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Prostate tumor cells with cancer progenitor properties have high telomerase activity and are rapidly killed by telomerase interference

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Abstract

Background—Cancer progenitor cells (CPC) have been postulated to promote treatment resistance and disease progression in prostate and other malignancies. We investigated whether the enzyme telomerase, which is active in cancer cells and in normal stem cells, plays an important role in CPC which can be exploited to neutralize these cells.

Methods—We used flow cytometry and assays of gene expression, clonogenicity and invasiveness to isolate and characterize a putative CPC subpopulation from freshly-resected human prostatectomy specimens. Telomerase activity was measured by qPCR-based Telomeric Repeat Amplification Protocol (TRAP). Telomerase interference was achieved by ectopic expression of a mutated telomerase RNA construct which reprograms telomerase to generate "toxic" uncapped telomeres. Treated cells were assayed for apoptosis, proliferation in culture, and xenograft tumor formation.

Results—CPC in prostate tumors expressed elevated levels of genes associated with a progenitor phenotype and were highly clonogenic and invasive. Significantly, CPC telomerase activity was 20 to 200-fold higher than in non-CPC from the same tumors, and CPC were exquisitely sensitive to telomerase interference which induced rapid apoptosis and growth inhibition. Similarly, induction of telomerase interference in highly-tumorigenic CPC isolated from a prostate cancer cell line abrogated their ability to form tumor xenografts.

Conclusions—Human prostate tumors contain a CPC subpopulation with markedly elevated telomerase activity which renders them acutely susceptible to telomerase interference. These findings offer the first tumor-derived and *in vivo* evidence that telomerase may constitute a CPC "Achilles heel" which may ultimately form the basis for more effective new CPC-targeting therapies.

Keywords

cancer progenitor cell; telomerase targeting; prostate cancer; cancer stem cell

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Introduction

Cancer progenitor cells (CPC) are self-renewing, highly tumorigenic cancer cells recently identified in a broad spectrum of malignancies and implicated in tumor formation, therapy resistance and disease dissemination [1-2]. In prostate, as in other cancers, various strategies have been developed to enrich and isolate CPC from tumors, including flow cytometry for specific cell surface markers [3-10], isolation of a side population [11], and formation of cell spheres [5] or holoclones [12-13]. In addition, cancer cell lines, human xenograft tumors [5-8,10] and mouse models [14-15] have also been commonly used to characterize prostate CPC. The mostly robust prostate CPC markers to emerge from this collective work have been integrin α2β1, CD44 and CD133 [3-10]; these cell surface antigens enrich for a subpopulation of prostate cancer cells with an embryonic stem-like gene expression profile and with high clonogenic, metastatic, and tumorigenic potential relative to marker-negative cells [3-4,6-10,16-17].

In light of CPC's high tumorigenicity and therapy resistance, it has been suggested that truly effective new cancer treatments should specifically aim to target this tumor subpopulation [1-2,18]; however, there are as yet no effective CPC-targeting treatments. Telomerase activity is a recognized hallmark of malignancy which has been explored extensively by our group and others for its therapeutic and biomarker potential [19-22]. Whereas benign, terminally differentiated tissues have extremely low levels of telomerase [23], malignant cells from a variety of cancers have significant telomerase expression and activity that correlate with malignant potential and high tumor initiating ability [24-26]. In addition to its role in cancer, telomerase recently has been found to play an equally important function in *normal* stem cell function, inducing stem cell activation in human and mouse epidermal, gastrointestinal, hematopoietic, neuronal, and reproductive niches [27]. Given its dual roles in carcinogenesis and stem cell activation, we speculated that perhaps telomerase activation plays an equally critical role in CPC and hence may constitute an attractive therapeutic strategy for targeting these cells.

To address this question, we investigated the relative telomerase activity and expression levels within cancer cell subpopulations isolated from freshly resected human prostate tumors and from prostate cancer cell lines. Specifically, we used FACS to isolate integrin $\alpha2\beta1^{high}CD44^{+}$ cells, previously reported to possess a cancer progenitor-like phenotype [3-4,6-7,28-29]. When isolated from prostate tumors in our studies, these cells expressed higher levels of self-renewal genes and were more clongenic and invasive *in vitro* than $\alpha2\beta1^{high}CD44^{-}$ cells from the same tumors. Similarly, when isolated from the DU145 prostate cancer cell line, $\alpha2\beta1^{high}CD44^{+}$ cells expressed CPC-like properties *in vitro*, as well as high tumor initiation *in vivo* compared to $\alpha2\beta1^{high}CD44^{-}$ cells. Remarkably, both in tumors and cell lines the putative CPC ($\alpha2\beta1^{high}CD44^{+}$) possessed markedly elevated levels of telomerase expression and activity compared with bulk non-CPC ($\alpha2\beta1^{high}CD44^{-}$). Therefore, we tested whether CPC were susceptible to telomerase interference, a therapeutic strategy that specifically exploits the presence of high telomerase activity [19,22,30-31]. Telomerase interference is a two-pronged approach consisting of: 1. telomerase RNA with a mutated template region (MT-hTer), and 2. siRNA against endogenous wild-type telomerase RNA. Ectopic co-expression of these two constructs from a single vector (MT-hTer/siRNA) effectively substitutes MT-hTer for wild-type hTer, thus reprogramming telomerase to encode incorrect telomeres. The altered "toxic" telomeres elicit a brisk DNA damage response and rapid apoptosis in cells with high levels of active telomerase. In the present study, MT-hTer/siRNA effectively reprogrammed the active telomerase of prostate CPC to induce rapid apoptosis and abrogate tumor initiation, thus underscoring the therapeutic potential of targeting CPC with telomerase interference.

Materials and Methods

Tissue collection, processing and cell culture

Prostate tumors freshly resected from prostate cancer patients at USC Norris Comprehensive Cancer Center were examined, inked, graded and staged by a pathologist in a de-identified, IRB-approved protocol. Cells were obtained as described previously [3-4] with minor modifications: Briefly, tissue was minced with scalpels and digested in DMEM/F12 (50:50 mix) media supplemented with 8.75 μg/ml liberase blendzymes 3 (Roche Applied Science, Mannheim, Germany) and 1μg/ml DNAse I (Invitrogen, Carlsbad, CA) overnight at 37 °C in a shaker incubator. Epithelial organoids were separated from the stromal fraction by unit gravity centrifugation and disaggregated into single cell suspension by incubation with trypsin/EDTA **(**Mediatech, Manassas, VA) for 5 min at 37°C. A portion of the cells were stained and analyzed by flow cytometry. The rest were plated on collagen coated plate (BD Pharmigen, San Diego, CA) and incubated at 37°C for 1 hr to enrich for an $\alpha_2\beta_1$integrin$^+$ cell population as described previously [3]. Non-adherent cells were verified to be $\alpha_2\beta_1$integrin$^-$ CD44$^-$ (Supplementary Figure 1B). The adherent cells were collected by incubation for 5 min with accutase (Innovative Cell Technologies, San Diego, CA) for FACS. Tumor cells were maintained on collagen coated plate in CnT52 (PCT Prostate Epithelium Medium, Low BPE (Human), Millipore, CA) at 37°C, 5% CO_2. To further molecularly validate the cancer identity of CPC, GSTP1 promoter methylation was confirmed in a subset of specimens and compared to adjacent benign tissue (Supplementary Table 1). Human prostate cancer cell line DU145 was cultured in RPMI 1640 with fetal bovine serum (FBS, 10%, Omega) at 37°C, 5% CO_2.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was used for determination of marker expression by FACSAria (Becton Dickinson Immunocytometry Systems). The following antibodies were used: integrin α2 (PE-CD49b) and FITC-CD44 (BD PharMingen, San Diego, CA); CD133/1 (allophycocyanin (APC) labeled; Miltenyi Biotec, Inc., Auburn,CA). Single cell suspension was obtained from freshly resected and digested prostate tumor samples as described above and resuspended in cell staining buffer containing 2% FBS and 5mM EDTA. Cells were stained with the above antibodies on ice for 20 min. Data were analyzed by FACSDiva.

Colony-forming assay and cell migration assay

To test clonogenicity, sorted cells were seeded on collagen coated plates, and colonies were counted after 21 days. To test invasiveness, cell migration assays were performed using Matrigel-coated 24-well inserts (BD Pharmigen, San Diego, CA) per manufacturer's instruction. Briefly, 10^4 cells were placed in the upper chamber with CnT52 medium while the lower chamber was filled with CnT52 containing 10 ng/ml SDF-1 (R&D systems, Minneapolis, MN). Non-migrated cells in the upper chamber were removed following fixation and staining of cells in the lower chamber. Migrated cells were analyzed using a Zeiss Imager.Z1 microscope with Axiovision software at 20X magnification.

RNA extraction, reverse transcription, and PCR

Total RNA was extracted by RNAqueous-micro Kit (Applied Biosytems Inc, Foster City, CA). First strand cDNA was synthesized using the RETROscript reverse transcription kit (Applied Biosytems Inc, Foster City, CA). PCR primers were described previously [7,32] and in Supplementary Table 2. GAPDH was used as internal control.

Quantitative PCR – Telomeric Repeat Amplification Protocol (qPCR-TRAP) and telomere length assay

Telomerase activity from cell extracts was analyzed using real-time PCR based telomeric repeat amplification protocol (TRAP) as described [33] and in Supplementary Table 3. Briefly, cells were lysed in TRAPeze® 1X CHAPS Lysis Buffer (Millipore, Temecula, CA). Cell lysate from 1000 cells was added for each reaction for each sample. The iQ5 optical system software version 2.0 was used to analyze the results. To determine telomere lengths, genomic DNA was extracted using Qiagen DNeasy Blood & Tissue Kit (Qiagen), and relative telomere lengths were analyzed in triplicate by real time PCR (Biorad MyIQ) as described previously using T and S primers [22].

Virus production and infection

Lentivirus was generated and cells were infected as previously described [19,30]. Briefly, 12 μg of lentiviral vector, along with 3 μg of pMD.G and 9 μg of pCMVdR8.91 plasmids were cotransfected into 293T cells by using the calcium phosphate co-precipitation method. Virus-containing medium was harvested 48 and 72 hr after transfection and filtered through 0.45 μm filter. Cells were seeded at 10^5 cells/well in 6-well plate overnight before adding lentiviruses packaged from various constructs supplemented with 8 μg/ml polybrene. After overnight infection, medium was changed and GFP signal was confirmed at 48 hours post infection to ensure >90% transduction (>90% of cells GFP+).

Cell growth curve and apoptosis assay

Sorted cells were seeded at 10^5 cells/well in 6 well plate and infected with control or MT-hTer/siRNA lentivirus. Cell proliferation was measured by cell counting using a hemocytometer at subsequent time points after infection. Apoptosis was analyzed at day 4 post infection with MT-hTer/siRNA by TUNEL assays performed following the protocol described in In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN) and analyzed on BD LSR-II.

Subcutaneous tumor xenografts

All experiments were approved and performed following the rules of the Institutional Animal Care and Use Committees at University of Southern California. 6-8 week old, male SCID mice were purchased from NIH. DU145 cells were infected overnight with control or MT-hTer/siRNA lentiviruses and cultured at 37°C, 5% CO_2 for 1 day after changing media. For each mouse, 5000 cells were resuspended in media, mixed with 50 μl ice-cold matrigel (BD biosciences, San Jose, CA) and placed on ice until inoculation. 1 ml insulin syringe was used for subcutaneous inoculation onto the flank of each mouse (5 mice per treatment group). The growth of tumors was observed and recorded as tumor volume by caliper measurement. Ninety days after inoculation, mice were sacrificed, and tumors were resected and weighed.

Statistical analysis

Performed in collaboration with USC/Norris Biostatistics Core. All experiments were conducted in triplicate with error bars representing standard deviation around the mean. Student's t-test was used to determine statistical significance when comparing mean values at one point in time (e.g. gene expression, % apoptosis, cell growth inhibition, relative telomerase activity in DU145 cells, tumor weights). Two-sided Wilcoxon matched-pairs signed rank test was performed by using Graphpad Prism5.0 software to compare statistical significance of telomerase activity and telomerase (hTERT) mRNA level for patients' sample as well as tumor growth from DU145 cells over time.

Results

Prostate tumor cells expressing integrin α2β1^{high}CD44^{+} have a cancer progenitor cell (CPC) phenotype

Freshly resected human prostate tumors (Table 1 and Supplementary Figure 1) were disaggregated, digested, and dissociated into single cell suspensions as described previously [3-4] and in Materials and Methods. Cells were analyzed by FACS for expression of integrin $\alpha_2\beta_1$ (CD49b), CD44 and CD133, surface markers widely reported in prostate cancer to enrich for CPC properties such as elevated expression of self-renewal genes, high *in vitro* clonogenicity and invasiveness, and increased tumor initiation after selection from xenografted human cell lines [3-4,6-7,29]. In all tumor samples, we found α2β1^{high}CD44^{+} cells, which constituted 0.7% to 9.2% of all cancer cells (Table 1 and Supplementary Figure 1). In contrast, minimal CD133 expression was observed (Supplementary Figure 1), consistent with previous reports [28]. In all experiments, transient collagen adherence [3] was used to enrich for integrin α2β1^{+} cells, followed by FACS to further select for an α2β1^{high}CD44^{+} subpopulation; purity (>98%) was confirmed by re-analysis with FACS (Supplementary Figure 1).

To confirm the previously-reported CPC properties of the tumor-derived FACS-sorted cells, we conducted phenotypic analyses which revealed that – compared with the non-CPC majority of tumor cells (α2β1^{high}CD44^{-}) – the α2β1^{high}CD44^{+} subpopulation expressed significantly higher mRNA levels of genes associated with self-renewal, proliferation and invasiveness, such as beta-catenin, NANOG, Oct3/4, SMO and Bmi1; conversely, mRNA levels of genes associated with a differentiated prostate phenotype, such as androgen receptor (AR) and prostate specific antigen (PSA), were significantly lower (Figure 1A). Functionally, α2β1^{high}CD44^{+} subpopulations from all 8 tested tumors proliferated in culture, with three of these generating discrete colonies at an average of 44 colonies per 1000 cells seeded after 21 days in culture; in contrast, α2β1^{high}CD44^{-} cells from all 8 tumors tested did not proliferate in culture and produced no colonies (Figure 1B). Moreover, α2β1^{high}CD44^{+} cells from 4 of 8 tumors migrated across the membrane in a matrigel invasiveness assay at an average of 39 migrated cells per 5 high powered fields after 24 hours (interestingly, the same subset of tumors yielded α2β1^{high}CD44^{+} cells which were both clonogenic and invasive); in contrast, none of the α2β1^{high}CD44^{-} cells from all 8 tumors tested were invasive (Figure 1C).

CPC have high telomerase expression and activity that can be targeted with telomerase interference

We measured relative telomerase activity from the putative tumor-derived CPC subpopulations (α2β1^{high}CD44^{+}) and non-CPC subpopulations (α2β1^{+}CD44^{-}) using qPCR-TRAP. Notably, we found that CPC had significantly higher (approximately 20-fold to 200-fold) telomerase activity than non-CPC in 6 of 6 tumors tested (Figure 2A, p=0.03). CPC also had significantly higher mRNA levels of hTERT, the chief determinant of telomerase activity (Figure 2B, p=0.008), whereas their mean bulk telomere lengths were not significantly different from those of non-CPC (Figure 2C).

Having observed the markedly elevated telomerase expression and activity of tumor-derived CPC, we reasoned that this subpopulation may be particularly susceptible to telomerase interference, ectopic introduction of telomerase RNA with a mutated template region (MT-hTer/siRNA) that reprograms the telomerase enzyme to add incorrect telomeres, resulting in telomeric uncapping, DNA damage, and apoptosis [19,22,30]. We infected the tumor-derived CPC with lentivirus expressing either MT-hTer/siRNA or vector control. Two days after infection, MT-hTer expression by qPCR was ~6-fold compared to vector control

(Figure 3A), and a modified qPCR-TRAP assay to detect mutant telomeric repeats showed a 5-fold increase in mutant telomerase activity (Figure 3B); together, these data confirmed successful MT-hTer/siRNA expression and reprogramming of telomerase activity in the CPC subpopulation. Importantly, telomerase interference resulted in 80% apoptosis by day 4 of infection and ~95% cell growth inhibition by day 6 (Figure 3C-D). Hence, the high telomerase expression and activity of tumor-derived CPC rendered them acutely susceptible to telomerase interference.

CPC derived from a prostate cancer cell line have high tumorigenicity that is abrogated by telomerase interference

Having observed the marked efficacy of telomerase interference *in vitro*, next we tested whether this approach could also inhibit tumor formation *in vivo*. Currently there is no robust model for direct implantation and growth of human tumor-derived prostate cancer cells; therefore, we investigated tumor initiation *in vivo* using $\alpha2\beta1^{high}CD44^{+}$ cells isolated from the DU145 prostate cancer cell line, a strategy previously shown to enrich for CPC properties [6-7,29]. Consistent with previously published cell line data [6-7,29] and similar to our primary tumor results, the DU145-derived putative CPC subpopulation ($\alpha2\beta1^{high}CD44^{+}$) had significantly higher self-renewal gene expression (Figure 4A) and was significantly more clonogenic and invasive (Figure 4B) than the non-CPC ($\alpha2\beta1^{high}CD44^{-}$) subpopulation. Further mirroring our findings in primary tumors, telomerase expression and activity of DU145-derived CPC were double those of non-CPC (Figure 4C), and telomere lengths were not significantly different (Figure 4D). Next, we tested the *in vitro* effects of telomerase interference (MT-hTer/siRNA) in the CPC fraction relative to the non-CPC fraction of DU145 cells, a direct comparison which had not been possible with primary tumor derived cells, because primary tumor-derived non-CPC did not propagate in culture. Notably, ectopic expression of MT-hTer/siRNA in DU145-derived CPC caused a significant 3-fold inhibition of proliferation relative to vector control by day 6 after lentiviral infection (Figure 5A). In contrast, proliferation of DU145-derived non-CPC was *not* significantly inhibited by telomerase interference, possibly because the lower telomerase expression and activity of these cells provided less substrate to be reprogrammed by MT-hTer/siRNA (Figure 5A).

We investigated the *in vivo* impact of telomerase interference on the tumor initiating ability of CPC and non-CPC subpopulations derived from the DU145 cell line. MT-hTer/siRNA or vector control were ectopically overexpressed by lentiviral infection in CPC and non-CPC, and 5000 cells were inoculated subcutaneously into SCID mice (4 groups, 5 mice per group). In the vector control groups, CPC generated measurable tumors in 5 of 5 mice by day 45 with mean wet weight of 0.72 g at excision on day 90, whereas non-CPC generated measurable tumors in only 3 of 5 mice by day 61 with mean wet weight of only 0.32 g at excision on day 90 (i.e. fewer and smaller tumors with greater lag time), thus confirming the greater baseline tumorigenicity of CPC relative to non-CPC (Figure 5B, C). Notably, both subpopulations formed no tumors over 90 days of follow-up when ectopically expressing MT-hTer/siRNA, suggesting that telomerase interference effectively abrogated tumor initiation *in vivo* (Figure 5B, C).

Discussion

Telomerase activity is considered a nearly universal characteristic of cancer cells, an assumption that may exist because early surveys of telomerase activity were conducted indiscriminantly from lysates of entire cancer populations [25], and because the oncogenic role of telomerase was demonstrated by ectopically introducing the enzyme into unselected cell populations [34-35]. Contrary to this model of homogeneous telomerase activation, studies of normal tissue stem cell compartments have demonstrated a unique role for

telomerase in stem cell activation [36-37], raising the possibility that perhaps telomerase plays a parallel unique role in so-called cancer progenitor (CPC) or tumor initiating cells. In support of a unique telomerase role in CPC, ectopic overexpression of telomerase in cancer cell lines has indeed been shown to enhance tumor initiation, perhaps reflecting a potentiation of the CPC phenotype [24,26]. Hence, we sought to determine whether telomerase activity in prostate tumors and cell lines is not uniformly distributed as previously assumed, but rather is focused predominantly in the CPC subpopulation where it can be used to neutralize these cells.

A variety of experimental systems have been described previously for the study of prostate CPC, resulting in a wide array of observations. For instance, a luminal epithelial stem cell was shown to be a cell of origin for prostate cancer using a mouse model in one study [38], while in another report only basal cells from primary benign human prostate tissue could initiate prostate cancer in immunodeficient mice [39]. Moreover, direct inoculation of primary prostate tumor subpopulations into mice as proof of a CPC phenotype has not been achieved as is done routinely in several other tumor types [40-44]. Given this background of multiple models and reports, we elected to conduct our study using primary human prostate tumors enriched for $\alpha2\beta1^{high}CD44^{+}$, the most robust and consistently cited markers for a CPC phenotype in the prostate cancer literature [3-4,6-7,10,29]. Functionally, CD44, the primary receptor for hyaluronic acid (HA), plays critical roles in cancer cell adhesion, migration and drug resistance, consistent with a CPC phenotype [45]. This choice of markers was further reaffirmed by a recent report wherein integrin $\alpha6^{high}CD44^{+}$ cell spheres generated *in vitro* from human prostate tumor tissues were successfully implanted (along with rat urogenital sinus mesenchyme) into a new strain of highly immunocompromised NOD-SCID/IL2rγNull mice, further substantiating the stem-like phenotype of this population [28].

In our present study, prostate tumor-derived $\alpha2\beta1^{high}CD44^{+}$ cells expressed elevated levels of genes associated with self-renewal and also were more clonogenic and invasive than bulk unselected cells. Similarly, DU145-derived $\alpha2\beta1^{high}CD44^{+}$ cells also had CPC-like *in vitro* properties as well as increased tumorigenicity *in vivo* relative to $\alpha2\beta1^{high}CD44^{-}$ cells. Another study recently examined prostate cancer cell lines for CPC-like cells and observed this subpopulation to have elevated telomerase activity which was inhibited with an anti-telomerase oligonucleotide [13]. Our present studies sought to further advance this line of investigation in several ways: Comparing telomerase activity between CPC and non-CPC subpopulations from freshly resected human tumors, testing the impact of telomerase reprogramming (via MT-hTer/siRNA) on these tumor-derived subpopulations, and investigating the effects of telomerase interference on tumor initiation *in vivo* using a similar cell line-derived CPC subpopulation.

Strikingly, we found that a subpopulation of primary tumor cells with CPC properties had markedly higher telomerase activity and hTERT expression than non-CPC from the same prostate tumors. Moreover, the non-CPC fractions, which comprised the vast majority (>90%) of tumor cells, did not propagate *in vitro*, a finding that further highlighted the biologic dichotomy between the two subpopulations: The large non-CPC subpopulation had very low telomerase activity and was unable to proliferate in culture, whereas the small CPC subpopulation had very high telomerase expression and activity, did proliferate *in vitro*, and underwent rapid apoptosis with exposure to telomerase interference, a strategy which specifically reprograms and exploits telomerase to generate toxic uncapped telomeres. This dichotomy in telomerase phenotype was further borne out in the DU145 experiments, where telomerase interference significantly inhibited the proliferation of CPC but exerted minimal effects on non-CPC. One possible explanation for this differential effect was that CPC had much more telomerase available to be reprogrammed by MT-hTer/siRNA, although

contribution from other biological differences between CPC and non-CPC cannot be ruled out. Lastly, in the *in vivo* experiments, non-CPC derived from DU145 were unable to form tumors, whereas DU145-derived CPC had brisk tumor formation that was efficiently abrogated by telomerase interference with MT-hTer/siRNA.

Collectively, our experiments demonstrate that telomerase expression and activity are *not* a uniform phenotype common to all cancer cells as generally assumed, but rather are concentrated in a subpopulation of cells with CPC-like properties in prostate tumors and cell lines. Their highly active telomerase phenotype rendered prostate CPC exceedingly susceptible to telomerase interference, which induced apoptosis, inhibited proliferation, and abrogated tumor formation. Moreover, the potent effects of MT-hTer/siRNA on DU145-derived CPC (telomerase-high) versus its minimal effects on DU145-derived non-CPC (telomerase-low) suggests that telomerase interference may have a degree of therapeutic selectivity for CPC vis-à-vis their high telomerase. Therefore, targeting telomerase in this manner may constitute a promising new therapeutic strategy for neutralizing CPC in prostate and other cancers, ultimately leading to more effective control of tumor recurrence and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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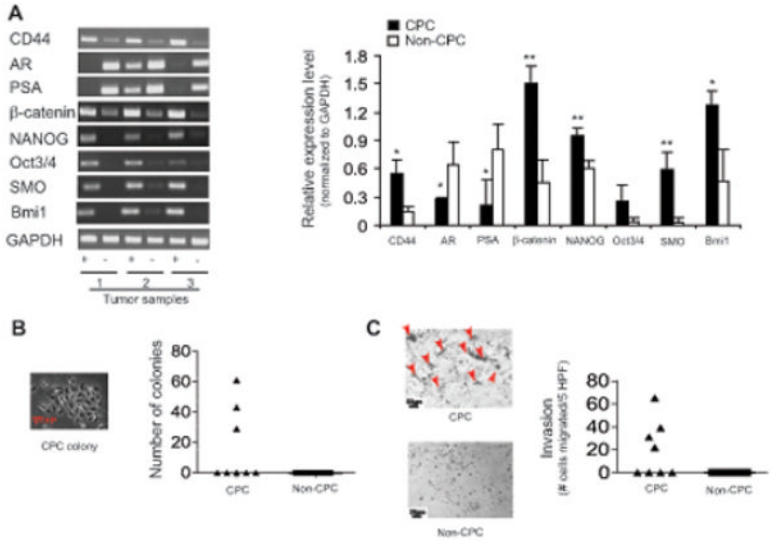

Figure 1. CPC phenotypes of cell subpopulations isolated from human prostate tumors

(A) Left: Gene expression (by RT-PCR) of cell subpopulations from 3 individual tumors ("+" = CPC, and "−" = non-CPC). Right: Semi-quantitative densitometric analysis of gel at left (mean values from 3 tumors). (B) Relative colony formation and (C) matrigel invasion of cell subpopulations from 8 individual tumors with sample micrographs (arrows on matrigel micrograph indicate cells that migrated across the membrane).

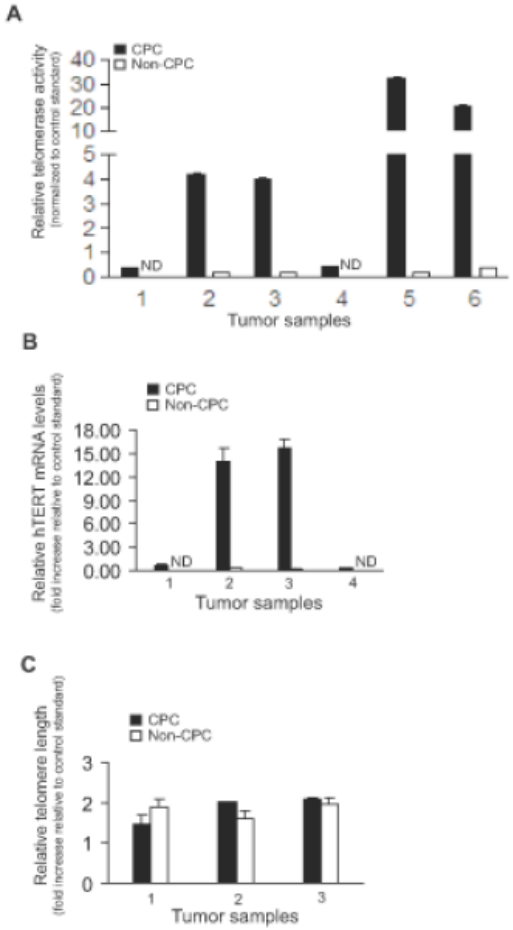

Figure 2. Telomerase and telomere characterization of CPC and non-CPC cell subpopulations isolated from human prostate tumors

(A) Telomerase activity of cell subpopulations from 6 individual tumors by qPCR-TRAP, normalized to a standard control telomerase activity from DU145 cancer cells (p=0.03). (B) Relative hTERT mRNA levels of cell subpopulations in 4 individual tumors (p=0.008). (C) Telomere lengths of cell subpopulations in 3 individual tumors, respectively. In 2A-B, “ND” = not detectable. In all panels, 1000 cells were used for each experiment, and assays were conducted in triplicate and compared using a two-sided Wilcoxon matched-pairs signed rank test of statistical significance.

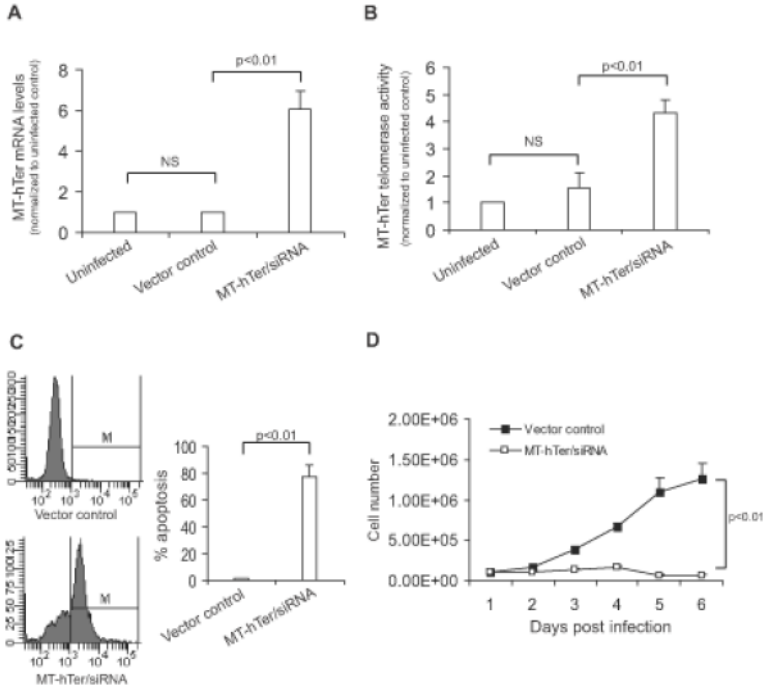

Figure 3. Induction of telomerase interference (reprogramming of telomerase) in tumor-derived CPC

(A) MT-hTer expression by qPCR and (B) MT-telomerase activity levels by MT-specific qPCR-TRAP 48 hours after infection with lentivirus expressing MT-hTer/siRNA. (C) Percent apoptosis by TUNEL assay at day 4 after treatment with MT-hTer/siRNA or vector control. (D) Proliferation after treatment with MT-hTer/siRNA or vector control. Data in A-D reflects biological triplicate experiments conducted using CPC cells isolated from 1 patient tumor. All experiments were also repeated in triplicate using 2 additional patient tumors with similar results (data not shown).

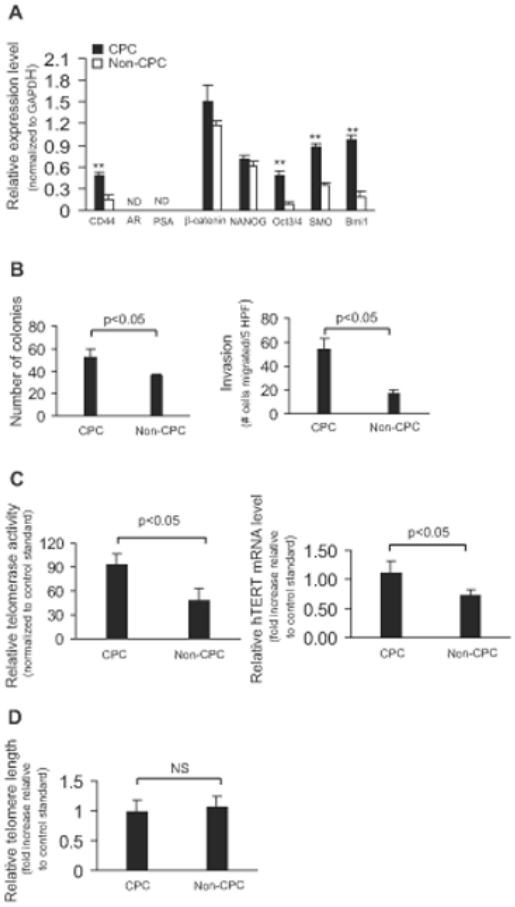

Figure 4. CPC phenotypes of cell subpopulations isolated from DU145 human prostate cancer cell line

(A) Relative gene expression and (B) Colony formation (left), and invasiveness (right) of CPC and non-CPC subpopulations. (C) Relative telomerase activity (left) and hTERT mRNA levels (right), and bulk telomere lengths (D) of CPC and non-CPC subpopulations normalized to control standard (LNCaP cell line).

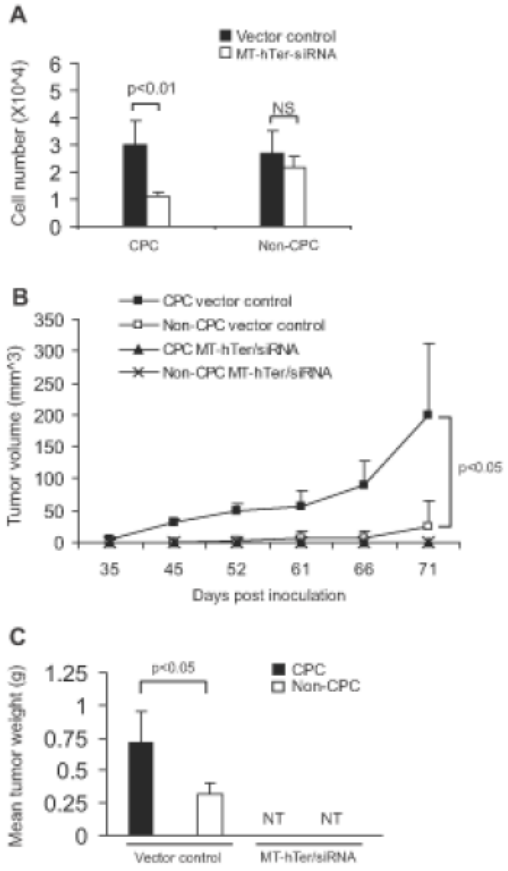

Figure 5. Effect of telomerase interference on *in vivo* tumor formation by DU145-derived CPC

(A) Relative growth inhibition of CPC and non-CPC subpopulations 6 days after treatment with MT-hTer/siRNA. (B) CPC were more tumorigenic than non-CPC, and tumor formation by CPC was abrogated by telomerase interference. (C) Mean tumor weights at excision on day 90 post inoculation (NT = no tumors).

Table 1

Clinical and histological features of prostatectomy specimens

Tumor	Pathologic TNM Stage	Gleason Score	PSA	% CPC
1	pT2bN0MX	3+4	6.76	7.0
2	pT4N1MX	5+5	3.71	0.7
3	pT3bN1MX	4+3	11.1	5.3
4	pT3bN0MX	4+5	9.32	3.5
5	pT3aN0MX	4+4	0.26	5.2
6	pT3aN0MX	3+3	8.67	6.4
7	pT3bN0MX	3+5	9.44	9.2
8	pT2cN0MX	3+5	5.14	5.2
9	pT2bN0MX	3+4	10.2	0.8
10	pT3aN0MX	4+3	19.7	6.6
11	pT3bN0MX	3+4	7.72	3.9
12	pT4N1MX	4+5	7.29	4.3
13	pT2aN0MX	4+4	6.77	2.8