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In Vitro Propagation and Characterization of Primary Prostate Tumor Stem/Progenitor Cells

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

by

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2009
The Thesis of Tuan T. Le is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2009
DEDICATION

To my mother, father, and brothers, for driving me to succeed in whatever I do, and believing in me when no one else will. Your love and support has made me the person I am today.
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This thesis, in part, has been submitted for publication December 2008 (Finones RR, Wu C, Yeargin J, Le T, Baird SM, and Haas, M. “Cultured Human Tumor Stem Cells and the Prostate Tumor Stem Cell Niche Generate Prostate Glands in Vivo”). The thesis author is the co-author of this paper.
ABSTRACT OF THE THESIS

In Vitro Propagation and Characterization of Primary Prostate Tumor Stem/Progenitor Cells

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Master of Science in Biology
University of California, San Diego, 2009

Professor Martin Haas, Chair

To better understand the underlying mechanism behind the recurrence of end-stage, androgen depletion independent prostate cancer (ADI), we have successfully characterized primary human prostate tumor cells in vitro. This thesis presents a reliable culture system that consistently propagates and maintains epithelial cultures taken directly from early-stage prostate adenocarcinomas. As a result of our methodology, we were able to put 70 prostate radical resection tissue samples into culture: 42 adenocarcinomas and 28 benign hyperplasias. All 42 early-stage cancers produced few (3-5) to many (400+) single epithelial colonies and expressed putative stem/progenitor cell markers CD44, integrin-α2β1, High MW CK 5/14, and CD133 along with embryonic transcription factors Oct4, Sox-2, and immortalization gene
Bmi-1. Every proliferating colony possessed a naturally formed prostate stem cell niche, which supplied the highly epithelial prostate progenitors. These presumed stem cell niches, we termed “Stem Cell Centers” (SCC), stained positive for stem cell marker CD133. Senescence was observed in all cultures in later passages due to the loss of the necessary SCC. Our methodology is optimal for reliably growing out early-stage prostate tumor stem/progenitor cells – which is essential in the understanding of lethal ADI progression – and allows for the development of better therapies and treatments for this metastatic disease.
INTRODUCTION

Prostate Cancer general facts

Prostate cancer is the most commonly diagnosed malignancy among men in the United States, with approximately 186,320 new cases projected in this year alone (1). Nearly 20% of patients initially diagnosed with prostate cancer acquire the lethal recurrent form of the disease, leading to the second most frequent cause of male cancer-related deaths in the U.S. (1).

The prostate’s primary function is the production of seminal fluid within the male reproductive tract. Epithelial glands and a fibromuscular stroma comprise the sex-specific organ. Within the glandular epithelium reside three cell types: basal, luminal secretory, and neuroendocrine. A subset of basal cells (<1%) contains epithelial stem cells, the main source for luminal cell differentiation (2). Luminal cells then secrete the necessary components of prostatic fluid, express androgen receptor (AR), and secrete prostate specific androgen (PSA) in an androgen-dependent manner. Since PSA is almost exclusively a product of prostate cells, it is commonly used as a tumor marker for diagnosis of prostate cancer and monitoring the effectiveness of treatment (15).

Prostate cancer as a distinct disease

Progressive prostate cancer is most commonly treated using androgen ablation therapy, causing initial regression in all cases due to the dependent nature of early-stage prostate cancer. The paracrine androgen signaling – mediated through the
androgen receptor (AR) in the prostate – allows for its cell survival. A growing cancer has a chance of assuming an androgen depletion-independent state through bypassing the normal androgen signaling processes and using alternate pathways to sustain and expand itself (3).

The clinical course of prostate cancer occurs in two phases. The first phase is an androgen dependent cancer responsive to castration and/or anti-androgen therapies preventing the synthesis of relevant androgens. The second phase is an androgen insensitive prostate cancer unresponsive to androgen ablation interventions. This lethal phenotype – termed androgen depletion-independent prostate cancer (ADI) – recurs despite any available treatments, eventually killing the patient (3). The mechanism behind the androgen-dependent to androgen-independent transition remains to be elucidated.

Upon gaining this lethal phenotype, almost 90% of cases result in bone metastasis, making it the leading cause of morbidity for this disease. The replacement of hematopoietic tissue with the invading tumor cells causes anemia and increases susceptibility to infection. Severe pain, fractures, and spinal cord compression are a few of the symptoms accompanying this advanced stage of this lethal disease (4).

**Characterization of the Prostate Stem Cell**

The epithelial prostate stem cell is a major player in prostate cancer development. Its characteristic robust longevity assures a continued presence during long latency between exposure and cancer development (5). The strongest evidence for the existence of such prostate stem cells comes from the remarkable regenerative
capacity of both human and rodent prostates (51, 52). Androgen withdrawal results in the apoptosis of the majority of luminal epithelial cells while the basal cells remain intact. Upon androgen replacement, the luminal cell layer successfully regenerates, suggesting that stem cells dwell within the basal layer. It has been shown that a small population of human prostate basal cells expresses the stem cell surface marker CD133 (5). These cells (<1% of basal cell population) are restricted to the $\alpha_2\beta_1^{hi}$ population, another previously established marker for epithelial stem cells in prostate epithelia (6).

Prostate epithelial stem cells exhibit important features characteristic of prototypical stem cells. Notably, these cells possess high in vitro proliferative potential, and were shown to self-renew while maintaining the prostate’s associated secretory activity (5). These properties are indicative of stem cells existing within the prostate.

Prostate stem cells can be isolated and characterized using some or all of the following phenotype: CD44$^+/CD133^+/\alpha_2\beta_1^{hi}$/high molecular weight CK $5^+$/or $14^+/p63^-$/AR$^-$/PSA$^-$ (7, 9, 10, 12, 13). This small population is androgen receptor negative, supporting the notion that these cells serve as a cancer target for mutagenesis (9). Stem cells and cancer cells have been known to share similar signaling pathways (10), further validating this claim. In either case, prostate stem cells do exist and are considered major players in the field of prostate cancer development and maturation.

**Tumor-initiating cells share properties with normal prostate stem cells**

Prostate cancer’s developmental origin arises from a stem cell-like metastasis-initiating cell (14), known as the prostate cancer stem cell (PrCaSC). Similar to a
normal prostate stem cell, these androgen-insensitive cells can proliferate and self-renew, contributing to phenotypically diverse populations (10). This established cancer stem cell hypothesis is based off of the observed self-renewing and differentiating capacity of tumors (8) procured through sequential mutagenesis of an existing progenitor or stem cell (11, 14). The subsequent malignant tumor is initiated and maintained by this minor population of transformed cells sharing similar biological properties to normal adult stem cells (8, 16).

Still, major differences exist between cancer stem cells and adult stem cells, particularly in their regulatory machinery. In a normal prostate stem cell pool, a balance between self-renewal and differentiation is strictly regulated. Its cancer stem cell counterpart lacks these homeostatic restraints, differentiating and proliferating in an uncontrolled manner (13).

As a result, cancer stem cells are able to resist apoptosis and drive the growth and metastasis of tumors through utilization of its proliferating, self-renewing, differentiating, and survival capabilities (17). Cancer inducing mutations within the prostate progenitor/stem cells stimulate tumor initiation and eventually metastasize in androgen-refractory prostate cancer (11). Deciphering the direct linear relationships of normal prostate progenitor/stem cells and the probable cell lineages arising from transformation, is essential in understanding the underlying mechanism in metastatic prostate cancer. Though prostate tumor epithelial cells must first be propagated and maintained in culture to even merit further consideration into metastasis causation, as will be demonstrated in this thesis.
**Progression of early-stage to late-stage ADI lethal prostate cancer**

The dependence on androgens for growth and survival mark the distinct difference between the early treatable prostate tumor versus its recurrent lethal form. As stated earlier, all prostate cancers initially rely on androgens, making androgen ablation therapy the central therapeutic intervention for progressive prostate cancer. This primary treatment has proven very effective in causing the initial regression (15). Yet, for some, tumor progression is inevitable.

Possible reasons pushing towards hormone independency include gene modifications. Genetic change and adaptation early on significantly help to facilitate the evolution of this disease (18). The general increase in mutation rate then amplifies the probability of the prostate cancer cell to grow independently of androgen. These mutations possibly mark an early event independent from the selective pressure inflicted by the androgen blockade (19).

The particular targeting of the androgen signaling pathway provided by androgen ablation therapy (20) can also drive recurrence. The cancer cells’ response to such treatment includes boosting its sensitivity to extremely low androgen levels or lowering its androgen activation threshold (21). Selection for clonal expansion of a promiscuous androgen receptor can enable non-androgenic molecules present in circulation to activate it (22), producing a similar outcome.

Also, the induction of complementary or alternative pathways can bypass the androgen-mediated pathway completely. Upon androgen deprivation, mutations up-regulating parallel pathways could be selected as a substitute for signaling cell survival (15). An additional scenario in this androgen-depleted environment is the propagation
of a pre-existing subpopulation of androgen-independent tumor cells. These putative malignant epithelial “stem cells” remain viable post androgen withdrawal – and continue proliferation – ultimately resulting in a relapse of ADI disease (2).

Prostate cancer comprises a heterogeneous mixture of cells varying in their dependence on androgen for growth and survival (15). A selective pressure – either before or after androgen ablation therapy – alters the relative frequency of these cells spawning outgrowths of androgen-independent cancers.

**Circulating/Disseminating Tumor cells as intermediate stage**

A necessary prerequisite for tumor metastasis involves the intermediate dissemination step of prostate cancer cells to secondary sites via the vasculature. Post radical prostatectomy, a general elevation of prostate specific antigen (PSA) circulating in the blood or lymph nodes indicates the presence of these cells, known as disseminated/circulating tumor cells (DTC/CTC). They are necessary but not sufficient for true clinical metastasis (23, 24).

In all cases, the hiatus evident in the progression of cancer after diagnosis and treatment is indicative of a state of cancer dormancy (25). The observed cease of proliferation in these DTC cells represents the activation of an adaptive response to a stressful non-propitious environment, such as radiation (26). These dormant cancer cells carry mutations allowing them to persist by completely withdrawing from the cell cycle, and/or by maintaining a constant balance between apoptosis and proliferation (27). One of the aims of this thesis is to sidestep this intermediary dormant stage
using techniques that will be discussed later. This will establish a clear relationship between early and late-stage disease cancer stem cells.

**Cell Senescence and the protective pathways**

Cell senescence, or replicative senescence, refers to the mortality of an aging cell. It is an evolved phenomenon that limits the number of cell divisions before entering a postmitotic state, thus acting as a fail-safe mechanism protecting against cancer development (28). Cancers require a continuously self-renewing population, similar to adult stem cells. Cancerous mutations inactivate the usual constraints on normal stem cell expansion, causing defects in self-renewal response signals that normally promote this senescent process (29).

Alternatively, oncogenic mutations may allow the progenitor progeny to proliferate – without entering the typical postmitotic differentiated state – and escape senescence. A majority of normal prostate stem cells differentiate into these progenitor cell targets known as transit-amplifying cells (TA), which ordinarily have a limited growing/differentiating capacity. The aberrant activation of self-renewal regulatory machinery in these TA cells imbues them with stem cell-like properties. Additional mutations can then accumulate in this newly created self-renewing pool of cells (17), ultimately leading to carcinogenesis.

Several protective pathways maintain this senescent phenotype. Four vital pathways in particular tightly regulate the mortality of cells: the Telomere-telomerase (TERT) link serving as an internal mitotic countdown clock (30), the p53/p21 pathway inducing growth arrest or apoptosis when necessary (31, 32), the tumor suppressing
Rb/p16 pathway (33) putting the brakes on major cell cycle growth signals, and the proto-oncogenic Ras/E2F pathway governing cellular proliferation (34). Cancer development emerges upon disruption of one or more of these crucial pathways.

Replicative senescence’s presumed role has been put to test using immortal Li-Fraumeni human fibroblasts, defective in the p53 allele (30). These immortal lines expressed no detectable p16 protein products, despite carrying an intact p16 gene. Expression of p16 and/or p21 successfully induced senescence in these cells, demonstrating the importance of the senescent mechanism in protecting these afflicted cells from an immortal cancerous phenotype.

Ribonucleoprotein telomerase (TERT) is an active player in the cell mortality arena. By catalyzing the addition of telomeric repeats to chromosomal DNA, telomeric loss from cell division is prevented, and therefore expands the cell’s life cycle. When senescent signals are triggered, telomerase activity halts. Constitutive telomerase expression aids in breaching the senescent restraints, and is consequently associated with a poor prognosis in various cancer types (35).

Overexpression of polycomb group gene Bmi-1 can also defy senescence in cells on the road to cancer (36). Bmi-1 delays senescence due to the negative regulation of one of its downstream targets, the INK4A-ARF locus encoding tumor suppressors p16 and p19arf. As a result, cell progression is no longer inhibited by p16’s presence (37), nor can p19arf prevent p53’s inactivation and degradation (38). For these reasons, Bmi-1 expression is observed to be elevated in progressive prostate tumors, exhibiting a marked predisposition toward metastatic dissemination (36).
Furthermore, Bmi-1’s effect has been shown to act in a dose-dependent manner in regulating the INK\textsuperscript{4a}-ARF locus (39). Doubling exogenous expression of this gene in transgenic mice lead to a significantly increased rate of tumorigenesis. Conversely, a two-fold reduction in Bmi-1 gene resulted in a considerable decrease of lymphomagenesis, ascribed to increased apoptosis (39).

**Heterogeneity of Prostate Cancer**

The heterogeneous mix of immortal (<1%) and mortal cells is a hallmark characteristic apparent in any malignant cancer. Defined as a heterogeneous disease (40, 41), prostate cancer is no different containing a small immortal stem cell-like pool together with a larger differentiated population composed of TA and neuroendocrine cells. Heterogeneity is also evident in androgen receptor expression, with frequent AR-positive and AR-negative tumor populations between and within the same patient (41).

A striking degree of heterogeneity especially exists in end-stage ADI prostate cancer. The numerous phenotypes – including a wide range in rate of tumor progression, proportions of PSA-expressing cells, degree of glandular differentiation, and variety of histological patterns (40) – demonstrate the highly diverse morphology, immunophenotype, and genotype within this hormone-refractory disease.

**Immortalization**

An essential step in the process of malignant transformation is immortalization acquisition. This necessary prerequisite to cancer induction circumvents the senescent
barrier and is the source behind the resultant heterogenetic phenotype (42). The
deregulation and disruption of the aforementioned senescent pathways by means of
immortalization, sufficiently recapitulates prostate carcinogenesis – maintaining a
stable telomere length while generating constitutive mitogenic signals. This thesis will
show the limited proliferative capacity of epithelial stem cells grown from early-stage
prostate tumors (PrTuSC) in vitro. We predict successful immortalization of these
early-stage cells will trigger the late stage transition to metastasis essential to the
understanding of the metastasis mechanism relevant to any epithelial-based
malignancy.

Immortalization, as previously mentioned, requires continuous telomere
maintenance. Yet, telomerase expression by itself is not adequate to induce
immortalization of human epithelial cells (43). A high-titer MSCV virus retroviral
packaging system (44, 45) modified with our own hTERT-expressing vector confirmed
this assertion, along with Susan Kasper’s and John Rhim’s findings (9, 46) – which
favor preexisting mutations when immortalized using only TERT. Particularly,
introduction of Bmi-1 in cooperation with TERT can sufficiently immortalize cells of
epithelial origin (47) with little chromosomal instability. The inactivation of the
Rb/p16\textsuperscript{INK4A} pathway using Bmi-1 combined with active telomerase expression has
virtually no affect on the cells’ differential program (48). Non-immortalized prostate
epithelial TERT-infected cultures were shown to consistently express high p16\textsuperscript{INK4a}
levels (49) indicating that spontaneous p16\textsuperscript{INK4a} silencing and telomerase activation
represent premalignant events.
An in-depth analysis outlining the in vitro propagation and characterization of human prostate tumor stem/progenitor cells (PrTuSC) will be the major aim of this thesis. By establishing a novel systematic approach to efficiently growing out these presumed tumor-initiating cells, a deeper understanding behind metastasis transformation can soon follow. This model can be useful to further unravel prostate cancer development and progression, and provide more effective treatment/prevention therapies of this highly prevalent disease.

A secondary goal in this thesis will demonstrate the successful in vitro immortalization of isolated prostate tumor stem/progenitor cells by Bmi-1 and TERT infection. Future in vivo metastasis studies can be conducted to firmly establish the early-to-late stage transition of these uniquely cultured immortalized cells.

Acknowledgement

This chapter, in part, has been submitted for publication December 2008 (Finones RR, Wu C, Yeargin J, Le T, Baird SM, and Haas, M. “Cultured Human Tumor Stem Cells and the Prostate Tumor Stem Cell Niche Generate Prostate Glands in Vivo”). The thesis author is the co-author of this paper.
MATERIALS AND METHODS

**RNA isolation and Reverse Transcription-PCR**

Total cellular RNA collected from monolayer and spherical prostate tumor cultures was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s instructions, and also treated with the RNase-free DNase set (Qiagen) to remove any genomic DNA contamination. Reverse transcription and amplification by polymerase chain reaction was accomplished in the same tube (1-step RT-PCR, Qiagen). 500 ng of total template RNA was incubated with: 1 µL of 1-step RT-PCR enzyme mix, 0.6 µM of each oligonucleotide primer, 400 µM of each dNTP, RNase-free water, and 5 µL of 5x 1-step RT-PCR buffer containing 12.5 mM MgCl₂, totaling 25 µL per RT-PCR reaction. Each reaction was run under the following thermocycling conditions (Applied Biosystems): preheated for 30 min at 50°C, annealed for 15 min at 95°C, then cycled 35 times at 1 min 95°C, 1 min 55°C, 1 min 72°C, and finally elongated for 10 min at 72°C. The RT-PCR products were analyzed by electrophoresis on 1.0% agarose gels with ethidium bromide and imaged by a UV transilluminator. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control in all reactions and mouse embryonic stem cell RNA (mES) was used as a positive control for the genes of interest. The following set of primers were used: Sox-2 forward 5’-CCCCCGCGGGCAATAGCA-3’, reverse 5’-TCGGCGCAGGGAGGAGATAC-3’; Oct-4 forward 5’-ACATCAAAGCTCTGAAAGAACT-3’, reverse 5’-CTGAATACCTTCCAAATAGAACCC-3’; Nanaog forward 5’-TCCAGTCCACCTTTGAATTCTTCTCC-3’, reverse 5’-
GAGTAATATCAGTTTCACAGATCTTCACAGG-3'; Rex-1 forward 5'-GC GTACGCAAATTTAAGTCCAGA-3', reverse 5'-
CAGCATCCTAAACAGCTCGCAGAAT-3'; hTERT forward 5'-
CGGAAGAGTGTCGAGGCAA-3', reverse 5'-GGATGAAGCGGAGTCTGGA-3';
Bmi-1 forward 5'-GGAGACCAGCAAGTATTGTCCTTTTG-3', reverse 5'-
CATTGCCTGGGCATCGTAAG-3'; GAPDH forward 5'-
ACCACAGTCCATGCCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

**Tissue resources and generation of primary cell cultures**

The samples obtained for these experiments originated from patients undergoing a radical prostatectomy procedure from a neighboring Veterans Affairs hospital. Informed consent was given in accordance with the IRB-approved guidelines for human subjects prior to collecting the primary human prostate samples used. Samples were processed and prepped for tissue culture the same day the samples were received. Proper preparation of the samples included washing twice in PBS, steriley cutting into small pieces (comparable to minced garlic), and digested at 37°C overnight in 150 U/ml collagenase (Sigma-Aldrich) in growth medium stock (without supplemental growth factors, bovine pituitary extract (BPE), or CaCl₂). After single cell digestion the primary tumor samples were centrifuged and frozen down at ~10⁷ cells/ml in 90% fetal bovine serum (Omega Scientific) 10% DMSO (Sigma-Aldrich), stored in -80°C until ready for use.

6-well tissue culture-treated plates (Corning) were pre-coated with laminin at 10 mg/ml (Sigma-Aldrich) in PBS for 1 hour at 37°C before use. Unattached residual
laminin was removed by two subsequent PBS washes. Recipe of growth medium for maintenance of prostate tumor samples in culture is as follows: keratinocyte serum-free medium (Gibco) supplemented with 10 ug/ml basic FGF (R&D), 40 ng/ml EGF (R&D), 58 ug/ml BPE (Gibco), 1 mM CaCl2, 0.025 wt % BSA (Sigma-Aldrich), 40 mM L-glutamine (Gibco), 12.5 ug/ml Gentamycin (Abraxis), and 2.5 ug/ml mM Fungizone (Omega Scientific).

The initial culture for each cell type began with rapid thawing of ~10^7 cells into DMEM with 10% FBS, inactivating the DMSO preservative, then centrifuged and resuspended into pre-warmed (to room temperature) growth medium. Optimal culture growth conditions included incubation in a 10% CO2, 5% O2 chamber at 37°C, along with daily fluid changes using room temperature growth medium. When confluence was reached (80-90%), cells were transferred using ATV solution (Gibco) and replated 1:3. ATV solution was deactivated using DMEM (Gibco) supplemented with 10% FBS (Omega Scientific), L-glutamine, Gentamycin, and Fungizone at the same concentrations as growth medium.

**Immunocytochemistry**

Cultured cells were grown directly onto coverslips coated with laminin using growth medium. These cells were fixed in 2% paraformaldehyde in PBS for 10 minutes, and permeabilized in PBS + 0.5% TritonX-100. Coverslips were blocked in 5% donkey serum, and then stained as instructed by manufacturer’s protocol. Primary antibodies were incubated overnight at 4°C using CD133 (Milteny Biotech), High molecular weight cytokeratin 5/14 (Dako, M0630), α2β1 integrin (Abcam), CD44
(SCBT). The appropriate phycoerythrin or AlexaFlour-conjugated secondary antibodies at a dilution of 1:750 for CK 5/14 and CD133 and 1:500 for all other markers (Jackson, Invitrogen) were subsequently incubated with the coverslips, and mounted in Prolong gold antifade with DAPI (Invitrogen). Images were captured with a Zeiss LSM 510 confocal. To show specificity of staining, the following controls were included for each corresponding marker: omission of primary antibody and isotype controls to mouse and rabbit antibodies (Jackson).

**Flow cytometry**

Selectively pooled colonies – derived from apparent stem cell colonies found in the initial confluent cultured plates– were transferred to 12-well plates (Corning) coated with laminin, and grown until reaching 80% confluency using growth media. The samples were then washed with PBS, trypsinized, and pelleted, ready to be either fixed with 2% paraformaldehyde for 20 minutes in permeabilization buffer (eBiosciences) for CK5/14, or stained live for CD133, α2β1, and CD44 labeling using cold BSA in HBSS for 30 minutes. Staining with primary antibodies proceeded (same antibodies used for immunocytochemistry), followed by secondary antibody labeling using both AlexaFlour488 (Invitrogen) and PE (Jackson Research Laboratories). Propidium iodide was used to counterstain all live cells. Cells were sorted and analyzed by flow cytometry (BD). Sort gates were defined in reference to negative and isotype controls corresponding to each staining condition (CellQuest).
**Growth and senescence assay**

Images were taken immediately when first signs of colony growth appeared. The following time-points were recorded using phase contrast (Leitz) following noted growth: 0 h, 24 hr, and 48 h. NIH ImageJ was used to quantify samples. Approximately $10^6$ cells were replated to 12-well plates (Corning) in growth medium after the first week of growth. This allowed for overnight tumor culture sphere generation, imaged by phase contrast. The senescence assay involved detection by beta-galactocidase. A previous staining protocol was followed (50). Cells were initially rinsed with PBS, fixed in 2 % paraformaldehyde/0.2 % glutaraldehyde, and stained with 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside, Sigma B4252) overnight at room temperature.

**Acknowledgement**

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RESULTS

Propagation of epithelial stem cells derived from primary tumor samples (early-stage disease)

Our culture system consistently and successfully produced stem/progenitor cell colonies derived from early-stage tumors. Benign prostate hyperplasia (BPH) samples on the other hand, failed to yield any signs of colonial growth.

Our selected growth medium was initially screened from over 36 media types. Seventy primary prostate samples were put in culture comprising of 42 diagnosed adenocarcinomas and 28 diagnosed benign hyperplasias. The adenocarcinoma samples generated single cell colonies after the initial 7 days in culture, and retained about a 20 hour cell doubling time until senescence. A single colony showed expansion to \(~10^9\) cells in approximately 30 population doublings. These tumor-derived samples in particular, gave wide ranges of single cell colonies, varying from very few (3-5) to hundreds of colonies per 50 mg vial of cells (\(~10^7\)). The lack of any observed colony formation in the BPH samples correspond to an absence of epithelial stem/progenitor cells entirely.

Tightly-packed epithelial cell colonies, designated as prostate epithelial cells (PrEp), were consistently observed from the early-stage tumor samples grown in our culture conditions (Figure 1). The proliferating colonies mostly consisted of a crowded epithelial morphology, similar to cobblestones. Upon reaching confluence, each culture was passaged accordingly. Growth of all 42 prostate tumor samples, without exception, quickly seized on or before the 8th passage, indicating a state of senescence.
These late-passage cells acquired a larger, more flattened morphology, distinctly different from its earlier epithelial form. A senescence-associated beta-X-galactosidase assay demonstrated the senescent state of these cells, as seen in figure 2A. The immortalized form, also taken from late passage cells, was used as the negative control in this experiment (figure 2B). We were able to immortalize our early-stage prostate epithelial cells with Bmi-1 and TERT MSCV viral infection.

The tumor-derived cell clones produced tightly adherent epithelial cells, arising from a semi-adherent center of tightly bound cell clumps. These “clumps” are presumed to house the resident prostate stem cell(s) responsible for the observed clonal growth and expansion. All colonies reflected this assumption, originating from such focal centers (figure 3). We will denote these apparent localized niches as stem cell centers (SCC), which appear to nurture the growth and maintenance of prostate stem cells. The tight epithelial cells immediately surrounding the SCC would be its differentiated progenitors, the transit amplifying cells (TA). Continued observation in the colonies’ growth patterns confirmed the niche’s role, actively producing more epithelial cells both in number and density, especially in the SCC’s immediate vicinity. The observed proliferation of the TA cells elsewhere in the colony, independent of the SCC entirely, lends support to its progenitor identity. Its single cells appeared morphologically more fibroblastic and propagated at a significantly slower rate, all signs indicative of a progenitor state. At confluence, repeated subcultures of these colonies were shown to slowly dilute out the niches, which resulted in eventual senescence at or before the 8th passage.
Marker expression of tumor-derived stem/progenitor cell cultures

Marker expression studies on early-passaged tumor samples (1\textsuperscript{st}-3\textsuperscript{rd} passage) were used to analyze the successful propagation of epithelial stem/progenitor cell colonies. Specifically, fluorescence activated cell sorting (FACS), confocal immunocytochemistry, and reverse-transcription polymerase chain reaction (RT-PCR) were employed in characterizing these single colonies.

Both FACS analysis and immunocytochemistry results confirmed the expression of putative early prostate stem/progenitor cell markers CD44, integrin-α\textsubscript{2}β\textsubscript{1}, High MW CK, and CD133 from the tumor-derived epithelial cells. Figure 4 shows the typical FACS data obtained from early-passage epithelial stem/progenitor cells grown in culture. Taken from different tumor samples (n=14), the cells expressed the following four stem/progenitor markers at ranges varying from: 96-98% for CD44, 99-100% for integrin-α\textsubscript{2}β\textsubscript{1}, 28-70% for High MW CK 5/14, and 1.4-56% for stem cell marker CD133. The wider range and relatively lowered expression of CD133 is due to the limited expression of the few stem cells residing in the SCC. The SCCs are rapidly lost upon sequential passaging, also explaining the varied result.

The immunocytochemistry data is consistent with FACS analysis, yielding high expression of stem/progenitor markers CD44, integrin-α\textsubscript{2}β\textsubscript{1}, and High MW CK in the bulk of the cultured epithelial cells (figure 5). Again, CD133 expression was limited only to the subset of densely localized cells comprising the SCC.

Figure 6 displays results compiled from various RT-PCR experiments. Early-passaged tumor epithelial cells were shown to express a set of embryonic stem cell transcription factors Sox-2 and Oct-4, along with immortalization-associated gene
Bmi-1 at comparable amounts to mouse embryonic stem cells (mES). The idea that this culture system promotes the growth of stem/progenitor cells is supported by these results. The samples did not express any detectable hTERT, Rex-1, or Nanog. All apparent transcription factors were uniformly expressed up to pre-senescence passages (passage 6-8).

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DISCUSSION

We have successfully developed a culture system that allows the robust propagation of epithelial cells derived directly from radical prostatectomies of diagnosed tumors. Consequentially, we stumbled upon the naturally forming niche (figure 3), designated stem cell centers (SCC), which drove the observed epithelial cell proliferation. These stem cell centers were necessary to maintain any one of these early-stage tumor colonies in vitro, providing the essential nutrients for the residing stem cells. A single epithelial colony underwent ~30 population doublings (10^9 cells) before reaching senescence (figure 2A). When this stem cell niche was lost from dilution, as we saw through subsequent passages, the stem cell maintenance dissolved as well. An abrupt cease in growth was consistently correlated with such outcomes producing a senescent state. All 42 adenocarcinoma tumor-derived cell lines not only successfully formed single cell colonies from SCCs, but also senesced upon loss of its mandatory niche.

Two distinct populations of epithelial cells were observed from our tumor-derived cultures. The first being the progenitor transit amplifying cells, distinguished by its flattened cobblestone morphology. Tightly bound prostate tumor stem cell clusters comprising the localized SCCs made up the second much smaller stem cell population. Our FACS analysis and immunocytochemistry results are consistent with these observations. The colonial majority, composed mainly of tight epithelial progenitor TA cells, showed strong expression of all three early progenitor/stem cell markers CD44, integrin-α2β1, and High MW CK 5/14. Restricted solely to the SCC,
the small stem cell minority accounted for the limited expression of stem cell marker CD133 (figure 5).

The prostate tumor-derived epithelial cells also expressed some important embryonic stem cell transcription factors correlating to its characteristic “stemness”. This “stemness” is linked to the epithelial cells’ observed ability to produce progenitor progeny capable of further differentiation. These cells did not express all embryonic stem cell transcription factors – mainly TERT, Nanog, and Rex-1 – because they are NOT pluripotent nor immortal by nature, but rather a slightly more differentiated prostate adult stem cell. Moreover, the overwhelming majority of progenitor progeny can distort the detectable amounts of these transcription factors, also contributing to the observed negative outcome.

We immortalized the same prostate tumor-derived cell line with exogenous TERT and Bmi-1 MSCV virus to use as a negative control for the senescence assay (figure 2B), and to observe the differences in gene expression under RT-PCR analysis (figure 6). Although Bmi-1 was already strongly expressed in all cells, the additional exogenous Bmi-1 expression leaped the cells into an immortal state. This can be due to the dose-dependent behavior of Bmi-1 (39). The added dose resulted in the near complete suppression of the INK4a-ARF locus. Along with the required TERT expression, both these genes enabled the cells to breach the senescence barrier. A more thorough in vivo analysis in the future is crucial to understanding the effects of such gene alterations and its relation to metastasis.

Through the successful characterization of our cultured cells, using the 4 key stem/progenitor cell markers, we have identified the core targets for sequential
mutagenesis in cancer progression. As described previously, these cells positively express a phenotype closely associated to the evasive prostate stem cell. Using our system, the necessary early tumor stem/progenitor cells can be easily obtained in the hunt behind ADI emergence.

Our robust tissue culture system serves as the foundation behind the understanding of metastasis in prostate cancer. With a means to consistently propagate and maintain such vital epithelial cells, new doors are now open to future novel approaches in cancer’s metastasizing etiology.

Initially, we expected the primary human prostate cells to propagate indefinitely in culture, as all cancer cells are invariably immortal. All samples senesced in vitro despite our expectations. Therefore these cells cannot be truly cancerous in nature, but rather early-stage predictors of an inevitable cancerous outcome. Since these cells came directly from diagnosed early-stage prostate adenocarcinomas, they do possess qualities characteristic of cancer cells, especially in their deregulation of vital genes such as Bmi-1 or Oct4. Our culture system provides a reliable source of natural genetically altered cells prone to further mutations capable of transforming into a true metastasizing immortal cancer cell, which over time will assuredly occur. Among all else, this methodology can deliver a new model in studying prostate differentiation, and certainly lay the foundation to solving the mysterious early-stage tumor to metastatic disease transition. Further important implications of this research involve more effective therapeutic measures and prevention treatments to this worldwide prevalent and lethal disease.
APPENDIX

Figure 1. Early-stage tumor derived epithelial cells. Shown is a phase contrast image of early-passage prostate epithelial cells derived from primary human tumor samples. These cells were grown and maintained using our customized growth media. These cells displayed a tight cobblestone morphology characteristic of epithelial cells.
Figure 2. Tumor-derived epithelial cells are senescent. A β-galactosidase senescence detection assay was performed on late-passage cultured human prostate cells. Cells acquired senescence upon loss of its stem cell niche, or Stem Cell Center (SCC). Images were captured using a phase microscope. (A) Image of tumor-derived sample P74 epithelial cells at passage #8. Cells showed strong β-gal staining, indicative of a senescent state. (B) Image of tumor-derived sample P74 epithelial cells immortalized with Bmi-1 and hTERT MSCV virus at passage 20, serving as a negative control. Relatively, immortalized cells showed mostly negative β-gal staining.
Figure 3. Appearance of naturally forming stem cell niche in single colonies, termed Stem Cell Center (SCC), at day 7. Phase-contrast images of various SCCs found in tumor-derived prostate sample P13 epithelial cells. SCCs became apparent after the initial 7 days in culture. A more elongated differentiated morphology was observed in the outer epithelial cells further displaced from the SCC.
Figure 4. Identification of putative prostate epithelial stem/progenitor markers in prostate tumor-derived cells. Fluorescence Activated Cell Sorting (FACS) analysis of prostate stem/progenitor markers CD133, CD44, High-molecular-weight cytokeratin (CK5/14), and Integrin α2β1.
Figure 5. Characterization of stem/progenitor markers in tumor-derived epithelial cells. Immunocytochemical staining of prostate cancer stem/progenitor markers: Integrin α2β1, CD44, High-molecular-weight cytokeratin (CK5/14), CD133. DAPI (blue) used to visualize cell nuclei. Bottom row shows both DAPI and individual cell marker merged together. Tumor-derived epithelial cells were strongly positive for three of the stem/progenitor markers Integrin α2β1, CD44 and High MW CK5/14. CD133 expression was localized only to the stem cell niche, or Stem Cell Center, as seen in the right most column. Negative and isotype controls for each marker were done accordingly (data not shown).
Figure 6. Reverse Transcription Polymerized Chain Reaction (RT-PCR) analysis of ES transcription factors shows partial “stemness” in prostate tumor-derived epithelial cells. Various early-passage prostate tumor-derived samples (P48-P74) were analyzed for mRNA expression of signature stem cell transcription factors. Mouse ES mRNA was used as a positive control (left most lane). Oct-4, Sox-2, and Bmi-1 were consistently expressed at similar levels to mES RNA, indicating a partial “stemness” associated with these epithelial samples. Immortalized P74 (with hTERT and Bmi-1 virus) was also analyzed for mRNA expression (right most lane). As expected, increased levels of hTERT and Bmi-1 were detected.
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