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Characterization of hTERT-immortalized Prostate-derived Stromal and Epithelial Cells: an Authentic *in vitro* Model for Tumor Microenvironment Studies

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Abstract

Tumor development begins with mutational changes to the genetic makeup of a cell; tumor progression is not solely determined by the mutated cell, but also by the tumor's microenvironment. Prostate cancer, a leading cancer diagnosed in men, has been determined to be highly influenced by its surrounding stroma, particularly fibroblasts. It has been demonstrated that cancer-associated prostate fibroblasts (CAFs) differ from normal-associated prostate fibroblasts (NAFs). However, human prostate cancer model systems have focused largely on prostate cancer epithelial cells. Currently, a need exists for a more physiologically relevant human cell model system to study prostate cancer progression within the context of its tumor microenvironment. In this study, we characterized three prostate-derived cells: CAFs, NAFs and prostate epithelial cells (PrEs); all three lines were immortalized by human telomerase reverse transcriptase (hTERT) alone and have been continuously passaged for more than 40 PDL in our hands. Our data shows that the hTERT immortalized CAFs proliferate faster than the NAFs; in addition, both CAFs and NAFs express fibroblast markers such as TE7 and alpha smooth muscle actin (α-SMA), while neither cell line expresses epithelial marker such as CK14. Both CAFs and NAFs also express elevated levels of α-SMA upon TGF-β stimulation. All three prostate-derived cells weakly express the prostate specific marker AR, and show similar markers staining after extended culture. Importantly, conditioned media collected from CAFs is more effective at promoting tumor cell growth than NAF conditioned media. In conclusion, CAFs, NAFs, and immortalized PrEs provide a very valuable model system for the study of prostate cancer cell progression and tumor microenvironment studies.

Introduction

Prostate cancer remains a common cancer with an estimated 180,000 new cases each year, representing about 11% of all new cancer cases in the US in 2016. Over two million men are estimated to be living with prostate cancer and men have a 13 percent chance of being diagnosed with prostate cancer at some point in their lifetime[1]. Mortality rates for prostate cancer have remained consistent over the past decade[2]. Further, treatment options have not changed although prostate cancer animal models are available for use in development of new treatments. In fact, a comprehensive analysis of genetically engineered mouse models identified that invasive prostate cancers are associated with the presence of fibroblast or myofibroblast cells. Additionally, these cells were later observed in a subset of human cancers[3]. It is known that prostate cancer progression results not only from genetic changes within a cell, but also by the tumor microenvironment[4], which includes the underlying connective tissue and fibroblasts, myofibroblasts, endothelial cells and immune cells. However, the mechanisms behind prostate cancer progression remain poorly understood, in part due to a lack of human cellular models. Developing human cell models is difficult, particularly human prostate cells which are difficult to grow continuously in culture. Thus, an *in vitro* human cell model that incorporates normal prostate cells and their surrounding stromal cells is vital to understanding prostate cancer progression mechanisms.

Results

1. The hTERT Immortalized Prostate Derived Cells Grow Continuously

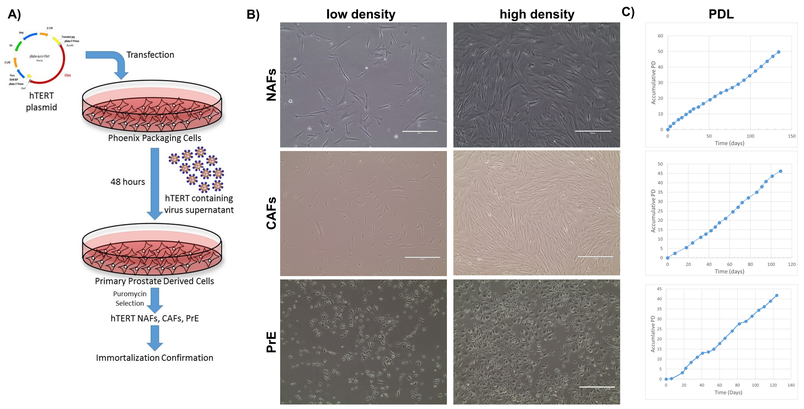

Figure 1. Prostate derived fibroblast and epithelial cells grow continuously in culture. **(A)** NAFs, CAFs and PrEs were immortalized by introducing hTERT[5] and selected with puromycin. Successful introduction of hTERT was confirmed by TeloTAGGG™ telomere length assay and telomeric repeat amplification protocol (TRAP) assay. **(B)** The selected clones grew continuously in culture for more than 40 population doublings (PD). **(C)** As depicted in the graphs, CAFs grow faster than NAFs, reaching 40 population doublings in under 100 days. Scale bar, 400μm.

2. Prostate-associated Fibroblasts Express Fibroblast Specific Markers

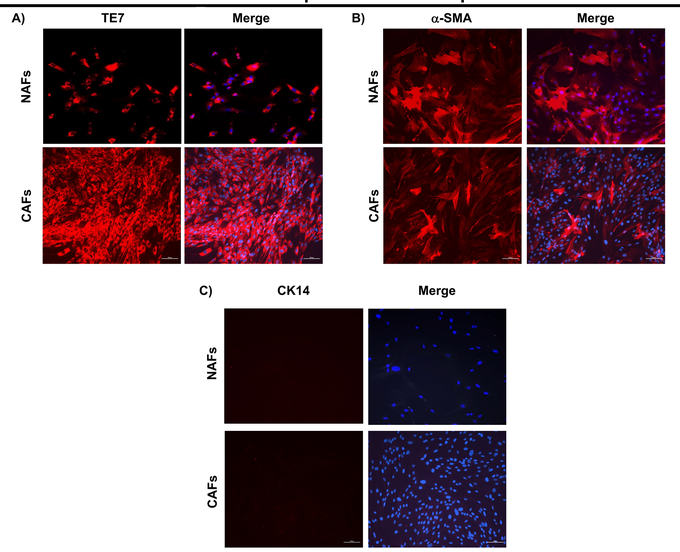

Figure 2. NAFs and CAFs express fibroblast specific markers. NAFs and CAFs cells were fixed using 4% paraformaldehyde, then immunostained with primary antibodies to **(A)** anti-human fibroblast (TE7), **(B)** alpha-smooth muscle actin (α-SMA), and **(C)** a cytokeratin 14 (CK14), then followed by staining with a secondary fluorescent antibody (red). The nucleus was stained with DAPI (blue), imaged with a fluorescent microscope, and a composite image was generated (Merge). Both cell lines positively stained for TE7 and α-SMA, but not for CK14, suggesting CAFs and NAFs are mesodermal derived human connective tissue. (N=5) . Scale bar, 100μm.

3. Normal Prostate Epithelium Express Epithelial Specific Markers

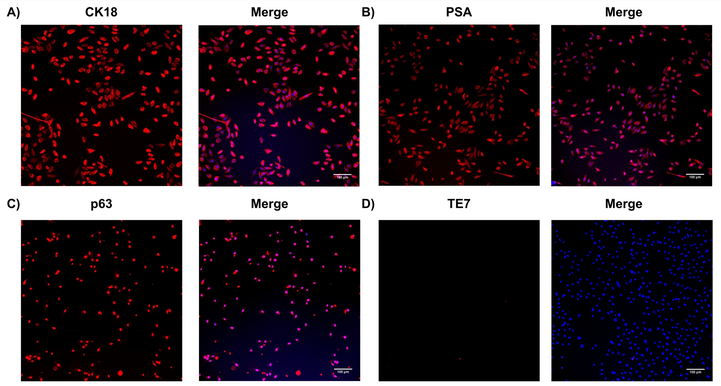

Figure 3. PrEs express epithelial markers, but do not express a fibroblast marker. PrEs were fixed using 4% paraformaldehyde, then immunostained with primary antibodies to **(A)** Cytokeratin 18 (CK18), **(B)** Prostate Specific Antigen (PSA), **(C)** p63 and **(D)** the anti-human fibroblast (TE7), then followed by staining with a secondary fluorescent antibody (red). The nucleus was stained with DAPI (blue), imaged with a fluorescent microscope, and a composite image was generated (Merge). PrEs show expression of CK18, PSA and p63, but do not express TE7, suggesting PrEs are of prostate origin. (N=2). Scale bar, 100μm.

4. α-Smooth Muscle Actin is Upregulated in Prostate Derived Fibroblasts

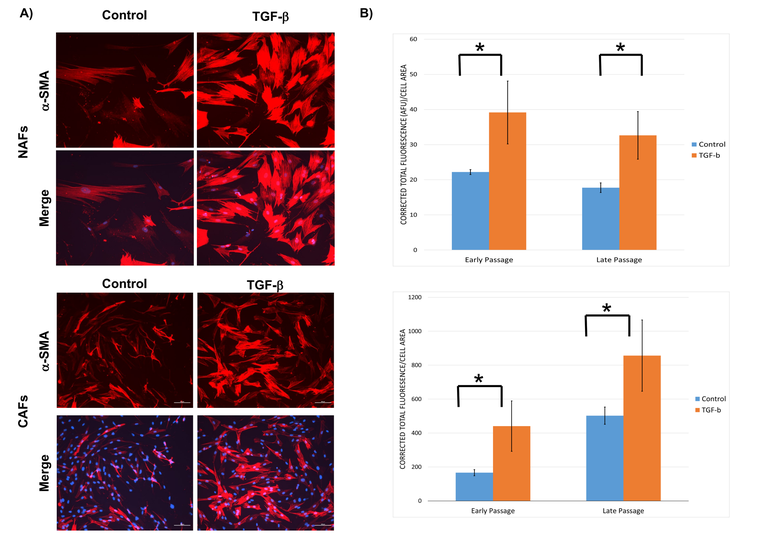

Figure 4. α-SMA upregulation in NAFs and CAFs cells when stimulated with TGF-β. NAFs and CAFs were untreated (control) or treated with 1ng/ml TGF-β. **(A)** After 24 hours, cells were fixed with 4% paraformaldehyde and immunostained with primary antibodies to alpha-smooth muscle actin (α-SMA), then followed by staining with a secondary fluorescent antibody (red). The nucleus was stained with DAPI (blue), then imaged with a fluorescent microscope and a composite image was generated (Merge). **(B)** Multiple fields of view were quantified for total cell fluorescence and normalized by the total area of the cells using ImageJ software. α-SMA levels increased in both early and late passage NAFs and CAFs cells after TGF-β stimulation. (N=3, Students t-test, * P<0.05). Scale bar, 100μm.

5. CAFs Conditioned Media Promotes Tumor Cell Growth.

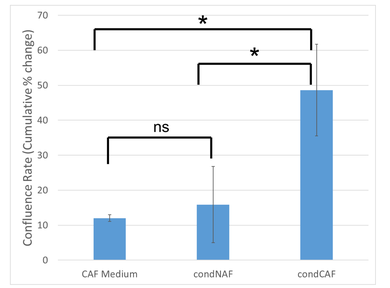

Figure 5. Conditioned medium from cancer-associated fibroblast enhances the growth rate of LnCap clone FGC cancer cells while conditioned medium from normal-associated fibroblast does not. Human prostate carcinoma cells, LnCap clone GFC cells (ATCC® CRL-1740™), were plated onto a 12-well plate at subconfluent levels, allowed to adhere, and then incubated in an Incucyte FLR© imaging system in the presence of four distinct growth mediums, including standard growth medium, conditioned normal-associated fibroblast (condNAF) medium, unconditioned cancer-associated fibroblast (CAF) medium, and conditioned cancer-associated fibroblast medium (condCAF). Growth was then monitored by confluence. The confluence rate was determined by the slope of the line during the exponential phase of growth. Percent change was calculated by normalization with growth in the standard growth medium. The data demonstrated that LnCap clone FGC cells have a significantly enhanced growth rate in condCAF medium, suggesting that cancer-associated fibroblasts, from which the medium was collected, may promote cancer cell growth. (N=3, Students t-test, ns = not significant, * P<0.05).

References

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Summary

- Prostate hTERT immortalized fibroblast and epithelial cells have been grown continuously for over 100 days without a change in growth rate or senescence.
- Normal-associated and cancer-associated fibroblasts express fibroblast specific markers, but do not express an epithelial specific marker.
- Prostate derived fibroblasts respond to stimulation with TGF-β, by increasing expression of α-SMA.
- Normal prostate epithelial cells express epithelial specific markers, but do not express a fibroblast specific marker.
- Cancer-associated fibroblast medium significantly enhanced the growth of cancer cells.
- These three characterized cell lines provide a human cell model for the study of prostate cancer progression.