



HAL
open science

Prostate tumor-initiating cells: A new target for telomerase inhibition therapy?

Calin O. Marian, Jerry W. Shay

► To cite this version:

Calin O. Marian, Jerry W. Shay. Prostate tumor-initiating cells: A new target for telomerase inhibition therapy?. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (4), pp.289. <10.1016/j.bbadis.2009.02.012>. <hal-00562900>

HAL Id: hal-00562900

<https://hal.science/hal-00562900v1>

Submitted on 4 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

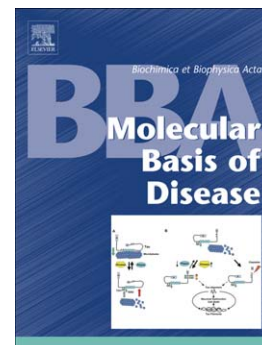
Prostate tumor-initiating cells: A new target for telomerase inhibition therapy?

Calin O. Marian, Jerry W. Shay

PII: S0925-4439(09)00046-5
DOI: doi:[10.1016/j.bbadis.2009.02.012](https://doi.org/10.1016/j.bbadis.2009.02.012)
Reference: BBADIS 62941

To appear in: *BBA - Molecular Basis of Disease*

Received date: 9 January 2009
Revised date: 20 February 2009
Accepted date: 23 February 2009



Please cite this article as: Calin O. Marian, Jerry W. Shay, Prostate tumor-initiating cells: A new target for telomerase inhibition therapy?, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.02.012](https://doi.org/10.1016/j.bbadis.2009.02.012)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Prostate Tumor-initiating Cells: A New Target for Telomerase
Inhibition Therapy?**

Calin O. Marian and Jerry W. Shay

University of Texas Southwestern Medical Center

Department of Cell Biology

5323 Harry Hines Boulevard

Dallas, TX 75390-9039

Correspondence: Calin O. Marian

Phone: 214-648-2684

Fax: 214-648-8694

Email: calin.marian@utsouthwestern.edu

Keywords: Prostate, Telomeres, Telomerase, Tumor-initiating cell, GRN163L

Summary

Conventional therapies for prostate cancer, especially in its androgen-independent form, may result in the survival of small populations of resistant cells with tumor-initiating potential. These “cancer stem cells” are believed to be responsible for cancer relapse, and therapeutic strategies targeting these cells are of great importance. Telomerase is a ribonucleoprotein enzyme responsible for telomere elongation and is activated in the majority of malignancies, including prostate cancer, but is absent in most normal cells. Putative tumor-initiating cells have significant levels of telomerase, indicating that they are an excellent target for telomerase inhibition therapy. In this review, we present some evidence for the hypothesis that conventional therapies (standard chemotherapy and/or radiation therapy) in combination with telomerase inhibitors may result in effective and more durable responses.

Introduction

Prostate cancer is the second most common malignancy found in men and is responsible for the highest rate of morbidity after lung cancer [1]. In most cases, localized prostate disease can be treated efficiently using surgery and androgen ablation therapy. However, the outcome for patients with metastatic disease remains poor [2]. Considering the advanced age for the majority of patients, the chemotherapy regimens have done little to improve median survival, and the lethality of the disease in patients with metastatic castrate-resistant disease remains high [3]. The Gleason classification of prostate tumors remains the best predictor for disease outcome, but more recently new molecular diagnostic techniques such as identification of TMPRSS2:ERG fusion transcripts [4, 5], Glutathione-S-transferase P1 (GSTP1) gene promoter hypermethylation [6, 7] and DD3 expression [8] can assist in early detection, prognosis, and monitoring of prostate cancer. In addition to diagnostics, current research in the prostate cancer field is focused on the establishment of new targeted therapies for the patients with metastatic disease. It is generally believed that cancer relapse in patients may be due to a small population of cells within the tumor mass which are resistant to conventional therapies.

The cancer stem cell hypothesis

The cancer stem cell hypothesis was described more than 150 years ago [9], but the modern revival of this concept arrived with the studies performed in leukemia, where it was shown that a single cell with the CD34+/CD38- phenotype had the capacity of

inducing the disease in NOD-SCID mice [10]. More recently, cancer stem cells have been isolated from solid tumors, first in breast cancer, then in neurological malignancies [11, 12]. The term “cancer stem cells” is still very controversial. Nevertheless, the general consensus is that these cells must have potent tumor initiation, self renewal and differentiation capacity [13]. The tumor initiation aspect refers to the capacity of these cells to form tumors in immunocompromised mice using very small numbers of cells. Self-renewal capacity is tested by serial transplantation experiments, where re-isolated cancer stem cells can be transplanted in secondary and tertiary recipients. The differentiation ability of these cells does not refer to multilineage differentiation but rather to the capacity of the resulting tumors to be a phenocopy of the original tumor. An important characteristic of cancer stem cells is their ability to survive various therapies by activating anti-apoptotic pathways, increasing activity of membrane transporters and high DNA repair capacity [14, 15]. It is important to point out that the definition of cancer stem cells does not imply the cell type from which these cells originated. This is the reason why for the purpose of this review we are going to use the term tumor-initiating cells. While the origin of tumor-initiating cells is highly debated, this review will focus on the intrinsic properties of these cells in prostate cancer, specifically on telomerase as both a biomarker and therapeutic target for this type of malignancy. The study of these populations of cells is very important, not only for the basic understanding of malignant transformation and pathogenesis, but also as a way to investigate and implement new therapies.

Isolation of prostate tumor-initiating cells

To some extent, the amount of knowledge about prostate tumor-initiating cells is still limited compared to that reported in other cancers types. This is due, in part, to the small amounts of primary tumors samples available for investigation, and the difficulty in distinguishing between normal and malignant prostate cells based on surface markers alone. Because prostate tumor-initiating cells are present in very low numbers within a primary tumor (usually less than 1%), the use of cancer cell lines provide an efficient alternative to clinical samples. The caveat is that one needs to validate any scientific knowledge derived from prostate cell lines with studies in primary tumor counterparts. Cell lines are usually grown in culture medium supplemented with serum and high Ca^{2+} , conditions that generally permits growth but also encourages differentiation. Some researchers are strong advocates of xenograft propagation of human tumors, but the mouse environment is very different from the human prostate stromal niche, especially when using subcutaneous or renal capsule inoculations, and some amount of differentiation is unavoidable. An alternative is the prostate orthotopic xenograft, but these are difficult to establish, with high rates of mortality. The combinatorial use of primary samples, xenografts and cell lines will likely provide the tools for the most rigorous scientific investigations.

There are several strategies to isolate prostate tumor-initiating cells. The most popular strategy employs the use of surface markers that share the same immunological profile with normal prostate stem cells. One of these surface markers is CD44, an adhesion molecule with multiple functions that appear to be important in tumor dissemination and metastasis [16-18]. One research group reported an in-depth study

using CD44^{high} cells isolated from various prostate cell lines [19]. These putative tumor-initiating cells were more proliferative, clonogenic, tumorigenic, and metastatic than the CD44^{low} cells. The CD44 cells also show properties of progenitor cells, such as BrdU label retention and expression of several “stemness” factors, such as Oct-3/4, BMI, β -catenin, and SMO. Moreover, while these cells were AR-, they had the capacity to differentiate into AR+ cells. The authors recognized that the CD44^{high} population of cells was still very heterogeneous and tried to further purify the tumor-initiating component using additional surface markers. In a subsequent study, it was shown that CD44^{high}/ $\alpha_2\beta_1$ integrin^{high} populations were more tumorigenic than CD44^{low}/ $\alpha_2\beta_1$ integrin^{low} populations when injected in immunocompromised mice and the authors proposed a tumorigenic hierarchy of prostate cancer cells based on the expression of these two markers [20].

Based on the similarities between mouse prostate and breast stem-like cells, another study sought to determine if a population of CD44+/CD24- cells identified tumor-initiating cells in the LNCaP prostate cancer cell line [21]. These cells were present at a very low level in the population (0.04%), and show increased clonogenic and differentiation capacity. Importantly, very low numbers of CD44+/CD24- cells were capable of forming tumors in NOD/SCID mice. These cells were also able to grow as spheroids in attachment-independent conditions and possessed an invasive gene signature.

Another important stem cell marker is prominin-1 (CD133), a pentaspan membrane protein with unclear function [22]. Collins et al., used a CD44/ $\alpha_2\beta_1$ integrin^{high}/CD133+ phenotype to isolate tumor-initiating cells from primary prostate biopsies [23].

The cells isolated with these markers have a high clonogenic and proliferative capacity, are highly invasive through matrigel and capable of differentiation.

The percent CD133⁺ cells is low after the initial purification from primary tumor samples. Using the prostate cancer cell line DU145 we also find low numbers of CD133⁺ cells (Table 1). CD133⁺ cells isolated from primary tumors or DU145 cells can be placed back in culture, using both serum-supplemented media and adherent conditions or chemically defined media and attachment-independent conditions (spheroids). Regardless of the media and culture conditions used, the percentage of cells expressing CD133 remains very low (less than 1%), without any apparent enrichment over time in culture. This indicates that the culture conditions commonly employed *in vitro* for the propagation of prostate tumor stem cells do not allow the enrichment of this rare population of cells. This is in stark contrast with brain tumor stem cells, where some degree of positive enrichment is possible when isolated CD133⁺ cells are placed in culture [12]. Importantly, these prostate experiments indicate that the biology of CD133⁺ cells in primary tumor samples and cancer cell lines might be similar, and if true, the prostate cancer cell lines can be used as a valuable source of research material.

A recent study confirmed the significance of CD133 as a marker for both normal and tumor-initiating prostate cells [24]. Within several androgen receptor positive (AR⁺) human prostate cancer cell lines, CD133⁺ cells were found at low frequency and were able to self-renew, generate heterogeneous progenies and were capable of an unlimited proliferation capacity. The authors of this study also speculated that CD133 may function differently between normal and cancer prostate cells and that malignant CD133⁺ cells are originating from a malignantly transformed intermediate cell. Finally it was confirmed

that in addition to CD133+, the CD44/ $\alpha_2\beta_1$ integrin^{high}/CD133+ population from the DU145 prostate cancer cell line [25] had high capacity of self-renewal and differentiation as well as strong proliferative and tumorigenic potential.

Another popular method to identify tumor-initiating cells is the isolation of the “side population” (SP). The SP cells are isolated based on the ability of cells to retain Hoechst dye, and in the LAPC-9 prostate cancer cell line the SP cells were shown to be more tumorigenic than the corresponding main population [26]. The LAPC-9 SP cells possessed other stem cell properties such as capacity of differentiation in vivo, as well as the ability to sustain subsequent transplantation. Additional information about stem cell surface makers (e.g. CD133, CD44, and $\alpha_2\beta_1$ integrin) was not provided by this study.

A different strategy adopted to identify tumor-initiating cells is based on their capacity to form holoclones – tightly packed clones with specific morphology that contain self-renewing cells and have been hypothesized to contain tumor-initiating cells [27]. The other two types of clones formed by epithelial cells (meroclones and paraclones) do not have the sustained proliferation capacity required for tumor initiation. Holoclones derived from the PC3 prostate cancer cell line were shown to contain stem-like cells that could initiate serially transplantable tumors [28]. In contrast, meroclones and paraclones did not proliferate and failed to initiate tumor development. Perhaps not surprising, the holoclones had high levels of CD44, $\alpha_2\beta_1$ integrin and β -catenin expression, whereas meroclones and paraclones show reduced expression of these stem cell markers. However, CD133 expression was not reported in this study.

In our experiments, we examined by immunofluorescence imaging the signature of DU145 prostate cancer cells grown at clonal density in attachment-independent

conditions (spheroids). The attachment-independent conditions exert even more strain on the cells, and because spheroid formation was used extensively to enrich for stem cells, the clonogenic spheroid formation assay probably identifies the population of cells that have the highest tumorigenic potential. We specifically focused on common tumor-initiating cells markers such as CD44 and CD133. CD44 is present at high levels in the majority of DU145 cells, regardless of culture conditions (monolayer or spheroids). The CD133+ cells were also clearly identified in the spheroids, but as the spheroids grew in size, the CD133+ population did not proliferate at the same rate as the CD44 cells within the spheroids (data not shown). While these spheroids were grown in serum-supplemented media (that usually promotes differentiation), the use of serum-free defined media also did not enrich the CD133+ population.

In summary, the study of prostate tumor-initiating cells is still an evolving field but the results to date suggest that a series of several surface and/or metabolic markers may be needed to identify prostate cancer-initiating cells.

Telomeres, telomerase and prostate cancer

Telomeres are nucleoprotein complexes that cap the ends of human chromosomes [29]. As the cells divide, the telomeres shorten by approximately 50-100 base pairs with each division [30]. In addition, single-strand breaks of telomere DNA caused by oxidative damage can lead to telomere attrition [31, 32]. Telomeres present a specific end-replication problem, recognized as early as 1970's [33, 34], and a specialized cellular enzyme, called telomerase is responsible for telomere extension [35]. Telomerase is

active in proliferating cells of the skin, gastrointestinal system and blood [36-38]. While normal prostate cells lack telomerase activity [39], telomerase is detected in the majority of prostate cancer samples, being absent or present at low levels in benign prostate hyperplasia (BPH) [40-45]. More significant is the fact that majority of the prostate cancer samples have much shorter telomeres than the corresponding normal or BPH prostate samples [39, 46].

Prostate cancer cells have robust telomerase activity and several commonly used prostate cancer cell lines show a significant TRAP signal (Figure 1A). Similar to the results observed in primary tumor samples (data not shown), these cell lines have relatively short telomeres (Figure 1B). The cell lines used were derived from different metastatic sites and cultured in serum-supplemented media, but prostate cancer primary cells cultured in our lab have shown the same characteristics (high telomerase activity and short telomeres) (data not shown). This is in contrast to normal prostate epithelial cells which are telomerase (TRAP) negative (Figure 1A).

Androgen ablation in a rat model leads to activation of telomerase and further treatment with androgen reverses the effects and led to the down-regulation of telomerase in these animals [47]. Similar results were obtained in a primate model [48]. In sharp contrast, telomerase regulation by androgens is reversed in prostate cancers. Telomerase activity is up-regulated upon androgen stimulation and in clinical specimens telomerase activity is significantly reduced after complete androgen ablation [49, 50]. Recent studies have shown that in prostate cancer cells the catalytic protein component of telomerase (hTERT) promoter is the target of down-regulation by AR in cooperation with p53 [51]. These experiments appear to reveal a mechanism for the protective role of androgens in

normal prostate and suggest that prostate cancer cells might escape this mechanism by mutations in the AR. There is general agreement that normal adult prostate stem cells are AR⁺ but whether AR is expressed in tumor-initiating cells is still a controversial topic. While some authors support the idea that prostate tumor-initiating cells do not express AR [52, 53], recent evidence supports the notion that prostate tumor-initiating cells are AR⁺ [24, 54].

Chromosomal instability is an essential feature of prostate cancer, being detected as early as prostate intraepithelial neoplasia (PIN), the earliest recognizable form of the disease. Using a high resolution, quantitative in situ method of investigation for telomere length [55], it was shown that the telomere length of high grade PINs were considerably shorter than adjacent normal cells [56]. The telomere shortening was restricted only to the luminal compartment, suggesting that the basal cells may not be the source of neoplastic transformation. This report supported previous studies showing that a subset of PIN cells activate telomerase, become immortal and eventually progressing to fully invasive adenocarcinoma [41]. Combining this theory of neoplastic transformation with the evidence provided by the presence of AR in prostate tumor-initiating cells [24, 54], we favor a model of prostate cancer in which telomere shortening and telomerase activity play a central role (Figure 2). Under the influence of chronic inflammation on a background of sustained telomere shortening, a subset of transit amplifying cells of intermediate phenotype will encounter severe chromosomal instability. To escape from the blockade induced by critically shortened telomeres, telomerase activation and subsequent cellular immortalization, combined with other mutations, leads to the development of prostate cancer. For example, cells in high grade PIN lesions that have

escaped replicative senescence have unlimited proliferative capacity to accumulate additional mutations leading to prostate cancer.

Telomerase activity in prostate tumor-initiating cells

Telomerase in normal stem cells is highly regulated and generally expressed at low levels. The major function of the enzyme in the normal stem cell compartment is believed to be the partial maintenance of telomere homeostasis during self renewal [57, 58]. Importantly, all human adult stem cells (and normal somatic cells) that have been examined progressively shorten their telomeres with increased age. This telomere loss mechanism may have evolved as an important anticancer mechanism by placing limitations on cells that accumulated harmful mutations and preventing their clonal expansion. This suggests that fully maintaining telomere length in normal cells may increase the risk of developing cancer because cancer cells cannot enter replicative senescence and will ultimately achieve indefinite proliferation potential.

While there is no direct evidence about the levels of telomerase activity in prostate stem cells, the expectancy is that these cells will also have low levels of telomerase activity. The only experiments that addresses the telomerase activity in isolated tumor-initiating cells of solid tumors came from the experiments performed in breast cancer, where the data presented shows that breast tumor-initiating cells have telomerase activity at similar levels with the main tumor mass cells, and importantly, that these cells have relatively short telomeres [59]. Experiments performed in our lab show that putative prostate tumor-initiating population of cells isolated from different cell lines

have significant telomerase activity (Figure 3). In the PC3 cell line, the surface markers CD44 and CD133 were used to isolate populations of cells that exhibit telomerase activity at the same level with the negative (low) fraction and main population. Two cell lines (LNCaP and PC3) that differ in terms of androgen response are shown for the CD133 marker, suggesting that telomerase is universally expressed in tumor-initiating cells isolated from cancer cell lines containing putative cancer-initiating markers. Experiments are under way to establish the presence of telomerase in tumor-initiating cells isolated from primary prostate tumor samples, as well as from distant metastatic sites in patients with advanced disease. We expect that prostate tumor-initiating cells isolated from these sources will have active telomerase, these expectations being consistent with the properties of brain tumor-initiating cells (Marian et al., unpublished data).

Circulating prostate tumor-initiating cells

Metastasis is the major cause of death in patients with advanced prostate cancer, the most frequent site for metastatic lesions being the bone [60]. There are still many unanswered questions about the metastatic process, but for the purpose of this review we are interested in how the tumor-initiating cells and circulating tumor cells (CTC) fit in the general scheme of cancer progression. It was already hypothesized that a subset of tumor-initiating cells are responsible for distant metastasis [61, 62]. Support for this hypothesis comes from the inherent properties of tumor-initiating cells. Only cells that have high plasticity and the capacity to form tumors will be responsible for the formation of

metastatic lesions. These cells need to adapt to a new specific niche and the heterogeneous nature of tumor-initiating cells make them ideal candidates for this task. Detection of CTCs has improved with the advent of automated systems, some of which are approved for clinical use. One of these systems can be used as a survival predictor in patients with prostate metastatic cancer [63]. Very significant from the perspective of this review is the discovery that CTCs in the patients with advanced prostate cancer have significant levels of telomerase activity [64]. In the same study, telomerase activity was also detected in 23% of patient CTC specimens all of which had undetectable serum PSA levels. This suggests the potential applications of this technique not only for early diagnostic but also for treatment monitoring. If circulating tumor cells contain small populations of tumor-initiating cells, it becomes important to determine if these cells are telomerase positive. If telomerase activity is maintained in tumor-initiating cells after dissemination, these cells will most likely have significant levels of telomerase activity. If these cells are telomerase negative, the up-regulation of telomerase activity will most likely occur after the initial period of quiescence commonly associated with disseminated cancer cells. Nevertheless, without telomere maintenance by telomerase, metastatic lesions cannot proliferate to a significant level that makes them dangerous for the patient. The presence of telomerase in prostate CTCs can also serve as a direct monitoring tool for telomerase inhibition therapy. The access to blood samples is more feasible and less invasive than the alternative bone marrow biopsies.

Telomerase as a therapeutic target for prostate cancer

Telomerase is also an attractive target for cancer therapy. The enzyme is present in majority of cancer cells analyzed but absent in almost all normal somatic cells, making telomerase inhibitors highly specific and telomerase a universal oncology target. Moreover, because normal cells have longer telomeres compared to cancer cells, the toxicity of these inhibitors in normal tissues is minimal. Several strategies have been employed for targeting telomerase, and several reviews have been written on the subject in recent years [65, 66]. Despite a multitude of pre-clinical studies, only two of these strategies to the present time have led to drugs that are currently in clinical trials. The first strategy targets the functional RNA (*hTR* or *hTERC*) component of the telomerase enzyme with N3'-P5' thio-phosphoramidate oligonucleotides [67]. The 13-mer compound used in these studies, GRN163L, is an antagonist that has high affinity to the *hTR* sequence and acts as an enzyme inhibitor (not as an antisense approach targeting mRNA). Once GRN163L is bound to the *hTR* component, it blocks access of *hTERT* (the catalytic protein component of telomerase) and prevents the assembly of an active telomerase enzyme. This leads to telomerase inhibition and progressive telomere shortening eventually leading to telomere uncapping and cell death. GRN163L is currently in Phase I and I/II clinical trials in several hematological and solid tumor malignancies. While not tested specifically for prostate cancer in clinical trials, this telomerase antagonist along with its un-lipidated precursor (GRN163) was shown to be effective in prostate xenograft models, and thus may become an effective therapy for prostate cancer [68-70].

A second strategy employs active telomerase immunotherapy directed towards the *hTERT* catalytic component. The presence of telomerase-specific cytotoxic T

lymphocytes has been discovered in some patients, suggesting that the immune system can elicit a response to telomerase-presenting cells even in the absence of vaccination [71]. In a clinical trial initiated in patients with prostate metastatic cancer, the subjects were inoculated with dendritic cells transfected with an mRNA encoding a chimeric lysosome-associated membrane protein-1 (LAMP) hTERT protein which allows a concomitant CD8⁺ and CD4⁺ T cell response. For the patients involved in this study, the vaccine had a significant impact on PSA levels and also led to a transient elimination of the PSA-expressing circulating tumor cells [72]. Another clinical study with prostate cancer patients revealed a significant induction of hTERT-specific T lymphocytes in response to inoculations with dendritic cells pulsed with a HLA-A2-restricted hTERT I540 peptide and keyhole limpet hemocyanin (KLH) [73]. Both these studies, along with clinical trials initiated for other malignancies [74] have shown minimal side-effects and no adverse effects on normal bone marrow stem cells. These cancer vaccination studies appear very promising, and some of the telomerase vaccines are moving forward to Phase II and III clinical trials.

Telomerase inhibition in prostate tumor-initiating cells

Previous reports have shown that GRN163L inhibits telomerase activity and eliminates the clonogenic potential of tumor-initiating cells from several different multiple myeloma (MM) cell lines. The same results were observed when using primary clinical samples where tumor-initiating cells isolated from the bone marrow of patients with MM were exposed to GRN163L [75]. In our lab we established the presence of

active telomerase in putative prostate tumor-initiating cells isolated from prostate cell lines and we proceeded to show that the telomerase inhibitor GRN163L is able to inhibit the enzymatic activity in these cells with the same efficiency as in the main population (Figure 3). Telomerase inhibition induced progressive telomere shortening in tumor-initiating cells at the same rate found in the cell line from which they were derived (data not shown). After the telomeres become critically short, the cells will enter apoptosis and die (data not shown). These experiments suggest that GRN163L may be a valuable therapy for the treatment of prostate cancer, its unique mode of action being able to target the elusive putative tumor-initiating cells that are usually resistant to conventional therapies.

Assuming the same telomere shortening dynamics are maintained *in vivo*, we propose a therapy regimen that combines conventional approaches (surgery and chemotherapy/radiotherapy) with telomerase inhibitors (Figure 4). While the standard therapy will have a de-bulking effect relatively fast, sustained telomerase inhibition will lead to critical telomere attrition and ultimately cell death in the small populations of cells that survive the first therapeutic intervention, including the tumor-initiating cells. The effect of this drug on normal stem cells in the organism should be minimal due to their slower proliferation rates, lower telomerase activity, and longer telomeres. This should provide an ample therapeutic window in which the shorter telomere-bearing tumor cells will be eliminated. After the telomerase inhibitor drug is removed, telomerase activity will return to normal levels in the proliferating cells.

Direct telomerase inhibitors are not the only agents that can be used with this therapy strategy, and vaccines targeting telomerase positive cells should be equally

efficient, unless tumor-initiating cells have special mechanisms to escape detection by the immune system. Preliminary data from clinical trials show that the vaccine might target and eliminate the cancer cells found in circulation [73], but based on the available data there is no direct evidence if the vaccines are targeting specifically tumor-initiating cells. This brings up a very important issue related to the availability of biomarkers to monitor telomerase inhibition therapy in prostate cancer. Because telomerase inhibitors will act promiscuously on all the cells in the organism, several cell types are available to assess therapy efficiency. The less invasive approach makes use of peripheral mononuclear blood cells (PBMCs). Activated leukocytes have low but detectable telomerase activity, therefore telomerase inhibition in this compartment can be used in various pharmacodynamic studies [76]. A more direct approach is to measure telomerase activity in CTCs or in disseminated cancer cells isolated from bone marrow aspirates.

Concluding remarks

The origin and identification of tumor-initiating cells is an exciting area of research, full of possibilities but also controversies that generate vivid arguments. The majority of these arguments are generated by the theoretical concepts associated with the “cancer stem cell” hypothesis and by some technical issues that make the isolation and characterization of these cells problematic [77, 78]. Some of these concerns are valid and more investigations are required to address these issues. However, the most valuable application of this scientific knowledge should be the discovery of new therapeutic strategies for the treatment of cancer. Telomerase inhibition might be one of these novel

targeted therapies, and due to the fundamental role of telomerase in most malignancies, we propose that telomerase therapeutics may also target tumor-initiating cells. If the source of tumor initiation is eradicated by targeting the ability of cell to maintain the end of the chromosomes, the quest for a cure might also come to an end.

ACCEPTED MANUSCRIPT

References

- [1] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray, M.J. Thun, *Cancer statistics, 2008*, CA: a cancer journal for clinicians 58 (2008) 71-96.
- [2] I.F. Tannock, R. de Wit, W.R. Berry, J. Horti, A. Pluzanska, K.N. Chi, S. Oudard, C. Theodore, N.D. James, I. Turesson, M.A. Rosenthal, M.A. Eisenberger, Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer, *The New England journal of medicine* 351 (2004) 1502-1512.
- [3] J.B. Aragon-Ching, W.L. Dahut, *Chemotherapy in Androgen-Independent Prostate Cancer (AIPC): What's next after taxane progression?*, *Cancer therapy* 5A (2007) 151-160.
- [4] G. Attard, J. Clark, L. Ambroisine, G. Fisher, G. Kovacs, P. Flohr, D. Berney, C.S. Foster, A. Fletcher, W.L. Gerald, H. Moller, V. Reuter, J.S. De Bono, P. Scardino, J. Cuzick, C.S. Cooper, Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer, *Oncogene* 27 (2008) 253-263.
- [5] B. Laxman, S.A. Tomlins, R. Mehra, D.S. Morris, L. Wang, B.E. Helgeson, R.B. Shah, M.A. Rubin, J.T. Wei, A.M. Chinnaiyan, Noninvasive detection of TMPRSS2:ERG fusion transcripts in the urine of men with prostate cancer, *Neoplasia* (New York, N.Y. 8 (2006) 885-888.
- [6] C.I. Suh, T. Shanafelt, D.J. May, K.R. Shroyer, J.B. Bobak, E.D. Crawford, G.J. Miller, N. Markham, L.M. Glode, Comparison of telomerase activity and GSTP1 promoter methylation in ejaculate as potential screening tests for prostate cancer, *Molecular and cellular probes* 14 (2000) 211-217.
- [7] C. Jeronimo, H. Usadel, R. Henrique, J. Oliveira, C. Lopes, W.G. Nelson, D. Sidransky, Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma, *Journal of the National Cancer Institute* 93 (2001) 1747-1752.
- [8] J.B. de Kok, G.W. Verhaegh, R.W. Roelofs, D. Hessels, L.A. Kiemeny, T.W. Aalders, D.W. Swinkels, J.A. Schalken, DD3(PCA3), a very sensitive and specific marker to detect prostate tumors, *Cancer research* 62 (2002) 2695-2698.
- [9] R. Virchow, *Cellular pathology*, R. M. De Witt, London, 1860.
- [10] D. Bonnet, J.E. Dick, Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell, *Nature medicine* 3 (1997) 730-737.
- [11] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, *Proceedings of the National Academy of Sciences of the United States of America* 100 (2003) 3983-3988.
- [12] S.K. Singh, I.D. Clarke, M. Terasaki, V.E. Bonn, C. Hawkins, J. Squire, P.B. Dirks, Identification of a cancer stem cell in human brain tumors, *Cancer research* 63 (2003) 5821-5828.
- [13] R.J. Ward, P.B. Dirks, Cancer stem cells: at the headwaters of tumor development, *Annual review of pathology* 2 (2007) 175-189.

- [14] M. Dean, T. Fojo, S. Bates, Tumour stem cells and drug resistance, *Nature reviews* 5 (2005) 275-284.
- [15] H. Ishii, M. Iwatsuki, K. Ieta, D. Ohta, N. Haraguchi, K. Mimori, M. Mori, Cancer stem cells and chemoradiation resistance, *Cancer science* 99 (2008) 1871-1877.
- [16] J.E. Draffin, S. McFarlane, A. Hill, P.G. Johnston, D.J. Waugh, CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells, *Cancer research* 64 (2004) 5702-5711.
- [17] D. Naor, S.B. Wallach-Dayana, M.A. Zahalka, R.V. Sionov, Involvement of CD44, a molecule with a thousand faces, in cancer dissemination, *Seminars in cancer biology* 18 (2008) 260-267.
- [18] H. Ponta, L. Sherman, P.A. Herrlich, CD44: from adhesion molecules to signalling regulators, *Nat Rev Mol Cell Biol* 4 (2003) 33-45.
- [19] L. Patrawala, T. Calhoun, R. Schneider-Broussard, H. Li, B. Bhatia, S. Tang, J.G. Reilly, D. Chandra, J. Zhou, K. Claypool, L. Coghlan, D.G. Tang, Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells, *Oncogene* 25 (2006) 1696-1708.
- [20] L. Patrawala, T. Calhoun-Davis, R. Schneider-Broussard, D.G. Tang, Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+ α 2 β 1+ cell population is enriched in tumor-initiating cells, *Cancer research* 67 (2007) 6796-6805.
- [21] E.M. Hurt, B.T. Kawasaki, G.J. Klarmann, S.B. Thomas, W.L. Farrar, CD44+ CD24(-) prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis, *British journal of cancer* 98 (2008) 756-765.
- [22] D. Mizrak, M. Brittan, M.R. Alison, CD133: molecule of the moment, *The Journal of pathology* 214 (2008) 3-9.
- [23] A.T. Collins, P.A. Berry, C. Hyde, M.J. Stower, N.J. Maitland, Prospective identification of tumorigenic prostate cancer stem cells, *Cancer research* 65 (2005) 10946-10951.
- [24] D.J. Vander Griend, W.L. Karthaus, S. Dalrymple, A. Meeker, A.M. DeMarzo, J.T. Isaacs, The role of CD133 in normal human prostate stem cells and malignant cancer-initiating cells, *Cancer research* 68 (2008) 9703-9711.
- [25] C. Wei, W. Guomin, L. Yujun, Q. Ruizhe, Cancer stem-like cells in human prostate carcinoma cells DU145: the seeds of the cell line?, *Cancer biology & therapy* 6 (2007) 763-768.
- [26] L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, D.G. Tang, Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic, *Cancer research* 65 (2005) 6207-6219.
- [27] M. Locke, M. Heywood, S. Fawell, I.C. Mackenzie, Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines, *Cancer research* 65 (2005) 8944-8950.
- [28] H. Li, X. Chen, T. Calhoun-Davis, K. Claypool, D.G. Tang, PC3 human prostate carcinoma cell holoclones contain self-renewing tumor-initiating cells, *Cancer research* 68 (2008) 1820-1825.

- [29] R.K. Moyzis, J.M. Buckingham, L.S. Cram, M. Dani, L.L. Deaven, M.D. Jones, J. Meyne, R.L. Ratliff, J.R. Wu, A Highly Conserved Repetitive DNA-Sequence, (Ttaggg)N, Present at the Telomeres of Human-Chromosomes, Proceedings of the National Academy of Sciences of the United States of America 85 (1988) 6622-6626.
- [30] C.B. Harley, A.B. Futcher, C.W. Greider, Telomeres shorten during ageing of human fibroblasts, *Nature* 345 (1990) 458-460.
- [31] P.A. Kruk, N.J. Rampino, V.A. Bohr, DNA damage and repair in telomeres: relation to aging, Proceedings of the National Academy of Sciences of the United States of America 92 (1995) 258-262.
- [32] T. von Zglinicki, R. Pilger, N. Sitte, Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts, *Free radical biology & medicine* 28 (2000) 64-74.
- [33] A.M. Olovnikov, A theory of marginotomy - The incomplete copying of template margin in enzymatic synthesis of polynucleotides and biological significance of the phenomenon, *Journal of Theoretical Biology* 41 (1973) 180-190.
- [34] J.D. Watson, Origin of concatameric T7 DNA, *Nature: New Biology* 239 (1972) 197-201.
- [35] C.W. Greider, E.H. Blackburn, Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts, *Cell* 43 (1985) 405-413.
- [36] K.J. Buchkovich, C.W. Greider, Telomerase regulation during entry into the cell cycle in normal human T cells, *Molecular biology of the cell* 7 (1996) 1443-1454.
- [37] K. Hiyama, Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M.A. Piatyszek, J.W. Shay, S. Ishioka, M. Yamakido, Activation of telomerase in human lymphocytes and hematopoietic progenitor cells, *J Immunol* 155 (1995) 3711-3715.
- [38] C. Harle-Bachor, P. Boukamp, Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes, Proceedings of the National Academy of Sciences of the United States of America 93 (1996) 6476-6481.
- [39] H.J. Sommerfeld, A.K. Meeker, M.A. Piatyszek, G.S. Bova, J.W. Shay, D.S. Coffey, Telomerase activity: a prevalent marker of malignant human prostate tissue, *Cancer research* 56 (1996) 218-222.
- [40] B.V. Kallakury, T.P. Brien, C.V. Lowry, P.J. Muraca, H.A. Fisher, R.P. Kaufman, Jr., J.S. Ross, Telomerase activity in human benign prostate tissue and prostatic adenocarcinomas, *Diagn Mol Pathol* 6 (1997) 192-198.
- [41] K.S. Koeneman, C.X. Pan, J.K. Jin, J.M. Pyle, 3rd, R.C. Flanigan, T.V. Shankey, M.O. Diaz, Telomerase activity, telomere length, and DNA ploidy in prostatic intraepithelial neoplasia (PIN), *The Journal of urology* 160 (1998) 1533-1539.
- [42] Y. Lin, H. Uemura, K. Fujinami, M. Hosaka, M. Harada, Y. Kubota, Telomerase activity in primary prostate cancer, *The Journal of urology* 157 (1997) 1161-1165.
- [43] Y. Lin, H. Uemura, K. Fujinami, M. Hosaka, Y. Iwasaki, H. Kitamura, M. Harada, Y. Kubota, Detection of telomerase activity in prostate needle-biopsy samples, *The Prostate* 36 (1998) 121-128.

- [44] C. Takahashi, I. Miyagawa, S. Kumano, M. Oshimura, Detection of telomerase activity in prostate cancer by needle biopsy, *European urology* 32 (1997) 494-498.
- [45] W. Zhang, L.R. Kapusta, J.M. Slingerland, L.H. Klotz, Telomerase activity in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic epithelium, *Cancer research* 58 (1998) 619-621.
- [46] C.A. Fordyce, C.M. Heaphy, N.E. Joste, A.Y. Smith, W.C. Hunt, J.K. Griffith, Association between cancer-free survival and telomere DNA content in prostate tumors, *The Journal of urology* 173 (2005) 610-614.
- [47] A.K. Meeker, H.J. Sommerfeld, D.S. Coffey, Telomerase is activated in the prostate and seminal vesicles of the castrated rat, *Endocrinology* 137 (1996) 5743-5746.
- [48] N. Ravindranath, S.L. Ioffe, G.R. Marshall, S. Ramaswamy, T.M. Plant, M. Dym, Androgen depletion activates telomerase in the prostate of the nonhuman primate, *Macaca mulatta*, *The Prostate* 49 (2001) 79-89.
- [49] K.A. Iczkowski, W. Huang, R. Mazzucchelli, C.G. Pantazis, G.R. Stevens, R. Montironi, Androgen ablation therapy for prostate carcinoma suppresses the immunoreactive telomerase subunit hTERT, *Cancer* 100 (2004) 294-299.
- [50] C. Guo, B.N. Armbruster, D.T. Price, C.M. Counter, In vivo regulation of hTERT expression and telomerase activity by androgen, *The Journal of urology* 170 (2003) 615-618.
- [51] U. Moehren, M. Papaioannou, C.A. Reeb, A. Grasselli, S. Nanni, M. Asim, D. Roell, I. Prade, A. Farsetti, A. Baniahmad, Wild-type but not mutant androgen receptor inhibits expression of the hTERT telomerase subunit: a novel role of AR mutation for prostate cancer development, *Faseb J* 22 (2008) 1258-1267.
- [52] A.T. Collins, N.J. Maitland, Prostate cancer stem cells, *Eur J Cancer* 42 (2006) 1213-1218.
- [53] D.G. Tang, L. Patrawala, T. Calhoun, B. Bhatia, G. Choy, R. Schneider-Broussard, C. Jeter, Prostate cancer stem/progenitor cells: identification, characterization, and implications, *Molecular carcinogenesis* 46 (2007) 1-14.
- [54] N. Sharifi, E.M. Hurt, W.L. Farrar, Androgen receptor expression in prostate cancer stem cells: is there a conundrum?, *Cancer chemotherapy and pharmacology* 62 (2008) 921-923.
- [55] A.K. Meeker, W.R. Gage, J.L. Hicks, I. Simon, J.R. Coffman, E.A. Platz, G.E. March, A.M. De Marzo, Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining, *The American journal of pathology* 160 (2002) 1259-1268.
- [56] A.K. Meeker, J.L. Hicks, E.A. Platz, G.E. March, C.J. Bennett, M.J. Delannoy, A.M. De Marzo, Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis, *Cancer research* 62 (2002) 6405-6409.
- [57] L. Harrington, Does the reservoir for self-renewal stem from the ends?, *Oncogene* 23 (2004) 7283-7289.
- [58] M.J. Greenwood, P.M. Lansdorp, Telomeres, telomerase, and hematopoietic stem cell biology, *Archives of medical research* 34 (2003) 489-495.
- [59] D. Ponti, A. Costa, N. Zaffaroni, G. Pratesi, G. Petrangolini, D. Coradini, S. Pilotti, M.A. Pierotti, M.G. Daidone, Isolation and in vitro propagation of

- tumorigenic breast cancer cells with stem/progenitor cell properties, *Cancer research* 65 (2005) 5506-5511.
- [60] K.M. Bussard, C.V. Gay, A.M. Mastro, The bone microenvironment in metastasis; what is special about bone?, *Cancer metastasis reviews* 27 (2008) 41-55.
- [61] T. Drewa, J. Styczynski, Can conception of prostate cancer stem cells influence treatment dedicated to patients with disseminated disease?, *Medical hypotheses* 71 (2008) 694-699.
- [62] S. Riethdorf, H. Wikman, K. Pantel, Review: Biological relevance of disseminated tumor cells in cancer patients, *International journal of cancer* 123 (2008) 1991-2006.
- [63] J.G. Moreno, M.C. Miller, S. Gross, W.J. Allard, L.G. Gomella, L.W. Terstappen, Circulating tumor cells predict survival in patients with metastatic prostate cancer, *Urology* 65 (2005) 713-718.
- [64] K. Fizazi, L. Morat, L. Chauveinc, D. Prapotnich, R. De Crevoisier, B. Escudier, X. Cathelineau, F. Rozet, G. Vallancien, L. Sabatier, J.C. Soria, High detection rate of circulating tumor cells in blood of patients with prostate cancer using telomerase activity, *Ann Oncol* 18 (2007) 518-521.
- [65] C.B. Harley, Telomerase and cancer therapeutics, *Nature reviews* 8 (2008) 167-179.
- [66] J.W. Shay, W.N. Keith, Targeting telomerase for cancer therapeutics, *British journal of cancer* 98 (2008) 677-683.
- [67] S. Gryaznov, A. Asai, Y. Oshima, Y. Yamamoto, K. Pongracz, R. Pruzan, E. Wunder, M. Piatyszek, S. Li, A. Chin, C. Harley, S. Akinaga, Y. Yamashita, Oligonucleotide N3' --> P5' thio-phosphoramidate telomerase template antagonists as potential anticancer agents, *Nucleosides, nucleotides & nucleic acids* 22 (2003) 577-581.
- [68] A. Asai, Y. Oshima, Y. Yamamoto, T.A. Uochi, H. Kusaka, S. Akinaga, Y. Yamashita, K. Pongracz, R. Pruzan, E. Wunder, M. Piatyszek, S. Li, A.C. Chin, C.B. Harley, S. Gryaznov, A novel telomerase template antagonist (GRN163) as a potential anticancer agent, *Cancer research* 63 (2003) 3931-3939.
- [69] B.S. Herbert, G.C. Gellert, A. Hochreiter, K. Pongracz, W.E. Wright, D. Zielinska, A.C. Chin, C.B. Harley, J.W. Shay, S.M. Gryaznov, Lipid modification of GRN163, an N3'-->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition, *Oncogene* 24 (2005) 5262-5268.
- [70] B.K. Canales, Y. Li, M.G. Thompson, J.M. Gleason, Z. Chen, B. Malaeb, D.R. Corey, B.S. Herbert, J.W. Shay, K.S. Koeneman, Small molecule, oligonucleotide-based telomerase template inhibition in combination with cytolytic therapy in an in vitro androgen-independent prostate cancer model, *Urologic oncology* 24 (2006) 141-151.
- [71] B. Minev, J. Hipp, H. Firat, J.D. Schmidt, P. Langlade-Demoyen, M. Zanetti, Cytotoxic T cell immunity against telomerase reverse transcriptase in humans, *Proceedings of the National Academy of Sciences of the United States of America* 97 (2000) 4796-4801.
- [72] R.H. Vonderheide, S.M. Domchek, J.L. Schultze, D.J. George, K.M. Hoar, D.Y. Chen, K.F. Stephans, K. Masutomi, M. Loda, Z. Xia, K.S. Anderson, W.C. Hahn,

- L.M. Nadler, Vaccination of cancer patients against telomerase induces functional antitumor CD8⁺ T lymphocytes, *Clin Cancer Res* 10 (2004) 828-839.
- [73] Z. Su, J. Dannull, B.K. Yang, P. Dahm, D. Coleman, D. Yancey, S. Sichi, D. Niedzwiecki, D. Boczkowski, E. Gilboa, J. Vieweg, Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8⁺ and CD4⁺ T cell responses in patients with metastatic prostate cancer, *J Immunol* 174 (2005) 3798-3807.
- [74] P.F. Brunsvig, S. Aamdal, M.K. Gjertsen, G. Kvalheim, C.J. Markowski-Grimsrud, I. Sve, M. Dyrhaug, S. Trachsel, M. Moller, J.A. Eriksen, G. Gaudernack, Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer, *Cancer Immunol Immunother* 55 (2006) 1553-1564.
- [75] A.A. Chanan-Khan, N. C. Munshi, M. A. Hussein, L. Elias, F. Benedetti, J. Smith, S. Khor and C. A. Huff, Results of a Phase I Study of GRN163L, a Direct Inhibitor of Telomerase, in Patients with Relapsed and Refractory Multiple Myeloma (MM) 50th ASH Annual Meeting (2008).
- [76] A.G. Bodnar, N.W. Kim, R.B. Effros, C.P. Chiu, Mechanism of telomerase induction during T cell activation, *Experimental cell research* 228 (1996) 58-64.
- [77] L. Vezzi, G. Parmiani, Limitations of the cancer stem cell theory, *Cytotechnology* 58 (2008) 3-9.
- [78] R.P. Hill, Identifying cancer stem cells in solid tumors: case not proven, *Cancer research* 66 (2006) 1891-1895; discussion 1890.

Figure legends

Figure 1. Prostate cancer cell lines have telomerase activity and short telomeres **A.** Telomere Repeat Amplification Protocol (TRAP) on cell lysates from different prostate cell lines. Normal primary prostate epithelial cells are telomerase negative. The internal amplification standard used for quantifying telomerase activity levels is indicated by an arrow. **B.** Terminal Restriction Fragment (TRF) assay on telomere DNA extracted from three different prostate cancer cell lines.

Figure 2. Telomere dysfunction and telomerase activation play an important role in prostate malignant transformation

Figure 3. Putative prostate tumor-initiating cells have telomerase activity which can be inhibited by telomerase inhibitors (GRN163L) Different populations of tumor-initiating cells were isolated by flow cytometry using surface markers (CD44 and CD133) and equal cell lysate amounts were used for the TRAP assay. The cells were pre-treated for 72 hours with the telomerase inhibitor drug (2 μ M) before sorting. HeLa cells were used as positive controls. The internal amplification standard is indicated by an arrow.

Figure 4. Telomerase inhibitors used in combination with standard therapies can target tumor-initiating cells Tumor-initiating cells escape conventional therapies and

are responsible for relapse but telomerase inhibition can target all the tumor cell fractions and lead to a durable response.

ACCEPTED MANUSCRIPT

DT

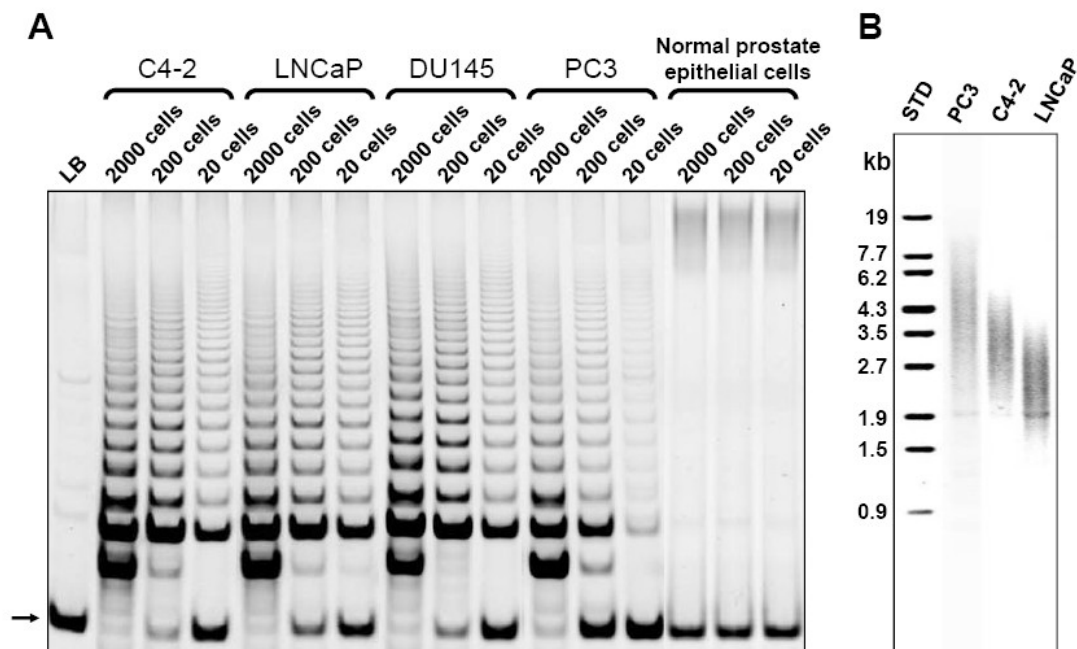
Percentage of DU145 CD133+ cells			
Days in culture	14	30	33
Monolayer*	0.19	0.19	0.18
Spheroids**	0.21	0.19	0.21

* The sorted CD 133+ cells were cultured in DMEM/199 media with 10% FBS
** The sorted CD133+ cells were cultured as spheroids in DMEM/F12 media

Table 1. The percentage of DU145 CD133+ cells is maintained at low levels in culture after initial FACS isolation in both monolayer and spheroid cultures

ACCEPTED

Figure 1



ACCE

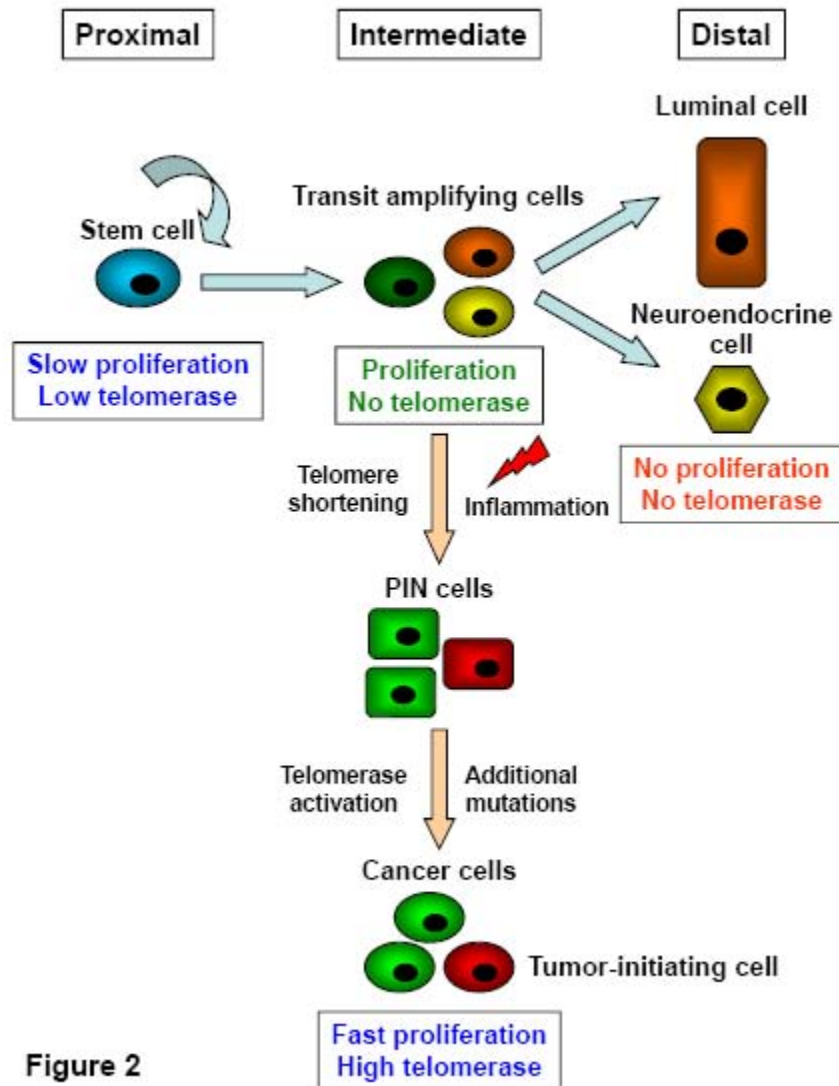
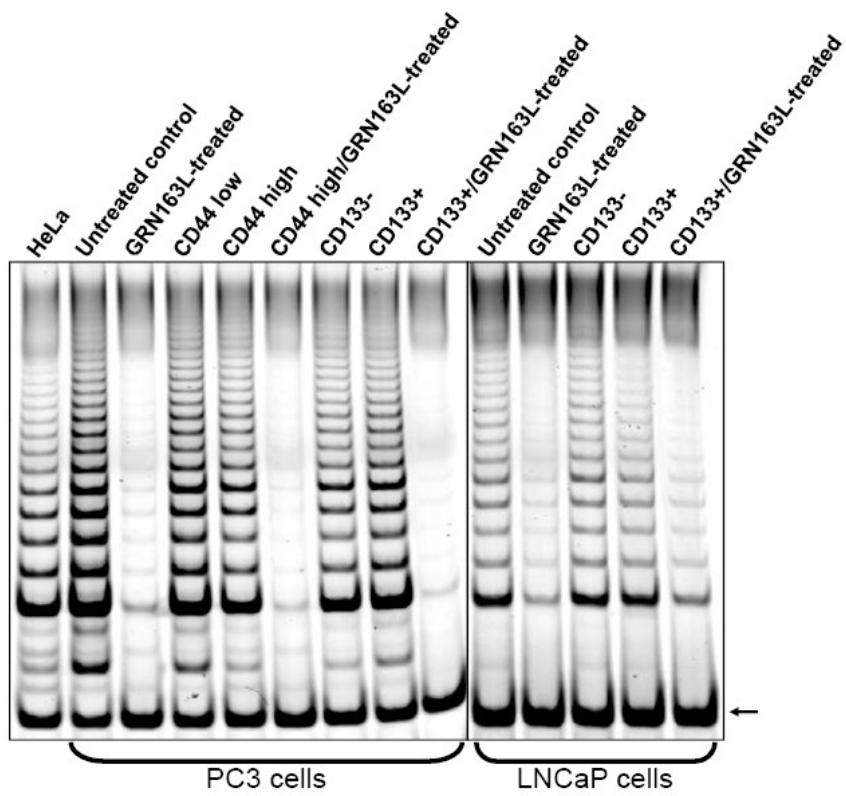


Figure 2

Figure 3



A

Figure 4

