptor still activates adenylyl cyclase and subsequent signaling with equal potency as CTRwt. However, the mutation abolishes CTR-ZO-1 interaction; and as result, prevents CT-induced disassembly of tight junctions. We have shown that the binding of CTR-ZO-1 is critical for proinvasive and metastatic actions of CTR on PC cells (Aljameeli *et al.* 2016). Present results confirm these earlier findings, but also provide a new evidence that the mutant CTR-ΔESS was much less potent in increasing CD44/CD133 expression as well as collagen adherence of PC-3 cell subpopulations as compared to CTRwt.

CD44 is a major stem cell antigen and functions as a receptor for hyaluronan and many other extracellular matrix (ECM) components (Ghatak *et al.* 2010). It also serves as a cofactor for growth factors and cytokines. Thus, CD44 is a signaling platform that integrates cellular microenvironmental cues with growth factor and cytokine

signals and transduces signals to the membrane-associated cytoskeletal proteins or to the nucleus to regulate a variety of gene expression levels related to cell-matrix adhesion, cell migration, proliferation, differentiation, and survival (Iczkowski *et al.* 2006, Iczkowski 2010). Accumulating evidence indicates that CD44, especially CD44v isoforms, are critical players in regulating the properties of stem-like cancer cells including self-renewal, tumor initiation, metastasis, and chemo/radioresistance. Significantly, CD44 has been identified as a typical stem-like cancer cell surface marker, individually or in combination with other marker(s) such as CD24, CD133, and CD34 (Shi *et al.* 2010, Yan *et al.* 2015).

Present results from this laboratory, that CTRwt induced a remarkable increase in CD44/CD133 expression in PC cells, are consistent with our earlier studies that CT induces a five-fold increase in the mRNA expression of the variant CD44v7-10, which favors fibronectin binding (Iczkowski *et al.* 2006). Integrins are known to play an important role in modulating the ability of tumor cells to adhere to adjacent cells and to the ECM and in PC metastasis (Thomas *et al.* 2007a, Desgrosellier & Cheresch 2010). For example, when PC cells lose androgen sensitivity after androgen ablation therapy, the tumors become highly invasive and metastatic, an effect that is partly mediated via the α6β4 integrins and an intact p38 pathway (Bonaccorsi *et al.* 2000). It is likely that CD44, especially v7/v9 variant, plays a key role in adhesion/invasiveness of PC cells because the silencing of CD44v9 expression led to significant reduction in Matrigel invasion of PC-3 cells (Iczkowski *et al.* 2005). This possibility is further corroborated by the report that CD44⁺ population from xenograft tumors and cell lines has enhanced proliferative potential and tumor-initiating

Table 3 Micrometastases in host organs.

Organs	PC3-CTRwt		PC3-CTRΔESS	
	Fast	Slow	Fast	Slow
Lungs	+++	++	++	+
Kidneys	+++	+	+	+
Femur	+++	–	+	–
Brain	++	–	–	–

Figure 8

Distant micrometastases in nude mice. (A) Representative photomicrographs of H&E stained tissues of nude mice (as labeled) implanted orthotopically with fast/slow adherent PC3-CTR-wt and PC3-CTRΔESS cells (Magnification 400×). Presence of micrometastases is indicated by arrows. (B) The presence of micrometastases in distant organs presented in Fig. 9A was confirmed by PSCA immunofluorescence. (C) Mean area of micrometastases was measured over total microscopic optical field (40×) for fast/slow adherent PC3-CTR-wt and PC3-CTRΔESS cells and represented as percentile of total area. * $P < 0.05$. Fast adherent (PC3-CTR-wt vs PC3-CTRΔESS); # $P < 0.05$ slow adherent (PC3-CTR-wt vs PC3-CTRΔESS); one-way ANOVA and Newman-Keuls test. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

Figure 9

Tumorigenicity of PC-3 cell subfractions: Effect of CTR. (A) Orthotopic tumors formed by PC-3v, PC-3CTRwt and PC-3CTRAESS cells in nude mice. PC-3 cells were transfected either with vector (v), CTR-wt or CTR-ΔESS constructs. The cells (1×10^6) were orthotopically implanted into the prostate of nude mice ($n = 6$ per group). Animals were killed 8 weeks after implantation, and prostate tumors were weighed (A). * $P < 0.01$ (PC-3v vs PC-3CTRwt); ^ $P < 0.01$ (PC3CTRAESS vs PC-3CTRwt); one-way ANOVA and Newman-Keuls test. (B) Representative photomicrographs of H&E stained prostate sections of nude mice implanted with fast/slow adherent PC-3-CTR-wt and PC-3-CTRAESS cells (Magnification 400×). The sections were also probed for prostate stem cell antigen immunofluorescence as described in Methods section. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0333>.

ability *in vivo* compared to CD44⁺ cells (Wang *et al.* 2013). The CD44⁺ cells are likewise AR⁺ and express higher mRNA levels of stemness genes such as *OCT3/4* and *BMI 1* (Lang *et al.* 2009).

CD133⁺ cells have been shown to display a more transient amplifying cell population phenotype with increased metabolic activity and proliferation, and a pro-inflammatory phenotype with high NFκB expression (Shepherd *et al.* 2008). Considering that PC-3CTRwt cells induced CD44/CD133 expression and also displayed significantly greater proliferation, invasion, tumorigenic, metastatic and repopulation activity, it is likely that CT may induce or activate these mechanisms through the induction of CD44/CD133 expression in PC-3 cells. However, CT has been shown to induce multiple signaling pathways such as Gαs, Gαq, Wnt/β-catenin as well as PI3/Akt/survivin pathways (Shah *et al.* 1994, 2009a, Thomas & Shah 2005). It is conceivable that the multiple effects of CT on proliferation, invasion, tumorigenicity, metastatic activity, chemoresistance and repopulation of tumor may be the outcome of the activation of multiple pathways as well as the induction of CD44/CD133. Our earlier studies have shown that the mutation of CTR-C PDZ-binding site is critical for GαS-mediated invasive, tumorigenic and metastatic activity of PC cells (Aljameeli *et al.* 2016). Present studies also demonstrate that the ΔESS mutation in CTR completely abolished CTR-induced invasiveness, tumorigenicity and metastatic activity of PC cells. However, PC-CTRAESS cells still retained their stimulatory effects on CD44/CD133 expression, collagen adhesion, cell proliferation and spheroid formation. Based on these results, it is possible to conclude that CTR-ZO-1 interaction-dependent junctional destabilization is

critical for invasive, oncogenic and metastatic activity. However, CTR-ZO-1 interaction may be only partly important for CD44/CD133 induction, cell adhesion, proliferation and spheroid formation. Based on the action of CT on CD44 mRNA expression, it is conceivable that CT-induced increase in the expression of CD44/CD133 precedes the increases in adhesion, proliferation and spheroid formation. Additional studies will be necessary to examine this phenomenon in more detail.

To summarize, present results have shown that the prevention of CTR-ZO-1 interaction by ΔESS mutation abolishes tumorigenic and metastatic actions of CTR. However, the mutation partially reduces the ability of CTR to induce CD44/CD133 expression, cell adhesion, proliferation and spheroid formation. Considering that CT plays a major role in the prevention of bone resorption as well as its direct effect on osteoclast function, present results that CT induces expression of stem cell antigens as well as other CRC characteristics of PC cells and our earlier results that CT is locally produced and significantly increases metastatic potential of PC cells by inducing EMT, raises a strong possibility that CT produced by metastatic PC cells may help cancer cells to colonize the bone tissue by increasing their cell adhesion and through induction of stem cell antigens.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0333>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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