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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of Cultured Early Human Prostate
Cancer Stem/Progenitor Cells

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

in

Chemistry

by

Melissa Jennifer Lee

Committee in charge:

Professor Daniel Donoghue, Co-Chair
Professor Martin Haas, Co-Chair
Professor Deborah Spector
Professor Hector Viadiu-Illaraza

2012

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Co-Chair

University of California, San Diego

2012

DEDICATION

In recognition of completing my Master of Science degree; for believing that I can accomplish anything; for inspiring me to continue doing well in school; for inspiring me to always do my best; for always being proud of me; and for always loving me, I would like to dedicate my thesis to my grandmother, Becky Lee.

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LIST OF ABBREVIATIONS

Aldehyde Dehydrogenase is ALDH

Androgen receptor is AR

Benign Prostatic Hyperplasia is BPH

Cancer-initiating cells is CICs

Estrogen Receptor alpha is ER α

Estrogen Receptor beta is ER β

Fluorescence Activated Cell Sorting is FACS

Normal Prostatic Epithelial Cells is NPrEpCells

Prostate Cancer is PrCa

Prostate cancer sample + ID number is Pr#

Prostatic intraepithelial neoplasia is PIN

Prostatic Specific Antigen is PSA

Prostate Tumor Cells is PrTuCells

Reverse Transcription Polymerase Chain Reaction is RT-PCR

Telomerase Reverse Transcriptase is TERT

LNCaP, VCaP, and PC3 are all prostate cancer cell lines

MCF-7 is a breast cancer cell line

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ABSTRACT OF THE THESIS

Characterization of Cultured Early Human Prostate Cancer Stem/Progenitor Cells

by

Melissa Jennifer Lee

Master of Science in Chemistry

University of California, San Diego 2012

Professor Martin Haas, Co-chair
Professor Daniel Donoghue, Co-chair

Androgen-dependent (“AD”) early prostate cancer often switches to a lethal, castration-resistant (CR) phenotype. We show that early, “AD” prostate cancers contain CR Cancer Initiating Cells (CIC) that drive its growth. We have isolated CR CIC cells from early PrCa, which possess (i) a high degree of self-renewal, (ii) differentiation ability into hormone-responsive prostate cells, (iii) potent tumorigenicity, (iv) the capacity to grow as “sphere” cells and (v) the potential to metastasize. The propagation of CR PrCa cells from localized AD-PrCa facilitates their characterization.

Characterization of CIC-spheres and their adherent progenitor Prostate Tumor Cells (PrTuC) utilized RT-PCR, FACS analysis, and immunofluorescent staining. The

markers analyzed include AR, ER α , ER β , TERT, TMPRSS2-ERG fusion RNA, and ALDH. PrTuCells and their CIC-sphere embodiment are unresponsive to physiological concentrations of steroid hormones.

PrTuCells and CIC-spheres express abundant AR mRNA but no AR protein; ER α is up-regulated and ER β down-regulated compared with normal human prostate epithelial cells. PrTuCells and CIC-sphere cells express the TERT gene; normal prostate epithelial are TERT-negative. The PrCa-associated fusion mRNA TMPRSS2-ERG was undetectable in PrTuCells. The stemness of PrTuCells and CIC-sphere cells was supported by their expression of aldehyde dehydrogenase (ALDH).

Propagation of PrCa CIC facilitates complete exome-sequencing, leading to the identification of causative/etiological events in PrCa initiation and potentially permitting the discovery of targetable entities on early human prostate cancer cells. Our work points to the existence of CR (“androgen-independent”) CIC in the early stages of human prostate cancer, representing a paradigm change with far-reaching consequences to the field.

CHAPTER 1: INTRODUCTION

Prostate cancer is expected to present with approximately 242,000 new cases in 2012. Of these new cases, an estimated 28,000 deaths will occur. With such a high incidence it represents the second-highest cancer-associated mortality rate in males and prostate cancer research is of utmost importance (1). The prostate is a fibromuscular and glandular organ in the male reproductive system that is made up of different epithelial types; luminal, basal, and neuroendocrine cells and also contains stromal/mesenchymal cells (2).

As men age, the size of their prostates increase. The exact mechanisms and why enlargement occurs are unknown, but are hypothesized to be due to increased exposure to circulating androgens. Benign enlargement of the prostate is known as benign prostatic hyperplasia (BPH). Research has been done with conflicting results as to whether BPH directly leads to prostate cancer (3). Differential gene expression analysis between prostate cancer and BPH has shown that the two diseases differed in the expression of 120 genes, hence, there are many differences between benign prostate hyperplasias and prostate carcinomas (4). Prostatic intraepithelial neoplasia (PIN) is a first step in prostate cancer and it is reliably diagnosed via routine pathological determination. PIN is characterized by luminal epithelial hyperplasia and a decrease in basal cells. Progression may then occur to prostate cancer, as the cells develop into adenocarcinomas. This progression is accompanied by an increase in luminal characteristics and possibly spreading outside the prostate proper and eventually metastasis (5).

Prostate cancer is pathologically defined in a series of stages. In stage I, the

cancer is found only in the prostate and cannot be seen through imaging. The prostate specific antigen (PSA) levels are higher than normal and the Gleason grade is less than 6. In stage II, the cancer is only found in both sides of the prostate, but is more advanced with higher PSA levels than stage I. Stage III of prostate cancer is characterized by spreading of the cancer outside the prostate onto the seminal vesicles and stage IV occurs when the cancer has spread to local tissue and organs (6).

To numerically classify prostate cancer (PrCa) stages, Gleason grades are numerical values that are assigned according to the severity of the PrCa. There are two Gleason scores ranging from 1 to 5 and are based on how much the cancer cells appear like normal prostate cells, with grade 1 being cells that look normal and grade 5 being cells that look least normal. The scores are assigned to two areas that are contained within the cancer. The two scores are then added to make a Gleason score (7).

A common form of treatment for prostate cancer has been chemical castration, actual castration, or androgen-deprivation. This procedure involves anti-androgen drugs that can stop the prostate cancer cells from utilizing testosterone that the testicles or adrenal glands have already produced (7). Depriving the prostate and the tumor of androgen causes the prostate and the cancer to shrink to an undetectable size. The common thought today is that prostate cancer, even in its earliest stages, is androgen-dependent and then undergoes an unknown mechanistic switch to become androgen-independent, castration resistant, and thus resistant to androgen treatments.

Though we have not sought evidence for this androgen-dependent to androgen-independent switch, we have derived data suggesting that within early, stage I and II

“androgen-dependent” tumors, there are androgen-independent cells that may be the source of prostate cancer recurrence after treatment. An old study conducted in 1987 by Walsh showed that a decrease in the concentration of circulating androgen caused a shrinkage in all of the patients' prostates. When androgen exposure was restored, the prostates returned to 99% of their original size. This restoration in prostate size leads us to the hypothesis that the prostate harbors an androgen-independent component that is not affected by androgen deprivation (8). The androgen-independent – i.e. castration-resistant - cancer cells have stem/progenitor characteristics and have thus been named Cancer-Initiating Cells (CICs).

Cancer is viewed as uncontrolled cell growth, so each of the cells within the tumor is able to divide and metastasize. Even though these are the characteristics of cancer cells, only a few of these cells are capable of completing all the steps required to drive tumorigenesis. These rare cells are cancer-initiating cells (9). CICs are capable of self-renewal, tumorigenesis, androgen-independence, and metastases.

The isolation and characterization of CICs has been accomplished in other cancers, such as breast, head and neck, testicular, and ovary (9, 10). However, all published work in the CIC field has isolated CICs from fully progressed and/or metastatic human cancers or from established cell lines which are progressed by definition. Isolation of CICs from early human cancers has not been described. Similar work on the isolation of CICs from early-stage human prostate cancer has yet to be done.

There has been some controversy over the origin of the prostate cancer cells. One suggests that luminal cells are the cells of origin of prostate cancer, as the cancer itself

seems to have luminal characteristics; however, if the cell of origin is a basal stem/progenitor cell as we have found, its differentiation would result in a luminal cell phenotype (11). Shen was able to identify a homeobox gene called Nkx3.1 that regulates prostate epithelial differentiation and marks stem/progenitor cells that play a role in prostate regeneration. These authors discovered that some rare luminal cells express Nkx3.1 and can self-renew in vivo without androgen (11). Others have found that basal cells also have potential stemness. Basal cells with AKT, ERG, and AR genes up-regulated were implanted into SCID mice and resulted in the development of adenocarcinomas; but when luminal cells were used, adenocarcinomas did not occur (12). We have used cells from early-stage prostate cancer (stage I and II) in order to grow spheres and isolate CICs. Since there is uncertainty as to the actual original stem cell, a set of markers specifically for these stem cells need to be established (13).

One form of the enzyme aldehyde dehydrogenase (ALDH) aids in ethanol metabolism by breaking down acetaldehyde into acetate and NADH (14). Through its metabolizing actions, ALDH promotes cell survival by detoxifying potentially cytotoxic molecules. It contributes to the drug resistance seen in stem and cancer cells (15). ALDH7A1 specifically, has been proven to promote bone metastasis. When knocked down, a decrease in stem/progenitor cells is observed, as well as, a decrease in migration and cloning ability of prostate cancer cells (16). In breast cancer, high ALDH activity has been used to identify cells that are capable of self-renewal and of generating tumors that duplicate the histological and non-uniform characteristics of the parental tumor (17). Recently, prostate cancer cells with high levels of ALDH have been shown to have high

cloning and migrating abilities in vitro. These cells are considered cancer-initiating cells and show increased tumorigenicity and metastases (18).

We have developed a novel two-step approach to produce cancer-initiating cells (CICs), based on the methodology Prins used with normal prostate cells (19). Unlike others, we have been able to routinely propagate and isolate CICs from early prostate carcinoma tissue (20). We have been able to isolate, grow, and characterize CICs from early (stage I and II) human prostate cancer. These processes have not been done before on early prostate cancer. The characterizations of these early stage prostate cancers are important to better understand their properties and to direct therapeutic research. These CICs are very important to study gene expression, receptors displayed, and biomarkers. Characterization of these CICs will lead to the development of methods and reagents for more intelligent methods of prostate cancer treatments capable of treating both the androgen-dependent and androgen-independent cells.

CHAPTER 2: MATERIALS AND METHODS

PLATING CELLS

OBTAINING SAMPLES

Samples used throughout these experiments were obtained from prostatectomy patients diagnosed with stage I and II prostate cancers. Frozen sections of the prostate cancer and the immediate surrounding tissue were harvested and histology examined. More than 120 samples have been collected with Gleason scores from 5 to 9; however, few were used in these experiments due to limited quantity and some did not produce colonies.

CELL CULTURES

These primary human prostate tumors were cut into small pieces within 60 minutes of being harvested and were digested with constant stirring in 150U/mL collagenase I (Sigma-Aldrich) in growth medium at 37°C overnight. The samples were then divided into 50mg or so of tissue and were frozen live in 90% FBS and 10% DMSO (Sigma-Aldrich) and placed in liquid nitrogen. When grown out, 6-well tissue culture plates (Corning) were coated with laminin (Sigma-Aldrich) in PBS at 37°C for 1 hour. The plates were then washed twice with sterile PBS. The prostate tumor samples were then grown in serum-free growth medium (keratinocyte serum free medium (Gibco)) with 40mM L-glutamine (Gibco), 12.5µg/mL gentamycin and 2.5µg/mL amphotericin B and was supplemented with 10ng/mL basic Fibroblast growth factor (bFGF) (R&D), 40ng/mL EGF (R&D), 58µg/mL Bovine Pituitary Extract (Gibco), 1mM CaCl₂, and

0.025% BSA (Sigma-Aldrich). Cultures were incubated at 37°C in 10% CO₂, 5% O₂, and medium changed every second day.

PLATING CELLS IN MATRIGEL

After epithelial cell colonies grew out as adherent cells (“Prostate Tumor Cells,” PrTuCells) on laminin in a first step that selects for epithelial cells and rejects mesenchymal/fibroblast cells, the cells were trypsinized and cultured as non-adherent cells suspended in Matrigel to yield spheres. Different numbers (10 to 50,000 cells) of cells were plated in 12-well clusters a 1:5 mixture of cells in serum-free medium to Matrigel. 1.5 to 2mL of medium was added to each well, which were fluid changed every other day, and were incubated at 37°C in 10% CO₂ and 5% O₂.

GROWTH CURVE/FACS

A 12-well Corning plate was covered with laminin and incubated at 37°C for one hour. Cells were trypsinized and 1000 cells plated in 96-well flat bottom plates coated with (100µg/mL) laminin using multi-pipettors. The CyQuant cell proliferation fluorescent assay was used to establish cell growth according to the manufacturer’s methodology. Assays were read in a PerkinElmer VICTOR Multilabel Plate Reader. Each time point was represented by 8 identical culture wells. Control cultures were grown in “medium 6”, other, identical cultures were grown in the presence of physiological concentrations (nM range) of dihydrotestosterone (androgen, A), estrogen (E), or A+E. The wells were fluid changed every other day. Every three days, samples

from each category were removed and were subject to FACS. The growth curves were then plotted.

RT-PCR

Total RNA isolation was carried out using Quiagen RNA isolation kits. Reaction samples were made using Bioline Real-Time one-step RT-PCR kits (25 μ L of 25X reaction mixture, 1 μ L of RNase inhibitor, 0.5 μ L of Reverse Transcriptase, 2 μ L of each forward and reverse primers, 0.25ng of RNA, and water to total 50 μ L of reaction).

Samples were amplified in a Thermocycler according to the following; AR, ER α , TERT - 1 cycle: 45°C (20 min), 95°C (1 min), X cycles: 95°C (10 sec), T_m (Table 1) (10 sec), 72°C (30 sec), hold at 4°C; ER β - 1 cycle: 45°C (20 min), 95°C (1 min), 45 cycles: 95°C (30 sec), 54°C (30 sec), 72°C (30 sec), 1 cycle: 72°C (5 min), hold 4°C (Table 1).

Multiple primers sets were designed and/or chosen from the published literature and amplification conditions were exhaustively screened to amplify the TMPRSS2-ERG fusion mRNA, using the VCaP prostate cancer cell line as fusion mRNA-positive control. Amplified samples were run with 10 μ L of dye on 1.6% agarose gel in 1X TAE.

Table 1. The sequences, number of cycles, T_m, amount of RNA, and the type of each primer used for RT-PCR are listed.

Primers	Sequence	Cycles
Androgen Receptor (AR) (21)	5'-CCTGATCTGTGGAGATGAAGCTTC-3' 5'-TGTCGTGTCCAGCACACACTACAC-3'	40 cycles, T _m : 60°C, 0.25µg RNA
Estrogen Receptor alpha (ER α) (22)	5'-TGCTTCAGGCTACCATTATGGA-3' 5'-TGGCTGGACACATATAGTCGTT-3'	40 cycles, T _m : 55°C, 0.25µg RNA
Estrogen Receptor beta (ER β) (23)	5'-CACCATCTAGCCTTAATTCTCC-3' 5'-CACACTTCACCATTCCCAC-3'	45 cycles, T _m : 54°C, 1µg RNA
Telomerase Reverse Transcriptase (TERT) (24)	5'-CGACATCCCTGCGTTCTTG-3' 5'-CAGCTCCCATTTTCATCAGCA-3'	40 cycles, T _m : 61°C, 0.25µg RNA
GAPDH (control) (24)	5'-CGACAGTCAGCCGCATCTT-3' 5'-TTCCCATGGTGTCTCAGC-3'	40 cycles, T _m : 60°C, 0.25µg RNA
TMPRSS2-ERG Translocation	5'-TAGGCGCGAGCTAAGCAGGAG-3' 5'-GTAGGCACACTCAAACAACGACTGG-3' (25)	35, 38, 40 cycles, T _m : 71, 72°C, 1µg RNA
	5'-CAGGAGGCGGAGGGCGGA-3' 5'-GGCGTTGTAGCTGGGGGTGAG-3' (18, 26, 27)	36, 37, 38, 40 cycles, T _m : 64, 65, 66°C, 1µg RNA

DNA SEQUENCING

Amplified samples were run on 10% non-denatured acrylamide gel: diluted 12% non-denatured acrylamide (48g acrylamide, 1.6g bis-acrylamide, 40mL of 10X TBE in water), 0.07g of solid ammonium persulfate, and 5µL of TEMED. Gel was run in 1X TBE. 5µL of RNase dye was added to each 50µL sample (already amplified). DNA ladder was prepared using 15µL of a 100 base pair DNA ladder pre-mixed with dye. Samples were loaded into every other well to avoid contamination. Gels were run at

190V, 38mA, and 6W for approximately 2 hours. Bands were removed from gel and eluted while rotating overnight with 750 μ L of Non-Denaturing Elution Buffer (0.1% SDS, 0.5M NH₄Ac, 10mM Mg(Ac)₂, and 1mM EDTA into MQ deionized water) in each sample tube. The samples were then centrifuged for 1 minute. The samples were then syringe filtered into new eppy tubes. N-butanol extractions were then done until about 100 μ L of sample remained. Then each sample had 300 μ L of 5M NH₄Acetate, 4 μ L MgCl₂, and then filled with 95% ethanol and mixed. The samples were then placed in -70°C for 1 hour. Samples were then spun down for 15 minutes at 4°C. Supernatant was removed and 300 μ L of 70% ethanol was added and spun for 15 minutes at 4°C. Supernatant was again removed. Samples were then placed on a dry vacuum for about 30 minutes. 20 μ L of Glass Distilled Water was added to each sample. 8 μ L of each sample and diluted primers were sent to UCSD Moore's Cancer Center for DNA Sequencing. Results were emailed back and sequences were compared to human gene sequences using <http://genecards.org>.

IMMUNOFLUORESCENCE SLIDE PREPARATION

PrTuCells were grown on acid washed (1N HCl, 60°C, three changes and overnight incubations) 22mm #1.5 glass cover slips in 6-well Corning cluster plates, 5 coverslips per well. The coverslips were coated with laminin, washed with PBS and the cells were plated on top. Primary (i.e. transfer #0) prostate cells and trypsinized cultured PrTuCells were seeded on the coverslip-containing 6-well clusters, making sure that the coverslips maintained a single layer without overlap.

Spheres grown in suspension in Matrigel (BD Biosciences) cultures were freed from the Matrigel by digestion with 1mg/mL dispase (UCSD Stem Cell Core), incubated at 37°C for 1 hour (until all the Matrigel had been digested). The spheres maintained themselves without falling apart into single cells. Spheres were slowly (~800XG, 10 min) pelleted. The pellets were resuspended in sterile 1X PBS and loaded into double slide makers in a Sheldon cytocentrifuge slide maker and spun at 240rpm for 12 minutes. The use of super-clean slides was a must. Cytocentrifuged cells on microscope slides were fixed for 10 minutes in -20°C methanol, air dried, and then kept at -20°C until use.

ANTIBODY STAINING

Adherent PrTuCells were grown on coverslips. CIC-sphere cells were deposited on microscope slides by cytocentrifugation. All cells were fixed in -20°C methanol for 10 minutes and kept at -20°C until use. Primary antibodies were used at 1µg primary antibody per test, by diluting 1.5µg antibody in into 25µL of 5% donkey serum in PBS. Staining was for 30-60 minutes at room temperature. Samples were rinsed three times in 1X PBS, then quickly with water. Once dry, the respective secondary antibodies conjugated with Alexa Fluor 488, at 1:100 dilutions were applied for 30-60 minutes at room temperature. Samples were rinsed three times in 1X PBS and then quickly with water. Once dried, 1 drop of ProLong Gold Antifade Reagent with DAPI was applied to each sample, covered, and sealed with clear nail polish. Pictures were then taken on fluorescence microscope, using the ImagePro program.

ALDH1A3 (ABGent), ALDH7A1 (ABGent), SSEA4 (Cell Signaling), AR (Santa

Cruz Biotechnology), ER α (abcam), ER β (GeneTex), PSA (GeneTex), ABC G2 (Chemicon International), vimentin (BD Pharmigen), CK 18 (Santa Cruz Biotechnology), E-Cadherin (unknown, IgG_{2b} mouse), Secondaries: IgG and IgM (Jackson Immunoresearch), IgG_{2b} (BD Pharmigen)

ALDEFLUOR test for Aldehyde Dehydrogenase detection

The fluorescent Aldehyde Dehydrogenase-Cell Detection Kit (Stem Cell Technologies, Vancouver) was used to determine ALDH activity in live cells. Growing PrTuCells and CIC sphere cells were trypsinized and resuspended in 1X PBS. For each determination of intracellular ALDH, three equal samples were prepared, (1) 0.5mL of cell suspension and 5 μ L of ALDEFLUOR Reagent, (2) 0.5mL of cell suspension, 5 μ L of ALDEFLUOR Reagent, plus 15 μ L of DEAB Reagent, a specific inhibitor of the ALDH fluorescent reaction (Stem Cell Technology), and (3) negative control – 0.5mL of cell suspension not further treated. The samples were then mixed and incubated at 37°C for 35 minutes. Following incubation, samples were centrifuged at 800rpm for 5 minutes. Supernatant was then removed and the cells kept on ice. The cells were resuspended in 100 μ L of ALDEFLUOR Assay Buffer and 5 μ L of propidium iodide was added to each tube. Tubes were stored on ice and taken to UCSD Moore's Cancer Center for flow cytometry. Flow cytometry was run by Dennis Young.

CHAPTER 3: RESULTS

PrCa tumor samples were obtained from patients undergoing prostatectomy to remove an early prostate carcinoma. All of the samples had Gleason scores 6 to 9 and are Stage I/II, i.e. carcinomas localized to the prostate (Table 2). The prostate carcinoma tissue was obtained and cultured as previously mentioned.

Table 2. Data about each of the samples used in this experiment are listed. Some of the data is unknown.

Lab ID	Primary Gleason Grade	Secondary Gleason Grade	Gleason Score	Sample Histology
5	?	?	?	Glandular hyperplasia and nodular hyperplasia, squamous metaplasia, chronic inflammation
9	4	5	9	Adenocarcinoma, high grade PIN and chronic inflammation
77	3	4	7	Adenocarcinoma
78	3	3	6	Adenocarcinoma
79	3	4	7	Adenocarcinoma
84	3	4	7	Adenocarcinoma
86	3	4	7	Adenocarcinoma
87	3	4	7	Adenocarcinoma
97	3	3	7	Adenocarcinoma
98	?	?	?	Prostate with mild hyperplasia changes, and chronic inflammation, and atrophy
99	4	4	8	Adenocarcinoma
100	3	3	6	Adenocarcinoma
105	3	4	7	Adenocarcinoma
106	3	4	7	Adenocarcinoma
108	4	3	7	Adenocarcinoma
109	3	5	8	Adenocarcinoma
ST	?	?	?	?

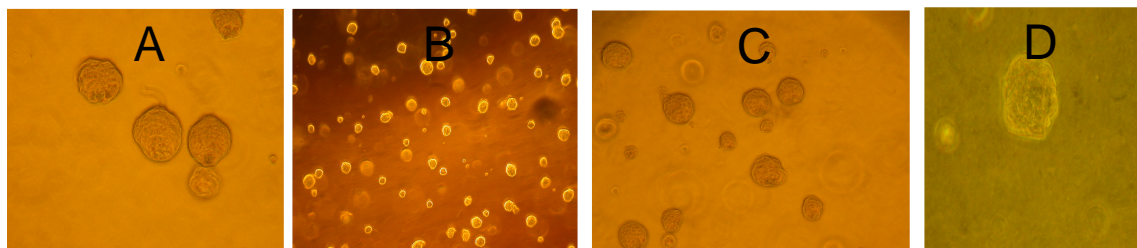


Figure 1. (1A) CICs/Prostaspheres growing on 3D Matrigel. (1B) After two-step propagation, few cells remain as single cells. Most CICs develop into prostaspheres. (1C) Different sizes of spheres can be seen. As time lapses, cells continue to divide to give larger spheres. (1D) Spheres divide to yield a maximum size of approximately 250 cells, upon which they start to differentiate from the center, as seen by the darker area.

SPHERES

A two step method has been developed to routinely propagate human prostate CICs (Figure 1A). The first step involves selective isolation by growth and selection of adherent epithelial Prostate Tumor cells (PrTuC) and the second consists of growing the PrTuCells as non-adherent spheres suspended in Matrigel, with the outgrowth of CIC spheres. After plating in Matrigel, > 90% of PrTuCells develop into prostaspheres provided that PrTuCells are replated in Matrigel after a limited number of transfers in adherent culture, as PrTuCells differentiate with the loss of stemness after 2-3 transfers at a split ration of 1:3. As seen in Figure 1B, very few of the PrTuCells remain single cells and refrain from developing into spheres of 100-250 cells each. Six days after plating of PrTuCells in Matrigel, the single trypsinized CICs have divided to develop spheres containing 4-6 cells each. By day 8, the spheres have become 8-12 cells each. After day 8, the CICs continue to divide to spheres containing up to 250 cells each (Figure 1C). After reaching a size of some 250 cells per sphere in 12-15 days, the center of the spheres start to differentiate and a limit of ~250 cells is reached. It is not clear whether differentiation is due to the limitation of nutrients into the tight spheres, or whether

paracrine feeding within the sphere is limited by intra-sphere diffusion. Paracrine crossfeeding has been shown in human embryonic cell colonies, but they are normally grown in a quasi-two dimensional format which allows more penetrant crossfeeding and the continued growth of the colonies to larger sizes (Figure 1D).

Sphere cells were trypsinized to single cells, mRNA extracted, and the cells were deposited onto microscope slides then fixed for RT-PCR analysis for gene expression and immunohistological (IH) staining. RT-PCR was done to determine the semi-quantitative level of expression of specific genes in young sphere-forming cells, as was IH staining. The RT-PCR results show that Pr#77 spheres, and spheres grown from ~40 other prostate tumors, express the telomerase reverse transcriptase (TERT) gene as well as, androgen receptors (AR), estrogen receptor α (ER α), and estrogen receptor β (ER β), but were TMPRSS2-ERG gene fusion negative. Compared with normal human prostate epithelial cells (NPrEpCells) - purchased from the LONZA company – sphere cells express high concentrations of AR mRNA but no AR protein, express high levels of the ER α receptor, equal to or higher than NPrEpCells, but express reduced levels of ER β mRNA and protein. CIC sphere cells also consistently express the TERT gene, unlike NPrEpCells grown from young donors, which are TERT negative. Finally, without exception, CIC sphere cells do not synthesize detectable levels of the TMPRSS2-ERG fusion gene. The lack of expression of TMPRSS2-ERG fusion mRNA has been pursued for three whole years using many different RT-PCR oligonucleotide sets, different temperatures, and cation/formamide concentrations.

CIC-sphere cells were tested for the expression of the receptor proteins AR, ER α ,

and ER β by immunohistological staining. Additionally, CIC sphere cells were tested for the expression of the ABCG2 protein (Figure 2A). ABCG2 is a detoxifying pump that actively removes toxins from stem/progenitor cells. This, and similar proteins constitute a defense mechanism of stem/progenitor cells against toxins, thus ensuring the viability of these crucial cells in the life cycle of the organism. Interestingly, and now finally understood, proteins like ABCG2 protect cancer cells against chemotherapeutic attack by the “multidrug resistance” mechanism, thus defeating complete therapeutic elimination of many cancers by protecting Cancer Initiating stem/progenitor Cells from chemotherapeutic drugs. Indeed, early (Stage I/II) prostate-derived CIC spheres express ample ABCG2, contributing to their characterization as “stem/progenitor” cells.

Inasmuch as the transporter protein ABCG2, alias BCRP, is a highly specific marker for stem cells, it is expressed to a considerable level in the cytoplasm of the prostate CIC sphere cells (Figure 2A), though its detection by immunofluorescence is not a reliable quantitative test. Surprisingly, prostate cancer CIC sphere cells also express ample levels of the vimentin protein, considered to be a marker found and specific for mesenchymal cells (Figure 2B). An explanation for this observation is readily apparent as the particular CIC sphere cells shown in Figure 2B were differentiated with 1 μ M retinoic acid for 12 days. Nevertheless, the expression of vimentin by CIC sphere cells needs to be verified in a fully controlled experiment and investigated further.

CIC sphere cells express little or no AR protein (Figure 2C), though they express ample AR mRNA, as described below. Similarly, CIC sphere cells express reduced amounts of the ER β receptor (Figure 2D) compared to the level of ER β expressed by

NPrEpCells (not shown). The consistent down-regulation of the ER β receptor by prostate cancer-derived epithelial cells compared with NPrEpCells is a theme that has been consistently reported by Gail Prins and is beyond the scope of the experiments reported in this thesis (32). Furthermore, the estrogen receptor alpha (ER α) is expressed to a high level, similar to its expression by NPrEpCells (Figure 2E and not shown). The prostate luminal cell differentiation marker CK18 is also seen in the CIC prostaspheres (Figure 2F) and, similar to the expression of vimentin, the cells that are shown to express CK18 in Figure 2F were differentiated with retinoic acid. CK18 expression in CIC prostaspheres needs to be followed up with a fully-controlled experiment in the future, as CK18 expression may be related to the degree of retinoic acid-induced differentiation of the particular samples stained and shown in Figure 2F.

Significantly, in extensive experiments performed on CIC spheres grown from different prostate carcinoma cells, PSA expression has not been found (Figure 2G). This significant finding may mean that the early cancer-inducing stem/progenitor cells in human carcinomas is blocked from differentiating with the expression of PSA, and is supported by a recent paper by Dean Tang who isolated PSA-negative cells from the human prostate cell line LNCaP. These cells were PSA-negative and consisted of Self-Renewing Long-Term Tumor-Propagating Cells that Resist Castration (28). Finally, CIC sphere cells were E-cadherin negative (Figure 2H).

These CIC sphere cells express the human stem cell-specific antigen SSEA4, express medium-levels of the ALDH1A3 isoform of the stem cell-specific enzyme aldehyde dehydrogenase (ALDH) and very high levels of the 7A1 isoform of this

enzyme, ALDH7A1. ALDH is a widely-recognized intracellular marker specific for stem cells (16) (Figures 2I, 2J, 2K).

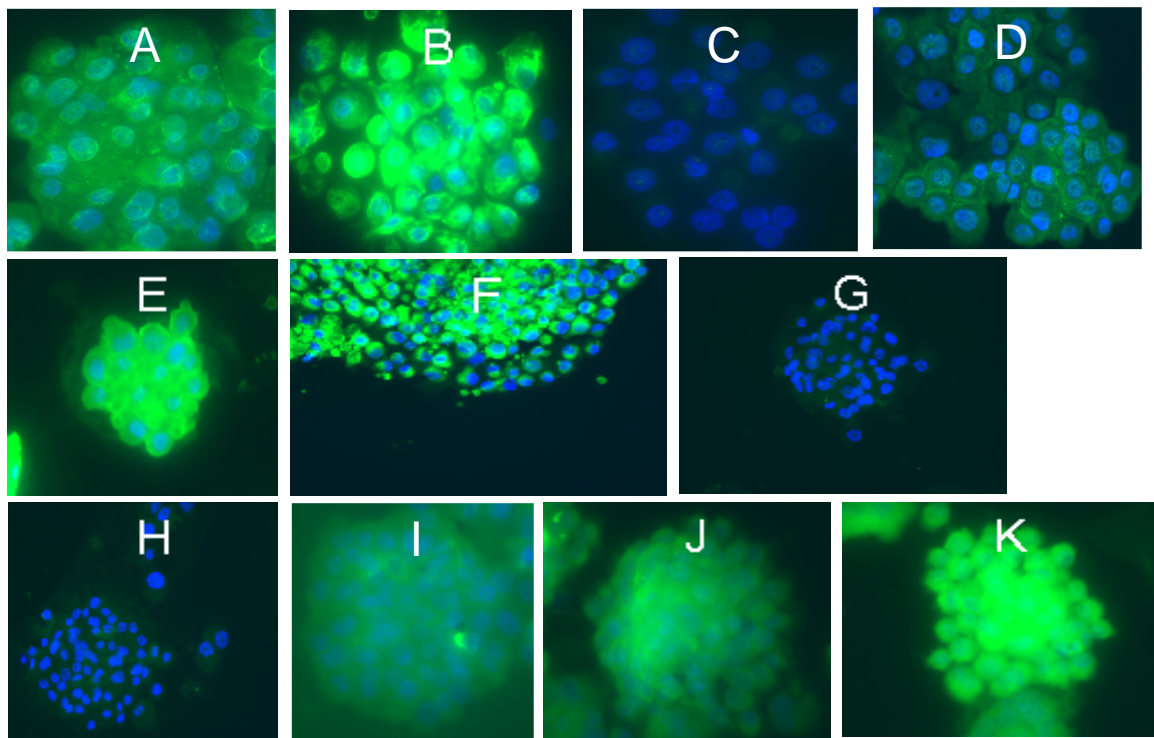


Figure 2. Immunohistological staining on undifferentiated prostaspheres. Specific proteins seen in green. Blue is the DAPI, which stains the nuclei. (2A) ABC G2 (1s exposure), (2B) Vimentin (250ms), (2C) AR (1s) is undetectable or unseen. (2D) ER β (500ms), (2E) ER α (1s), (2F) CK18 (500ms), (2G) PSA (1s), (2H) E-cadherin (1s), (2I) SSEA4 (1s), (2J) ALDH1A3 (750ms), (2K) ALDH7A1 (750ms).

Cultured CIC spheres were differentiated with retinoic acid (RA), a differentiating substance that has been shown to differentiate many different cell lineages. RA-induced CIC spheres were also characterized for the expression of differentiation antigens. The differentiated spheres expressed vimentin at a level higher than non-treated CIC spheres. Vimentin is specifically expressed by cells at the outer regions of the spheres, suggesting that the reagent (RA) did not diffuse throughout the entire “pingpong” ball of the spheres (Figure 3A). Whether this means that RA induced an actual mesenchymal phenotype in

the outer rim of the CIC sphere cells remain open to interpretation. These RA-differentiated spheres remained ALDH1A3 and ALDH7A1 positive (Figures 3B, 3C).

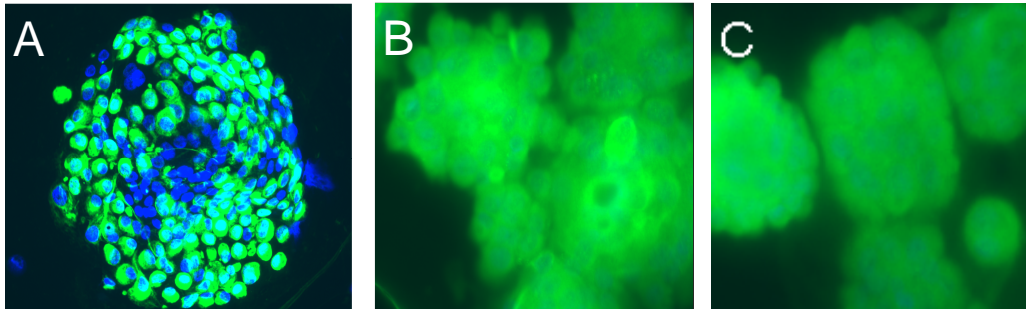


Figure 3. Immunohistological staining on retinoic acid induced differentiation. (3A) Vimentin (250ms), (3B) ALDH1A3 (750ms), (3C) ALDH7A1 (500ms).

TELOMERASE REVERSE TRANSCRIPTASE (TERT) POSITIVE

The first step in isolating and growing CIC-sphere cells from Stage I/II prostate cancers was their establishment as adherent cells in serum-free culture containing specific recombinant growth factors. These cells, designated PrTuCells were then characterized. Though not grown into suspension-culture prostaspheres, early cultures of these samples consist almost quantitatively of CICs as they can be grown into CIC spheres. We characterized these cells in various ways. A known characteristic of prostate cancer stem/progenitor cells is their ability to continuously replicate. TERT (telomerase reverse transcriptase) is a ribonucleoprotein that maintains the ends of DNA chromosomes, telomeres, from eroding with each cycle of DNA replication, with each cell doubling. Using RT-PCR, all the prostate carcinoma cell samples (n>30) grown into PrTuCell cultures were found to be TERT positive (Figure 4). PrTuCells grown from only one

prostatectomy patient, Pr#108, was found to be TERT-negative. Additionally, normal prostate epithelial cells grown from young donors were TERT negative, as expected. Since Pr#108 is TERT negative, the Pr#108 frozen cells may contain only normal prostate cells and no cancer cells. This assumption gains credence from the results obtained with NPrEpCells which are TERT-negative and is, of course, not a cancer cell source. In the RT-PCR amplification of TERT, the human cancer line NTERA and GAPDH were used as positive controls. Though several different PCR-amplification oligonucleotide sets were used to amplify TERT mRNA in these experiments, as well as a positive control – NTERA, to ascertain that the results indeed mean that PrTuCells express the TERT gene we purified and sequenced the amplified TERT DNA of PrTuCell #99 and obtained a full identity of the sequence of the amplified DNA with the corresponding sequence of the published human TERT gene (Figure 5).

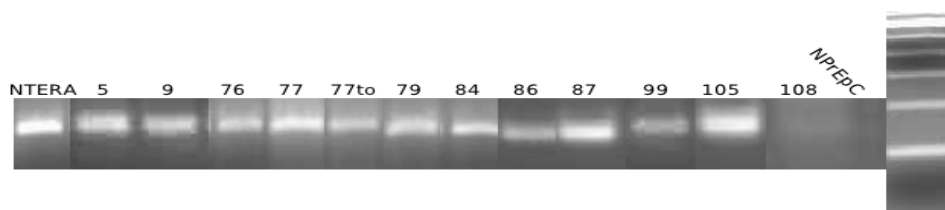


Figure 4. RT-PCR for TERT was run on a variety of samples, as shown. NPrEpC = normal human prostate epithelial cells from a normal, young donor, LONZA Inc.

TERT x PrTuCell #99

```

-----CATTTCCTGCGCGTCATCTCTGACACGGCC
          10          20          30
          :::::
TTTCATCAGCAAGTTTGAAGAACCCACATTTTCCTGCGCGTCATCTCTGACACGGCC
      3070      3080      3090      3100      3110      3120

          40          50          60          70
TCCCTCTGCTACTCCATCCTGAAAGCCAAGAACGCAGGGATGTCG-----
          :::::
TCCCTCTGCTACTCCATCCTGAAAGCCAAGAACGCAGGGATGTCGCTGGGGCCAAGGGC
      3130      3140      3150      3160      3170      3180

```

Figure 5. The TERT band of Pr#99 was sequencing according to the above protocol. The sequence matched that of the published gene. The number above the sequence is the number of base pairs matched and the bottom numbers are the number of bases within the whole TERT gene.

TMPRSS2-ERG GENE FUSION

Advanced human prostate cancer cases have been shown to harbor a specific gene fusion between an androgen-responsive promoter, TMPRSS2 and the oncogene ERG (26). Both genes reside on human chromosome #21 at a distance of about one million base pairs and in the same coding direction. A fusion mRNA is expressed in ~50% of human prostate cancer cases (26). Indeed, prostate cancer cell lines that harbor the fusion mRNA/protein express higher levels of the (weak) oncogene ERG (29). The notion that this, and associated gene fusions constitute an event in prostate cancer progression is widely accepted. However, some credible reports have shown that the TMPRSS2-ERG gene fusion is present specifically in early, non-progressed prostate cancers (30). Additionally, normal prostate epithelial cells that are grown in culture in the presence of high levels of androgen acquire the gene fusion (26, 31). Hence, in spite of significant “vested interests” in this gene fusion phenomenon, the jury is still out to determine what the fusion event means in the natural history of human prostate cancer. It is eminently

possible that the TMPRSS2-ERG fusion results FROM the presence of the carcinoma in an “androgen-bathed” organ, rather than being causal of its initiation or progression.

While the common notion is that the TMPRSS2-ERG defines human prostate cancer, this might be a fashionable error that may require further stringent causality testing. Indeed, only a small minority of human prostate cancer cell lines harbor the fusion protein, e.g. VCaP, and the view that “only fusion mRNA-expressing prostate cancers and cancer cell lines are in fact carcinomas” is probably erroneous, as we have found that all of the early carcinoma-derived PrTuCell cultures that we have tested lack a detectable fusion mRNA, even though the cells generate human prostate cancers when small numbers (1000 cells) of PrTuCells were transplanted into the anterior prostate of SCID mice. We subscribe to the view that the fusion mRNA event does not define prostate cancer but rather is induced by it.

In light of the fusion-marker controversy, it was important to test whether early, Stage I/II prostate carcinoma cells possess this gene fusion mRNA, in other words, whether the fusion event may be partially causal in the initiation of the carcinoma. Therefore we tested, using RT-PCR, whether a fusion mRNA is detectable in the PrTuCells grown from Stage I/II prostate cancers. Only short-time PrTuCell cultures were tested, as under the androgen-free culture conditions in “medium 6⁺⁺⁺”, cells harboring this fusion RNA in vivo may be lost from long-time cultures.

Since the published sightings of the TMPRSS2-ERG fusion RNAs have been limited to a small minority of cells in typical prostate carcinoma tissue, testing for the fusion RNA involves a problem of low-abundance transcript detection. Therefore, we

first established methods to detect the fusion RNA using a one-step RT-PCR test. Only the “MyTaq One-Step RT-PCR kit” of BIOLINE (Taunton, MA) proved capable of routinely detecting the low-abundance fusion message. Most RT-PCR detection kits available on the market were tried, using various published amplification oligo-pairs and amplification conditions (temperatures, cation concentration and formamide/DMSO addition). We used the VCaP cell line as a positive control in our TMPRSS2-ERG amplification experiments.

None of the 30 PrTuCell cultures tested for the TMPRSS2-ERG fusion mRNA showed evidence of the fusion species. (Figure 6, 14 shown). The primers used were taken from the scientific literature and yielded positive TMPRSS2-ERG-amplification results in their respective publications and with our positive mRNA control extracted from VCaP cells. The different amplification conditions used are shown in Table 1. To exclude the possibility that PrTuCells cultured in androgen-free “medium 6” might have lost TMPRSS2-ERG-positive cell populations that express the fusion mRNA, cultures were established in the presence of 10nM androgen and grown. None of the PrTuCell cultures grown in the presence of androgen showed evidence of detectable fusion transcript either (not shown).

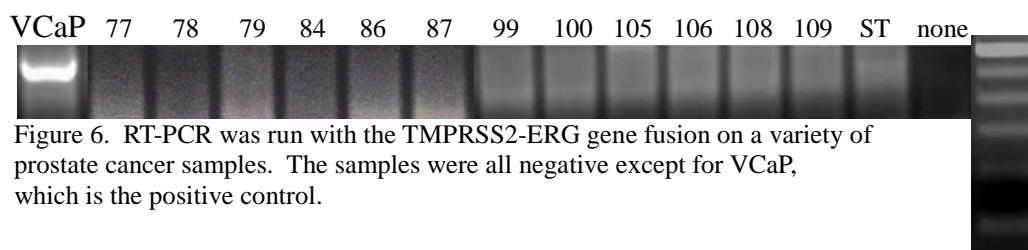


Figure 6. RT-PCR was run with the TMPRSS2-ERG gene fusion on a variety of prostate cancer samples. The samples were all negative except for VCaP, which is the positive control.

Thus we conclude that Stage I/II of prostate cancer-derived CIC cells that we have studied lack the TMPRSS2-ERG fusion transcript. Whether our work can be generalized

to suggest that the fusion event is a progression event in prostate carcinogenesis and is absent from the earliest stages of the carcinoma, or that the fusion event is generated BY the androgen-driven cancer and results FROM the same, is open to speculation.

ANDROGEN RECEPTOR (AR) EXPRESSION AND MISREGULATION

Recognizing the importance of the androgen receptor in prostate cancer as well as in the development of the normal prostate, the mRNA of the androgen receptor (AR) was amplified in a series of prostate tumor samples using RT-PCR (Figure 7A). All of the PrTuCell cultured samples used showed evidence for the transcription of the AR gene, though no quantitative AR-mRNA determinations were done. RNA extracted from Normal Prostate Epithelial Cells (NPrEpC) was also amplified by RT-PCR and the amplification band on the gel was somewhat weaker than that of the PrTuCell cultures. AR-expression in samples of cultured early prostate cancer cells – PrTuCells - should be compared with multiple sources of normal prostate epithelial cells - NPrEpC, as normal prostate is a reasonable control to the early cancer tissues studied. Multiple NPrEpCell cultures have not yet been studied, as the material is inordinately expensive and hard to come by. The LNCaP prostate cancer cell line and GAPDH served as the positive controls in AR-mRNA RT-PCR amplification experiments. The AR DNA band amplified from PrTuCell #109 was sequenced to ascertain that the oligo sets used indeed amplified an AR-gene sequence and no other, as shown in Figure 8.

Prostate tumor cells grown on coverslips in medium 6⁺⁺⁺ and fixed in ice-cold methanol were stained with antibodies specific for the human AR protein to estimate the level of expression of the AR protein in PrTuCells (Figure 7B-E). Compared to the

PrTuCell samples, the NPrEpCells (Figure 7F) express considerably more AR protein though AR-mRNA expression does not differ significantly. Other cultures of PrTuCell samples – not shown - express little or no detectable AR protein. While the methods employed are at best semi-quantitative, a trend appears clear, namely, that prostate tumor culture cells down-regulate translation of AR-mRNA, a finding that will be followed using quantitative methods in the future.

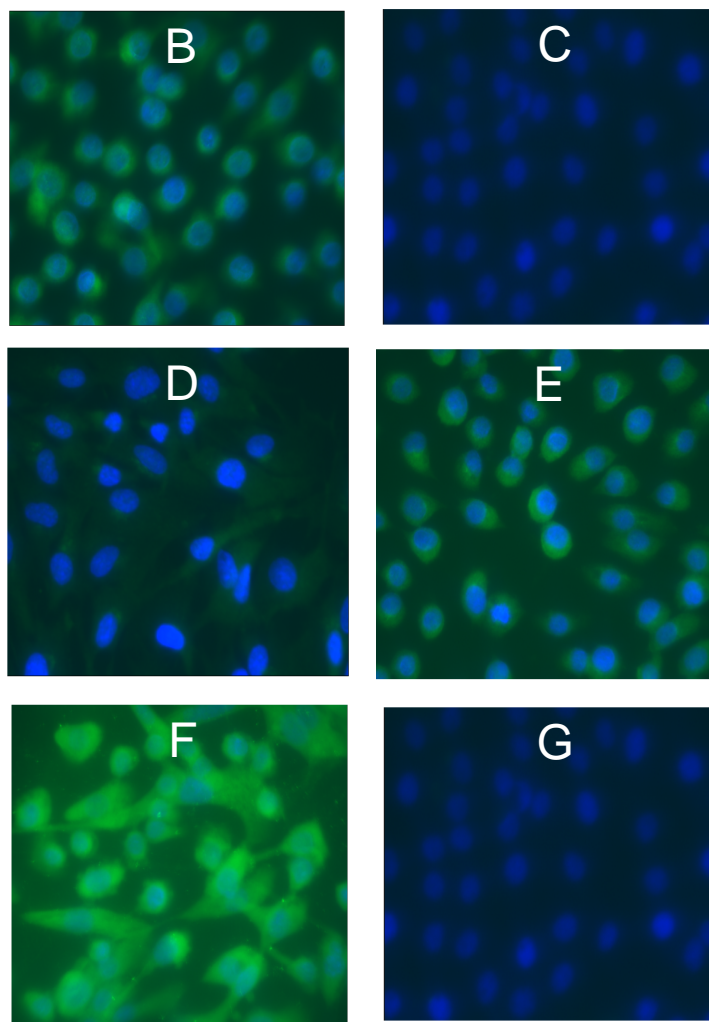
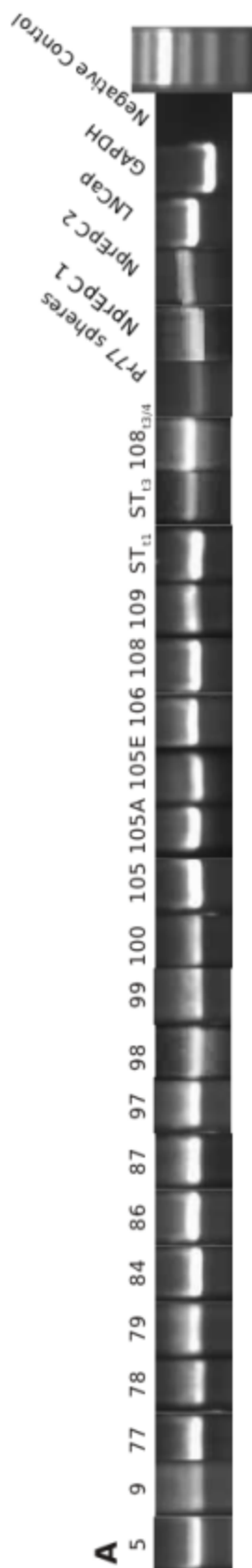


Figure 7. (7A), RT-PCR amplification of AR mRNA extracted from PrTuCell cultures. All of the cultures express AR mRNA, with some bands brighter than others. Positive controls LNCaP and GAPDH were run, along with a 100bp DNA ladder. (7B-F), Immunocytochemical staining of AR protein on Pr#9 (7B, 1s), Pr#87 (7C, 1s), Pr#105 (7D, 1s), Pr#108 (7E, 1s). (7F) Immunocytochemical staining of AR protein expressed by normal prostate epithelial cells NPrEpCells (250ms). Note the difference in photographic exposure between the fluorescently stained PrTuCells (1 second) and the NPrEpCells (250 milliseconds). (7G) Immunocytochemical of control (1s)

EXPRESSION OF THE ESTROGEN RECEPTORS

Even though prostate cancer is thought to be an androgen-based disease, estrogen is believed to play a role in the initiation and progression of the disease (32). The expression of the known estrogen receptors was investigated by PrTuCells and by their control NPrEpCells.

RT-PCR was performed on mRNA purified from PrTuCell cultures, to characterize the ER α gene in prostate tumor samples. As shown, (Figure 9A), all of the samples tested strongly expressed ER α mRNA. Though RT-PCR amplification is merely semi-quantitative, standard, equivalent amplification methods were used throughout, allowing a reasonable estimate of the expression of the ER α gene. The cells line MCF-7 and GAPDH were used as internal positive controls in these experiments. All cultured PrTuCells express much more ER α mRNA than the NPrEpCells. Immunofluorescent staining for ER α protein, while not strictly quantitative, shows that the expression of the ER α protein by PrTuCells is considerably higher than that expressed by the normal control cells used, NPrEpCells (Figures 9B-E).

Cultured PrTuCells were also characterized by RT-PCR for the expression of estrogen receptor beta (ER β) mRNA (Figure 10A). The level of ER β mRNA expressed by the cells appears to be significantly less than the expression of either ER α , AR or TERT mRNA, though all of the samples showed at least a slight band of the expected size on the gels. The PC3 cell line and GAPDH were used as positive controls in these experiments. Looking at ER β protein, all the PrTuCell samples synthesize significantly less ER β protein than the NPrEpCell cultures (Figures 10B-E).

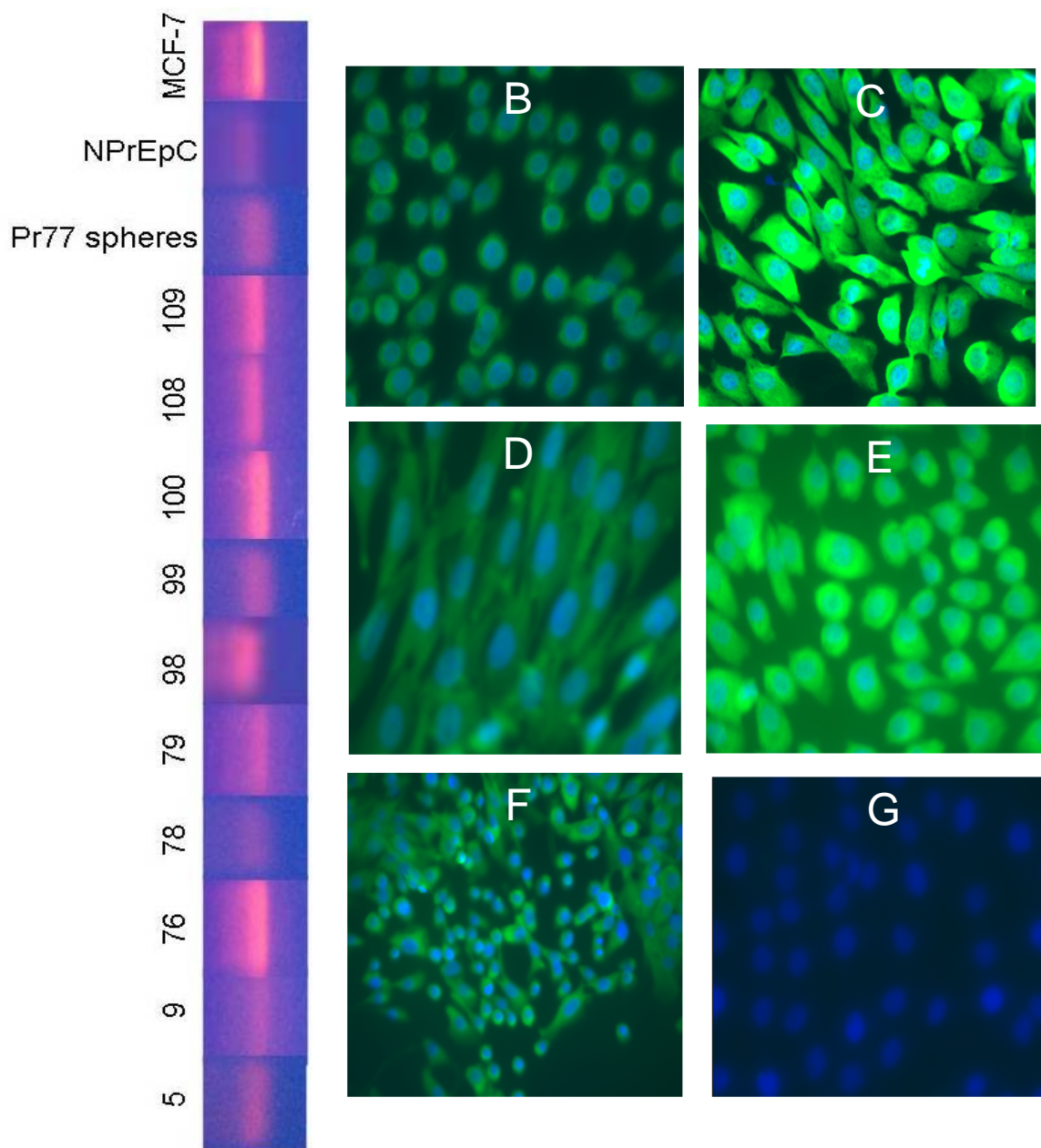


Figure 9. (9A) RT-PCR amplification of ER α mRNA expression by cultured PrTuCells. Pictures were taken through a different method due to technical circumstances. (9B-F) Immunocytochemical staining of the ER α protein on PrTuCells #9 (9B, 1sec), Pr#87 (9C, 1 sec), Pr#105 (9D, 1sec) and Pr#108 (9E, 1sec), (9F) Immunocytochemical staining of ER α on NPrEpCells (250ms), (9G) Immunocytochemical staining of control (1s).

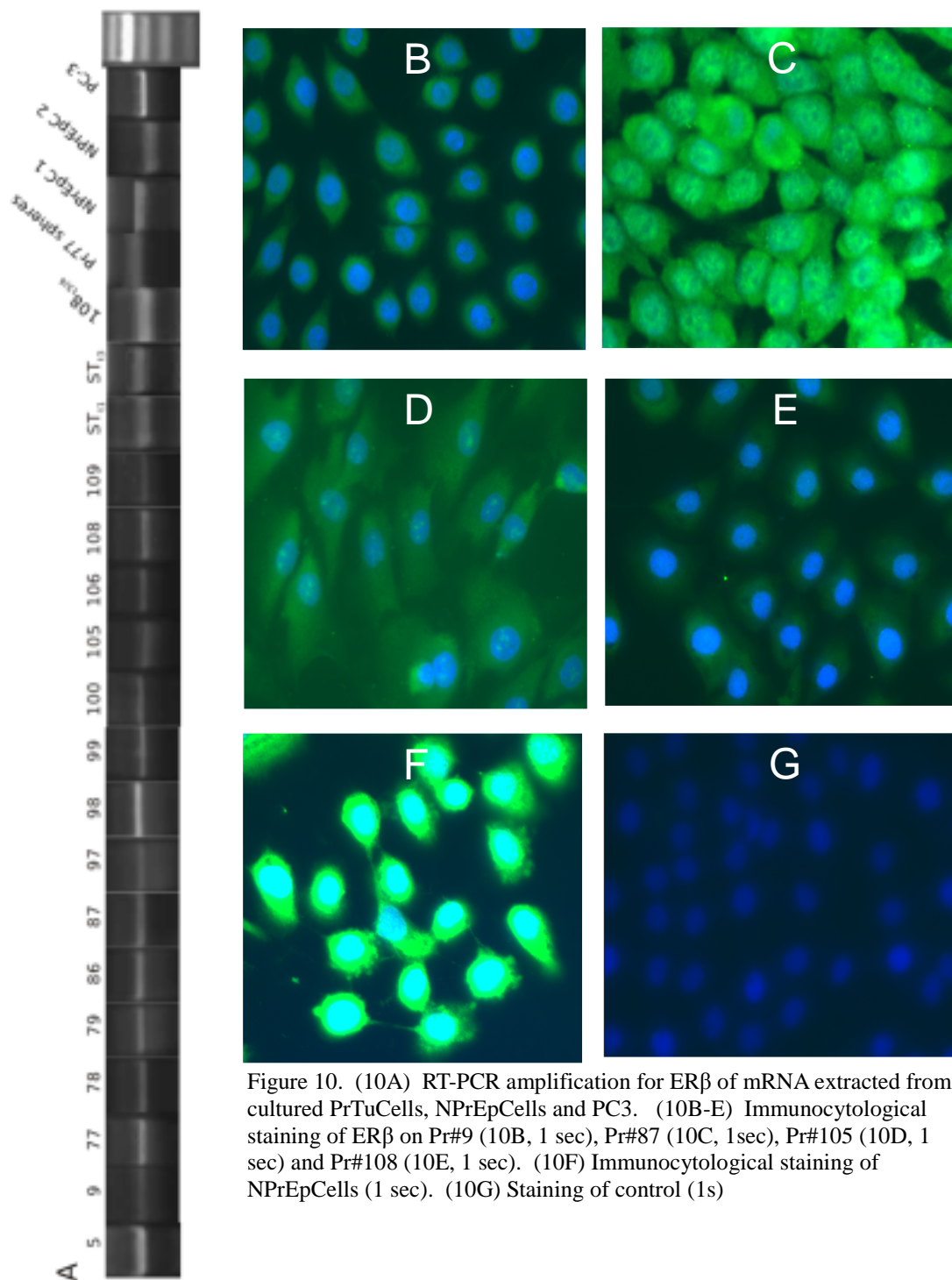
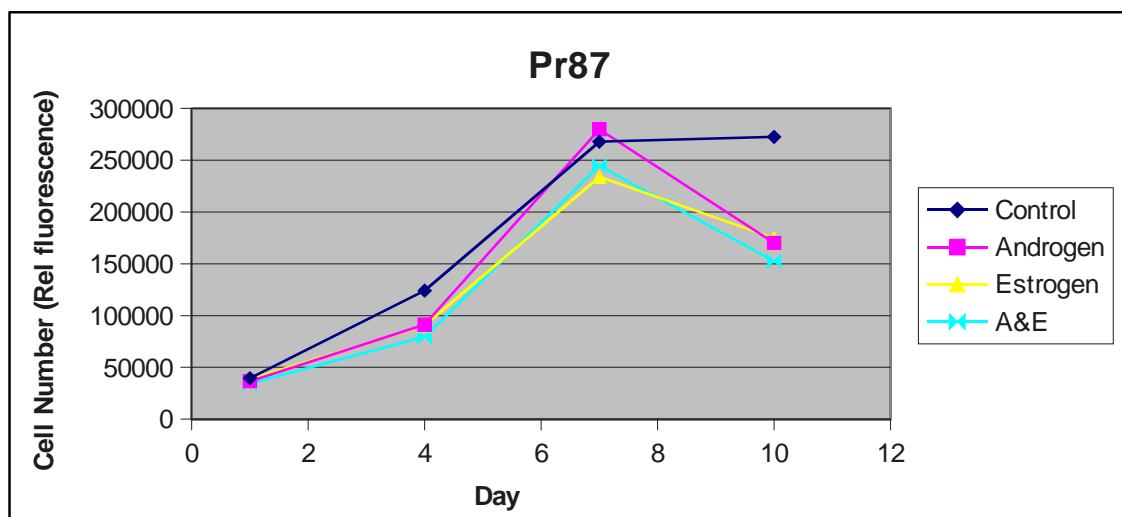


Figure 10. (10A) RT-PCR amplification for ER β of mRNA extracted from cultured PrTuCells, NPrEpCells and PC3. (10B-E) Immunocytochemical staining of ER β on Pr#9 (10B, 1 sec), Pr#87 (10C, 1sec), Pr#105 (10D, 1 sec) and Pr#108 (10E, 1 sec). (10F) Immunocytochemical staining of NPrEpCells (1 sec). (10G) Staining of control (1s)

GROWTH-RESPONSE of PrTuCells to ESTROGEN and ANDROGEN

Do the androgen or the estrogen receptors play a role in the proliferation or the differentiation /apoptosis of the PrTuCells, through their respective receptors ER α , ER β , and AR? To approach this question we grew PrTuCells in flat-well well microtiter plates in the presence of physiological concentrations of androgen (DHT), estrogen, or the two hormones together. These experiments were done with the hormones at concentrations of 1nM and 10nM. For each time point 8 wells were assayed of (no hormone added) control cultures, DHT-supplemented cultures, estrogen-supplemented cultures and the simultaneous supplementation of both hormones. The number of cells per well/culture was determined by staining the cells with a DNA binding dye using the Molecular Probes CyQUANT NF Cell Proliferation Assay Kit. Fluorescence was read in an automated PerkinElmer VICTOR Multilabel Plate Reader. Of the 5 PrTuCell cultures tested in this way, one result for PrTuCell #87 is shown in Graph 1 (other data in APPENDIX). There was no effect on growth rate of Pr#87 cells, neither acceleration nor growth reduction due to differentiation or apoptosis in response to the presence of either hormone.



Graph 1. Pr#87 cells were cultured in the presence of androgen, estrogen or the two hormones together. Cell growth was assayed by the CyQUANT fluorescent Cell Proliferation Assay of Molecular Probes.

This result was a surprise. After all, the cells express the respective hormone receptors. However, as shown below, the cells have significant characteristics of stem/progenitor cells, and without some degree of differentiation, they may not respond to extracellular hormone concentrations, as was in fact observed. Missing is a set of similar experiments done on NPrEpCells which also express the hormone receptors, though these express the ER β receptors – the receptor known to be active in differentiation/apoptosis of prostate epithelial cells – to a much higher level. These experiments have not yet been done due to the scarcity of human NPrEpCells.

COMPARATIVE ESTIMATE OF THE CONCENTRATION OF ER β

RECEPTORS ON PrTuCells and NPrEpCells

The level of staining of the ER β receptor protein, an estimate of the comparative number of ER β receptors on the surface of PrTuCells and their NPrEpCells as a control

was also measured by flow cytometry of ER β -antibody-labeled live cells. PrTuCell cultures of the following PrCa samples were tested, Pr#84, Pr#87, Pr#106, Pr#108, and Pr#109 (Figure 11). Fluorescent antibody staining was assayed by flow cytometry. As is shown in Figure 11, the peak of fluorescence intensity of ER β -antibody staining was ~20-30 fluorescence units, while normal prostate epithelial cells displayed a peak level of ~100-150. Indeed, the reduced expression of the ER β receptor and of its activity has been documented before (35). (Other receptors were measured, APPEDNIX)

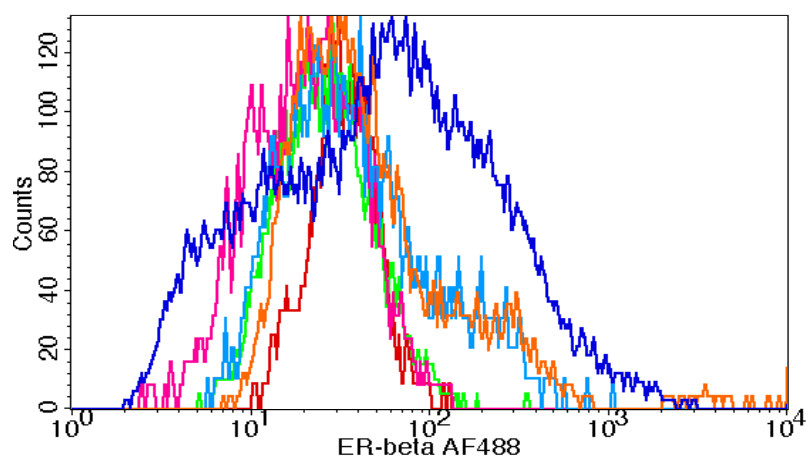


Figure 11. The FACS results of testing ER β on PrCa samples and NPrEpCells. Pr#9 (red), Pr#106 (green), Pr#108 (pink), Pr#109 (light blue), PrST (orange), and NPrEpCells (blue). The counts are the number of incidences detected and is graphed against the amount of fluorescence.

PrTuCells and CIC-SPHERE CELLS are STEM/PROGENITOR CELLS

In other work (in preparation) the potent cancer phenotype of PrTuCells and CIC-sphere cells has been documented by transplantation of small numbers of the cells into the anterior prostate of SCID mice and (immuno)-histologic analysis of the resulting cancers. Do PrTuCells possess characteristics of stem/progenitor cells, i.e. are the adherent PrTuCells a manifestation of Cancer Initiating Cells? Elsewhere, we have

shown that PrTuCells express a set of stem/progenitor markers, CD44, CD133, Integrin $\alpha 2\beta 1$ and CytoKeratin 5/14, which, as a group characterize stem/progenitor epithelial cells. Here we added to this group of cell surface stem/progenitor markers the expression of the intercellular enzyme Aldehyde Dehydrogenase, ALDH. In multiple publications, ALDH has been shown to be exclusively expressed in stem cells and in Cancer Initiating Cells (28).

Both the fluorescent test “ALDEFLUOR” of Stem Cell Technology, Vancouver, and fluorescent staining of fixed PrTuCells growing on coverslips, using antibodies specific for two different ALDH isoforms have been used. Antibodies to ALDH1A3, specific for human breast cancer CICs and ALDH7A1, an isoform specific for prostate stem cells have been used to characterize PrTuCells (33, 18).

PrTuCells were grown on cover slips, fixed and stained with polyclonal rabbit antibodies to ALDH1A3 and ALDH7A1 (ABGENT, San Diego, CA) can be seen in Figure 12, PrTuCells #87 stained much more robustly with the ALDH7A1 antibody (Figure 12B) than with the ALDH1A3 antibody, confirming the result presented by van der Hoogen (18). ALDH7A1 appears a little stronger than ALDH1A3. This result confirms the “stemness” of PrTuCells #87, and delineates the specific isoform of ALDH synthesized by PrTuCells #87. PrTuCell samples grown from other patient donors also stain more solidly with the antibody to ALDH7A1 than with other isotypes.

Figure 12C also shows that PrTuCells stain robustly with another stem cell-specific antibody, namely SSEA4, yet another stem cell-characterizing marker of PrTuCells grown from early human carcinomas.

Finally, PrTuCells were tested in the fluorescent ALDEFLUOR test kit developed by Stem Cell Technologies in Vancouver. Live cells stained with the reagent of this test and with the fluorescent reagent in the presence of a specific inhibitor of the reaction, DEAB, were shown to stain strongly for the presence of ALDH (Graphs 2A&B, 3A&B). The assay in the ALDEFLUOR test was by flow cytometry, performed with expertise by Mr. Dennis Young at the UCSD Cancer Center. PrTuCells grown in medium 6⁺⁺⁺ from patient samples Pr#76 and Pr#109 also were quantitatively shown to synthesize ample ALDH as assayed by the ALDEFLUOR-FACS test.

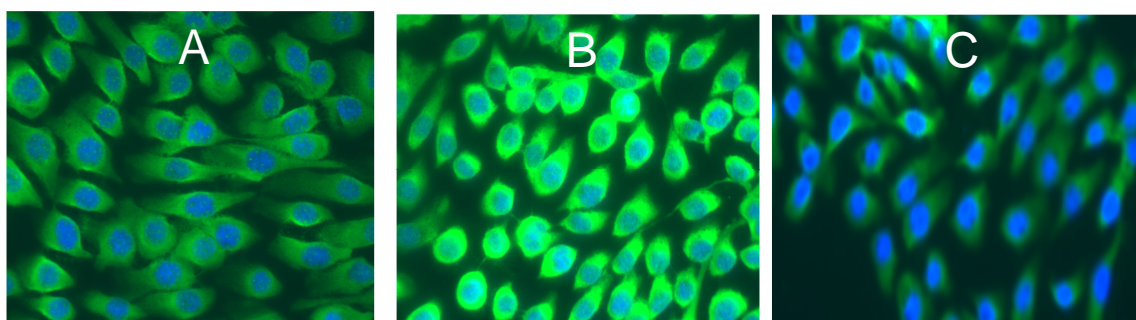
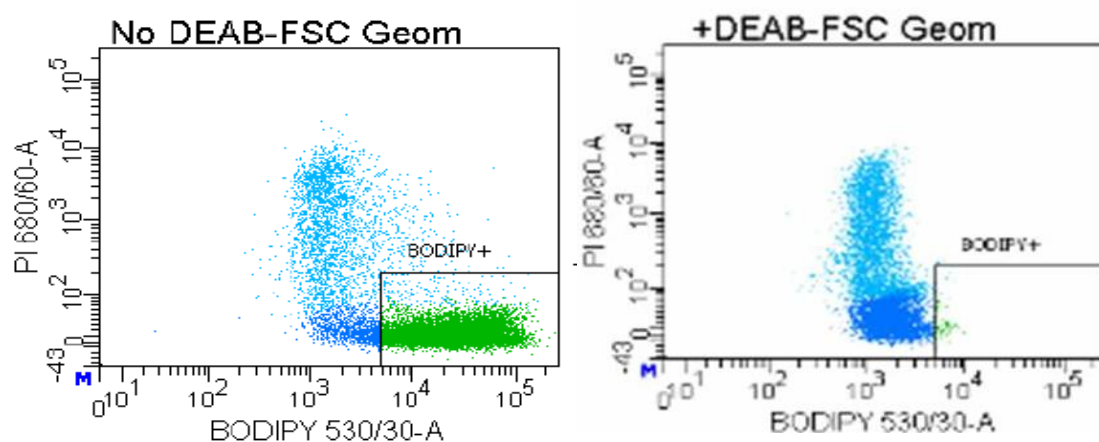
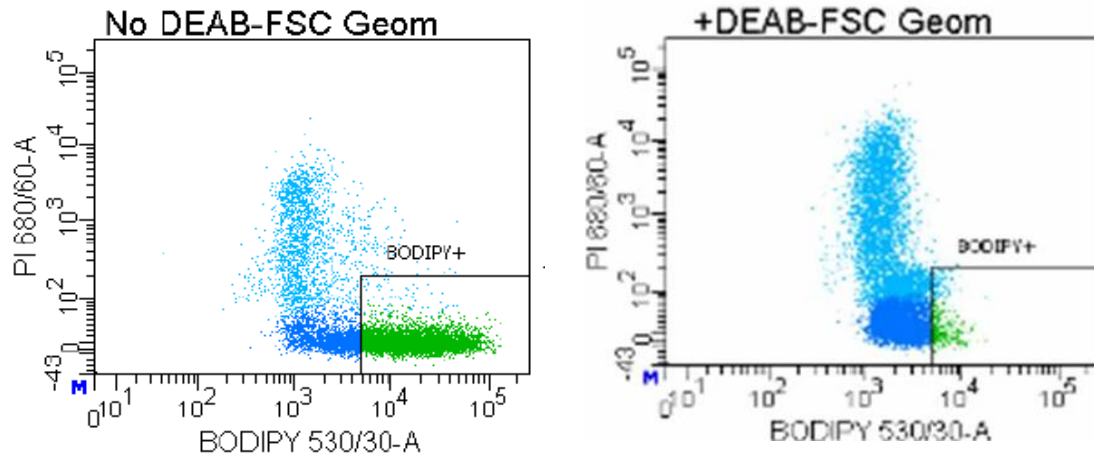


Figure 12. (12A) Immunohistological staining of ALDH1A3 on Pr87 (1s) (12B) Immunohistological staining of ALDH7A1 on Pr87 (1s) (12C) Immunohistological staining of SSEA4 on Pr87 (1s)



Graph 2A and 2B. (2A) Pr#76 positive (2B) Pr#76 negative control (with DEAB inhibitor). These cells were subject to FACS after reacting with Aldefluor. The green cells are the cells that stained positive for ALDH activity. The dark blue are the cells that were alive but not positive. The light blue cells are the cells that are dead. Quantitatively, can see ALDH positive cells.



Graph 3A and 3B. (3A) Pr#109 positive (3B) Pr#109 negative control (with DEAB inhibitor). These cells were subject to FACS after reacting with Aldefluor. The green cells are the cells that stained positive for ALDH activity. The dark blue are the cells that were alive but not positive. The light blue cells are the cells that are dead. Quantitatively, can see ALDH positive cells.

CHAPTER 4: DISCUSSION

We have developed methods to grow stem cell-like cancer cells (CIC, CSC) from carcinoma tissue of the earliest diagnosed prostate cancers, cancers that have not yet migrated from their initial location in the prostate and that are considered to be androgen-dependent or “castration-responsive”. The most important aspect of this work is that while these early, Stage I/II prostate cancers are widely *considered* to be androgen-responsive, the prostate tumor cells isolated and grown in our novel two-step methodology in serum- and hormone-free medium are androgen-independent, androgen-unresponsive, thus, “castration-resistant”. Small numbers of these cultured prostate tumor cells are potently tumorigenic in SCID mouse transplantations, generating human prostate cells with a short latency of only weeks (shown in other work, not here). We interpret these results to indicate that potentially lethal prostate cancer cells that are “castration resistant” – i.e. androgen-unresponsive and androgen-independent – are already present in the earliest stages of prostate cancer. Presumably, after further tumorigenic progression of these cells, they are the cells that become invasive and metastatic in their patient of origin – and become lethal and therapy-resistant on recurrence of the disease. Unlike the widely-held notion that early prostate cancers undergo “androgen/receptor-switching” while acquiring a castration-resistant phenotype, a form of castration-resistant cell pre-exist in at least 50% (n=52) of the Stage I/II prostate carcinomas that we have grown in culture and studied. Identification of castration-resistant cells in the earliest stages of prostate cancer turns the field of human prostate cancer inside-out and is of major scientific and biomedical importance as it

comprises an entirely new paradigm in human prostate cancer that is of far-reaching therapeutic consequence.

The purpose of this Masters Thesis work was to characterize these cultured, adherent prostate tumor cells (PrTuC) and their suspension sphere embodiment (CIC-spheres).

A summary of the results shows that the cells in question are of a stem/progenitor persuasion, express many well-established stem/progenitor markers, express basal/stem cell cytokeratin markers, are TERT-positive and lack the prostate cancer-associated TMPRSS2-ERG fusion mRNA. In addition, the cells are androgen-independent and androgen-unresponsive, in addition to being potentially tumorigenic *in vivo by themselves*.

Virtually all cancer cells express TERT and so do the PrTuCells/CIC that we have isolated from early cancers. Using PCR, others have found that ~100% of PrCa samples tested were positive for TERT, but TERT is not expressed in normal prostate and in BPH samples (34). TERT is an important marker to study because of its role in cell survival and its role in the continuous division of cancer cells (35). TERT mRNA has been shown to be present in all of our prostate tumor samples (Figure 4). Also, NPrEpCells are TERT negative. Our findings support others' results that PrCa samples with Gleason grades 6 to 9 are all TERT positive. By being TERT positive, it shows that these PrCa samples contain the gene that maintains telomeres and allow for the cells to continuously divide.

In spite of the expression of TERT by these potentially tumorigenic PrTuCells/CIC cells, they are not immortal in culture. In fact, while the cells are potentially tumorigenic *in vivo* when transplanted orthotopically into the native microenvironment, they senesce in

culture, suggesting that these androgen-independent prostate tumor cells depend on their microenvironment to express their sinister – immortal – program in vivo. This suggests that only after these cells have fully progressed to an invasive and metastatic phenotype will they become immortal in vitro. Supporting this notion is the fact that it has proven very hard to establish human prostate cell lines. Indeed, establishment in culture is a rare event, suggesting that the early malignant disease is fully dependent on its microenvironment, its stroma. The PrTuCells/CIC-spheres are not autonomously replicating cancer cells but microenvironment-dependent and potentially cancerous. This finding presents a novel question related to “cancer initiating cells”, CIC, a point that is discussed in the next paragraph. Additionally, if the mRNA fusion event TMPRSS2-ERG is in fact associated with prostate cancer invasion, metastasis and cancer cell progression – and poor prognosis – then the fusion event would be a contributor to the conversion of prostate cancer cells from a microenvironment-dependent/senescent state to an immortal phenotype. One attractive result of this assumption is that it is experimentally testable.

The isolation of CICs has been documented solely from progressed, metastatic cancer tissue from many human solid cancers. Starting with the work of the Ann Arbor group (9), and through publications documenting CICs from brain, liver, melanoma and other human solid cancers, the starting cancer tissue for CIC isolation were progressed cancers. Indeed, these human CICs were all stem/progenitor cells and possibly immortal in vitro. Our isolation by in vitro culture of CICs from very early human prostate cancers, CICs that possess many stem cell characteristics and constitute apparent microenvironment-dependent CICs, suggesting that the concept of CICs needs to be

broadened to include stem-like, microenvironment-dependent CICs in addition to a category of fully independent CICs. One would venture to suggest that the isolation and characterization of early prostate cancer CICs constitutes a new class of CICs, namely microenvironment-dependent CICs.

This hypothesis is supported by our finding that none of the early prostate tumor-derived cultured CICs possessed a detectable TMPRSS2-ERG fusion event.

Interestingly, Fine has reported that the frequency of this fusion event can be related to the cancer Gleason grade: the TMPRSS2-ERG fusion occurs most frequently in lower Gleason grades (less than 7) (30). Fine used FISH and correlated Gleason grade and tumor morphology. Their results could mean that since our samples are of higher Gleason grades, we do not see this TMPRSS2-ERG fusion.

The mechanism that triggers the gene fusion events as well as its incidence is not clear (31). Demonstrations of TMPRSS2-ERG fusion show that 59% of the fusion-positive prostate cancer cases had one of eight possible isoforms for this gene fusion (26). A potential source for this fusion event is androgen exposure (26, 31). An increase in ERG expression due to the fusion event leads to an increased expression of C-MYC, which is an oncogene closely associated with oncogenic cell transformation which prevents prostate epithelial cell differentiation (36). Using FISH, the fusion event is found to be induced through androgen receptor activity, as the TMPRSS2 and the ERG genes came together when AR activity was increased when compared to AR negative cells (31). This translocation may be associated with the aggressiveness of the prostate cancer; however, further research must be done to fully support this claim (26, 31).

Each of the PrCa-derived PRTuCells/CIC-sphere cells contains ample AR mRNA; however, there is little to no detectable AR protein. In NPrEpCells, there is less AR mRNA but more AR protein when compared to the early PrCa samples. These NPrEpCells show what the results would be if AR protein synthesis were functioning properly. When comparing PrTuCells #77 with Pr#77 sphere cells, there appears to be less AR mRNA in the latter though no AR protein is observed. This up-regulation of AR transcription seen in the PrCa samples could be due to the prostate trying and unable to produce more AR protein due to the malfunctioning of AR gene-translation. Some unknown mechanism could be blocking the translation of the increased transcription of AR mRNA. The fact that there is AR mRNA and no AR protein detected means that these samples, especially these CIC spheres are androgen-independent. No matter whether androgen is present or not, the translation from mRNA to protein is not occurring and this androgen-independence is already present in early stage I and II PrCa. *This observation appears to us worthy of serious follow-up and indeed we will pursue this discrepancy and its basis.*

Though historically thought to be primarily an androgen-dependent disease, evidence is mounting that estrogens play a role in the initiation and progression of prostate cancer. In an experiment to induce prostate cancer in SCID mice, the incidence of prostate cancer is 100% when supplemented with testosterone and estrogen; whereas, only 40% incidence occurred when supplemented with testosterone alone. This study shows that testosterone is necessary but not sufficient in the development of prostate cancer (32).

In our samples, there is very little ER β mRNA present in each of the tested PrCa samples and low/zero concentrations of ER β protein. Though difficult to rigorously compare, there is significantly less ER β mRNA than AR, ER α , and TERT mRNA. Looking at ER α , there is more ER α protein than ER β protein in the PrCa samples. There is also more ER α mRNA and protein expression in the PrCa samples than the NPrEpCells. ER α has been found in the stromal and basal prostate layers; whereas, ER β has been found on the stromal, basal, and epithelial layers (32). An increase in ER α expression is seen with an increase in Gleason grade and it mediates inflammation. ER β expression, on the other hand, is inversely correlated with Gleason grade and inhibits epithelial-mesenchymal transformation (32). Our results support these findings, as our prostate samples have down-regulated ER β and up-regulated ER α . In NPrEpCells, there is significantly more ER β protein compared to its expression in the PrCa samples. When compared to Pr#9, Pr#106, Pr#108, Pr#109, and PrST, the NPrEpCells has more ER β , as seen by flow cytometry as well.

Our FACS results show that there are no significant differences between the expression of the steroid hormone receptors when the samples are grown in the presence of androgen, estrogen, or both. These results show that the hormones do not have an effect on the growth or differentiation of the PrTuC samples. This could further support our notion that the PrTuCells grown from early PrCa samples constitute an embodiment of CIC/spheroids that are androgen-independent. Much further experimentation will be done because it is expected that since all of the PrCa samples contain the three receptors researched, they should respond to hormones. The differences due to hormones could be

difficult to detect and thus must be done again.

Several steps have been taken to characterize the PrTuCells and their CIC/spheroid counterparts with respect to their stem/progenitor cell properties. These early PrTuCell samples were previously transplanted orthotopically into SCID mice and produced tumors that were histologically identical to the patients' tumors. This first experiment shows that there are stem/progenitor cells within the samples.

The presence of the ALDH enzyme has been shown to identify stem cells (18). Hence, using the Aldefluor Assay kit, we have shown that our cultured PrTuCells contain and/or consist of prostate cancer stem cells (Graphs 2&3). We also stained the ALDH isotypes within the PrTuCells with antibodies specific for two specific ALDH isotypes. ALDH7A1 was expressed to a higher level in PrTuCells than ALDH1A3 (the ALDEFUOR kit is not specific for these isoforms of ALDH). All of these results attest to the stem/progenitor nature of the cultured PrTuCells. Also the cultured prostaspheres preferentially expressed the ALDH7A1 isoform. ALDH7A1 has been shown to be preferentially expressed in both localized prostate cancers, in disseminated and in matching bone metastases (18). Can we conclude that the cultured, castration resistant PrTuCells/CIC-spheres are an early manifestation of progressed prostate cancer cells that are lethal to the patients when they become invasive/metastatic? Though we are certainly implying that thesis, we have not yet proven this.

The PrTuCells/CIC-sphere cells express the ABCG2 gene. The ABCG2 product is a detoxifying pump of stem/progenitor cells that also asserts itself in cancers that have been exposed to chemotherapeutic drugs, asserting itself as one mechanism of cancer cell

multi-drug resistance. ABCG2 expression is, yet again, evidence for the stemness of the cultured/isolated PrTuCell/CIC-sphere cells.

The cultured/isolated PrTuCells/CIC-sphere cells are PSA negative. This antigen is important in diagnosing prostate cancer. Patient levels of the PSA serine protease increase when PrCa develops. This lack of PSA expression by our PrTuCells/CIC-sphere cells has caused us much consternation. However, recently a report has shown that CIC cells isolated from the progressed prostate cancer cell line LNCaP are PSA^{lo/-}, alleviating our concern (28).

The prostate cancer samples we used have Gleason scores assigned to them. Though these scores are characteristic of the samples, prostate cancer is a heterogenous disease, meaning that it is not uniform and can range in Gleason score depending on the parts of the cancer analyzed. This is why there are two grades given (the primary and the secondary) which are added together to give the Gleason score. Therefore, it is difficult and beyond this study to make conclusions about the correlations between the Gleason scores and our results. Out of 390 patients, Lattouf showed that even when samples are graded by up to 15 pathologists, Gleason grading is a poor predictor of pathological outcome, as over half of the tumor samples were either under-graded or over-graded (37). We hypothesize that the isolation of PrTuCells/CIC-sphere cells may, in time, acquire predictive clinical value inasmuch as they appear to be representative of the castration-resistant cells that are lethal to the patients.

Epithelial-mesenchymal transition is when the phenotype of PrCa samples undergo molecular changes from epithelial to mesenchymal. This transition is thought to

be the process that causes dedifferentiation and cancer progression toward a more malignant state (38). The PrCa samples used throughout this experiment are early prostate cancers (stage I and II). The transition occurs in cancers that are further progressed than our samples. Since our samples are not undergoing EMT, it was not discussed in this thesis.

Overall, this study has laid the foundation for further characterization of early prostate cancer stem/progenitor cells. The adherent PrTuCells that grow into CIC-prostaspheres in 3D culture are ALDH and SSEA4 positive, indicating stem/progenitor characteristics. They are also androgen-independent and are proven to appear in early prostate cancer samples. These samples also have ER α up-regulated and ER β down-regulated. Our results hold the promise of applying these newly-discovered cells to pre-clinical investigations, and, ultimately, to clinically-relevant procedures.

APPENDIX

PAGE 31, FACS DATA

Table 3. For Pr#84, FACS cell numbers shown according to the day the fluorescence was taken. Each set of growth conditions/hormones are listed.

Pr#84 Cell Number				
Day	Control	Androgen	Estrogen	A&E
1	77834	82169	82285	78101
4	136667	131126	98756	104171
7	126505	114236	103744	93803
10	141246	116744	127573	118563

Table 4. For Pr#87, FACS cell numbers shown according to the day the fluorescence was taken. Each set of growth conditions/hormones are listed.

Pr#87 Cell Number				
Day	Control	Androgen	Estrogen	A&E
1	39526	36634	38579	34342
4	124040	91300	90381	79395
7	267731	279597	233781	244572
10	272396	170119	174748	152540

Table 5. For Pr#106, FACS cell numbers shown according to the day the fluorescence was taken. Each set of growth conditions/hormones are listed.

Pr#106 Cell Number				
Day	Control	Androgen	Estrogen	A&E
1	27224	28239	27756	23392
6	72675	84355	63395	43792
11	69051	45248	49836	48460
20	28995	29701	59444	24201

Table 6. For Pr#108, FACS cell numbers shown according to the day the fluorescence was taken. Each set of growth conditions/hormones are listed.

Pr#108 Cell Number				
Day	Control	Androgen	Estrogen	A&E
1	95759	87623	67264	77325
6	88449	79490	72670	96919
11	181512	148347	129318	121165
20	113628	157884	123263	126713

Table 7. For Pr#109, FACS cell numbers shown according to the day the fluorescence was taken. Each set of growth conditions/hormones are listed.

Pr#109 Cell Number				
Day	Control	Androgen	Estrogen	A&E
1	88055	100662	105528	87158
6	133707	82737	76522	74840
11	91333	78249	67064	69676
20	117670	100277	104825	93193

PAGE 33, FACS for each receptor

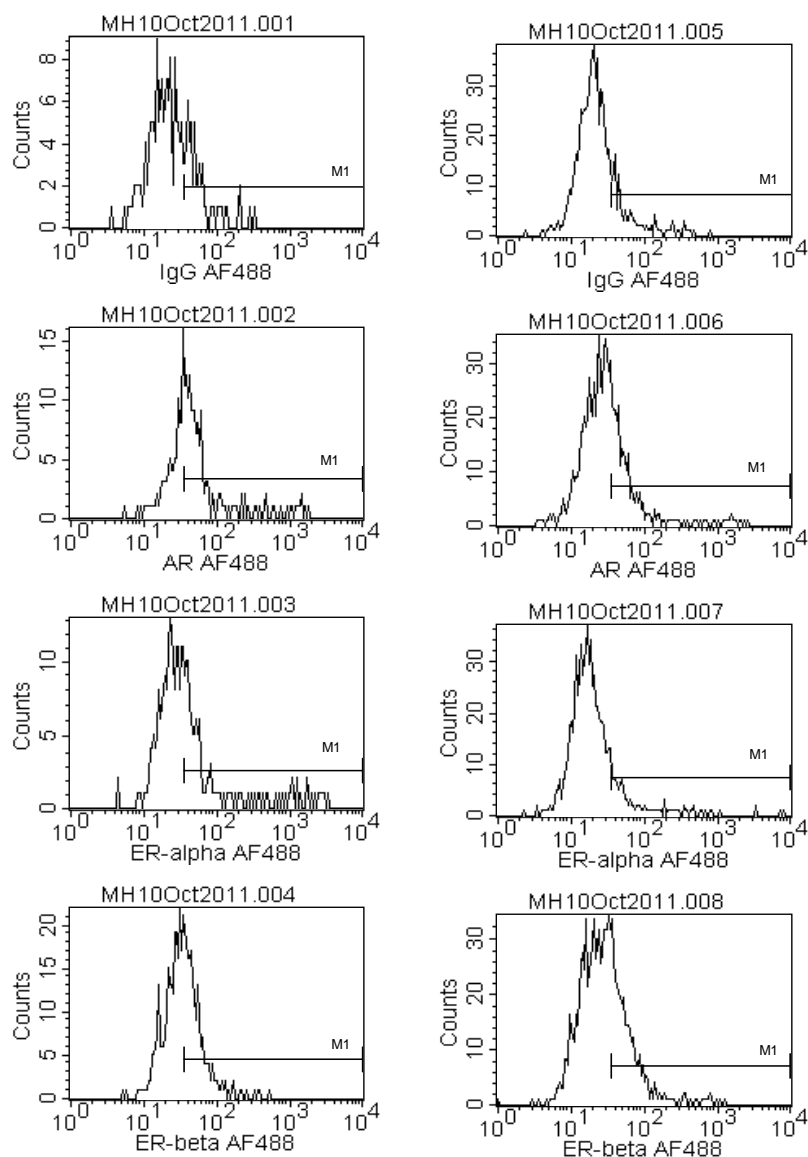


Figure 13. (Left) Pr#9, (Right) Pr#106. Using FACS, the amount of receptors that fluoresced are shown by the peaks and the areas under the peaks. Top is the control, next down is AR, next down is ER α , and the bottom is ER β .

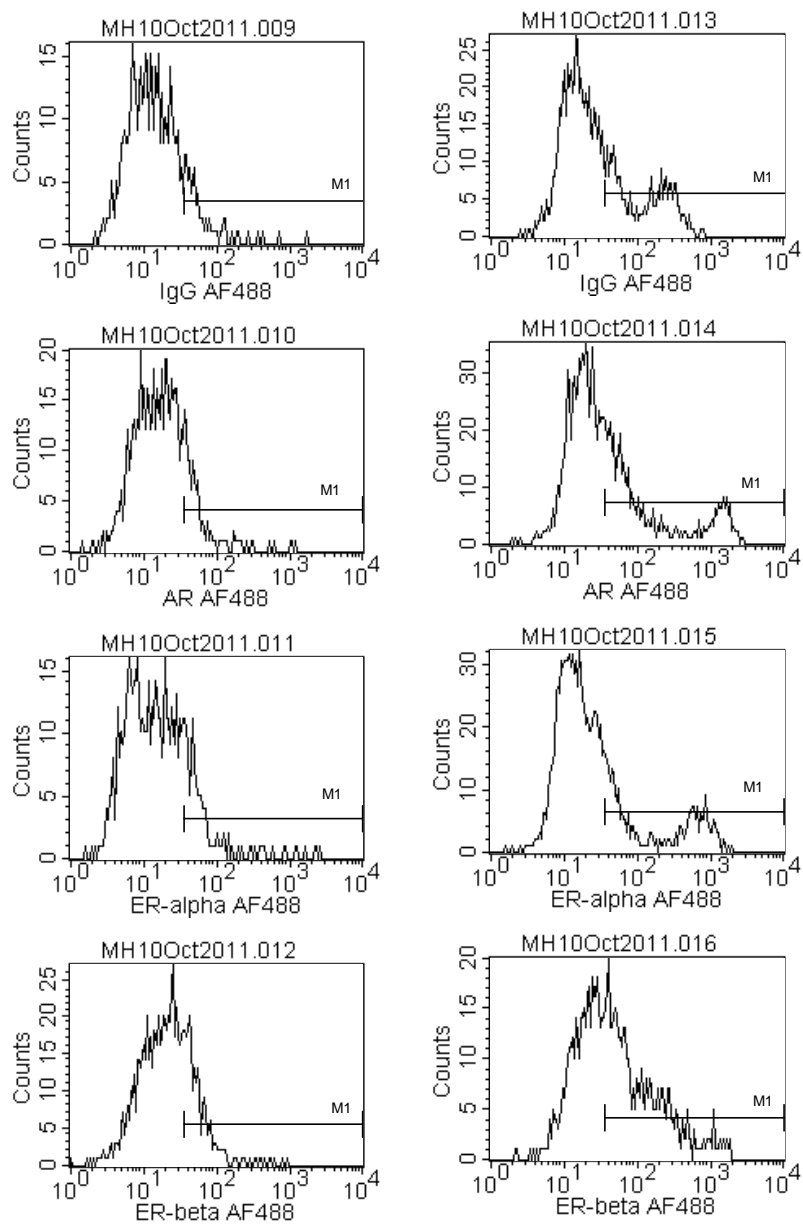


Figure 14.. (Left) Pr#108, (Right) Pr#109. Using FACS, the amount of receptors that fluoresced are shown by the peaks and the areas under the peaks. Top is the control, next down is AR, next down is ER α , and the bottom is ER β .

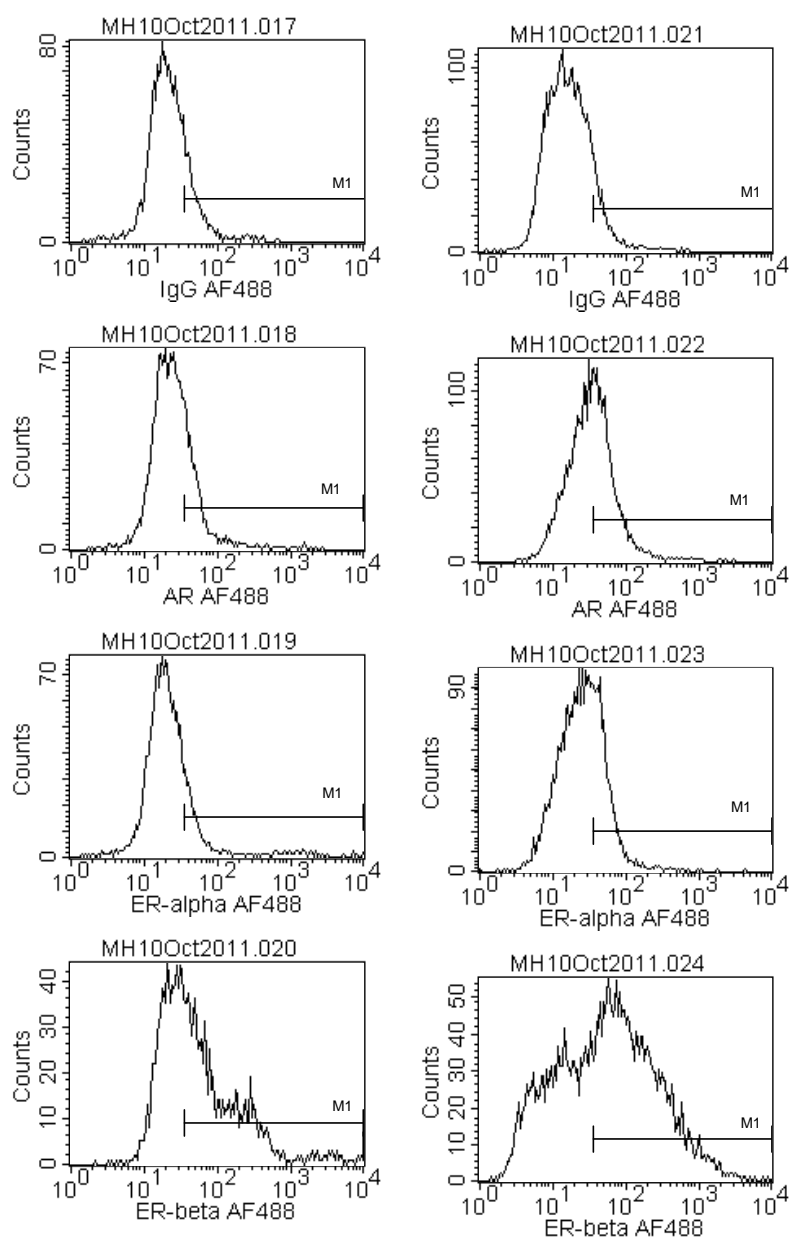


Figure 15. (Left) PrST, (Right) NPrEpCells. Using FACS, the amount of receptors that fluoresced are shown by the peaks and the areas under the peaks. Top is the control, next down is AR, next down is ER α , and the bottom is ER β .

REFERENCES

- (1) <http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-031941.pdf>, American Cancer Society website, accessed 3/28/2012 at 11:35pm
- (2) Snell, Richard S. Clinical Anatomy. 7th ed. Baltimore, MD and Philadelphia, PA: Williams & Wilkins, 2004.
- (3) Guess HA. “Benign Prostatic Hyperplasia and Prostate Cancer.” Epidemiologic Reviews 23.1 (2011): 152-158.
- (4) Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, Trent JM, and Isaacs WB. “Human Prostate Cancer and Benign Prostatic Hyperplasia: Molecular Dissection by Gene Expression Profiling.” Cancer Research 61.12 (2001): 4683-4688.
- (5) Shen MM, Abate-Shen C. “Molecular genetics of prostate cancer: new prospects for old challenges.” Genes & Development 24 (2010): 1976-2000.
- (6) <http://www.cancer.gov/cancertopics/pdq/treatment/prostate/Patient/page2> National Cancer Institute at the National Institutes of Health. Last modified 11/10/2011, accessed 2 may 2012 at 703pm.
- (7) Edited by Bostwick DG, Crawford ED, Higano CS, and Roach III M. American Cancer Society's Complete Guide to Prostate Cancer. Atlanta, GA: American Cancer Society, Health Promotions, 2005.
- (8) Peters CA, Walsh PC. “The Effect of Nafarelin Acetate, a Luteinizing-Hormone-Releasing Hormone Agonist, on Benign Prostatic Hyperplasia.” New England Journal of Medicine 317.10 (1987): 599-604.
- (9) Al-Hajj M, Wicha MS, Hernandez AB, Morrison SJ, Clarke MF. “Prospective identification of tumorigenic breast cancer cells.” PNAS 100.7 (2003): 3983-3988.
- (10) Hombach-Klonisch S, Paranjothy T, Wiechec E, Pocar P, Mustafa T, Seifert, Zahl C, Gerlach KL, Biermann K, Steger K, Hoang-Vu C, Schulze-Osthoff K, and Los M. “Cancer stem cells as targets for cancer therapy: selected cancers as examples.” Arch Immunol Ther Exp (Warsz) 56.3 (2008): 165-180.
- (11) Wang X, Julio MK, Economides KD, Walker D, Yu H, Halili MV, Hu YP, Price SM, Abate-Shen C, and Shen MM. “A luminal epithelial stem cell that is a cell of origin for prostate cancer.” Nature 461.7263 (2008): 495-500.

- (12) Goldstein AS, Huang J, Guo C, Garraway IP, and Witte ON. "Identification of a Cell of Origin for Human Prostate Cancer." Science 329 (2010): 568-571.
- (13) Rajasekhar VK, Studer L, Gerald W, Socci ND, and Scher HI. "Tumor-initiating stem-like cells in human prostate cancer exhibit increased NF- κ B signalling." Nature Communications 162.2 (2011): 1-13.
- (14) Berg JM, Tymoczko JL, and Stryer L. Biochemistry. 6th edition. New York, NY: W.H. Freeman and Company, 2007.
- (15) Alison MR, Guppy NJ. "Finding cancer stem cells: are aldehyde dehydrogenases fit for purpose?" The Journal of Pathology 22.4 (2010): 335-344.
- (16) Hoogen, Christel van den, Horst G van den, Cheung H, Buijs JT, Pelger RCM, and Pluijm G. "The aldehyde dehydrogenase enzyme 7A1 is functionally involved in prostate cancer bone metastasis." Clinical and Experimental Metastasis 28.7 (2011): 615-625.
- (17) Ginestier, Christopher, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott, Hayes D, Birnbaum D, Wicha MS, and Dontu G. "ALDH1 is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome." Cell Stem Cell 1 (2007): 555-567.
- (18) Hoogen C, Horst G, Cheung H, Buijs JT, Lippitt JM, Guzman-Ramirez N, Hamdy FC, Eaton CL, Thalmann GN, Cecchini MG, Pelger RCM, and Pluijm G. "High Aldehyde Dehydrogenase Activity Identifies Tumor-Initiating and Metastasis-Initiating Cells in Human Prostate Cancer." American Association for Cancer Research 70.12 (2010): 5163-73.
- (19) Hu WY, Shi GB, Lam HM, Hu DP, Ho SM, Madueke IC, Kajdacsy-Balla A, and Prins G. "Estrogen-Initiated Transformation of Prostate Epithelium Derived from Normal Human Prostate Stem-Progenitor Cells." Endocrinology 152.6 (2011): 2150-2163
- (20) Chen S, Principessa L, and Isaacs JT. "Human Prostate Cancer Initiating Cells Isolated Directly from Localized Cancer Do Not Form Prostatespheres in Primary Culture." Prostate (published online, not yet paper published, 6 MAR 2012)
- (21) Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, and French FS. "Dehydroepiandrosterone Activates Mutant Androgen Receptors Expressed in the Androgen-Dependent Human Prostate Cancer Xenograft CWR22 and LNCaP Cells." Molecular Endocrinology 11.4 (1997):450-459.
- (22) <http://pga.mgh.harvard.edu/primerbank/> Harvard primer bank.

- (23) <http://www.operon.com/tools/oligo-design-tools.aspx>. Eurofins mwg operon
- (24) Beattie GM, Lopez AD, Bucay N, Hinton A, Firpo MT, King CC, and Hayek A. "Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers." Stem Cells 23 (2005):489-495.
- (25) Rajput AB, Miller MA, De Luca A, Boyd N, Leung S, Hurtado-Coll A, Fazli L, Jones EC, Palmer JB, Gleave ME, Cox ME, and Huntsman DG. "Frequency of the TMPRSS2:ERG gene fusion is increased in moderate to poorly differentiated prostate cancers." Journal of Clinical Pathology 60 (2007):1238-1243.
- (26) Wang J, Cai Y, Ren C, and Ittman M. "Expression of Variant TMPRSS2/ERG Fusion Messenger RNAs is Associated with Aggressive Prostate Cancer." Cancer Research 66.17 (2006): 8346-8351.
- (27) Mani RS, Tomlins SA, Callahan K, Ghosh A, Nyati MK, Varabally S, Palanisamy N, and Chinnaiyan AM. "Induced Chromosomal Proximity and Gene Fusions in Prostate Cancer." Science 326.5957 (2009): 1230.
- (28) Moreb JS. "Aldehyde Dehydrogenase as a Marker for Stem Cells." Current Stem Cell Research & Therapy 3.4 (2008): 237-246.
- (29) Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, and Chinnaiyan AM. "Recurrent Fusion of TMPRSS2 and ETS Transcription Factor Genes in Prostate Cancer." Science 310.5748 (2005): 644-648.
- (30) Fine, SW, Gopalan A, Leversha MA, Al-Ahmadie HA, Tickoo SK, Zhou Q, Satagopan JM, Scardino PT, Gerald WL, and Reuter VE. "TMPRSS2-ERG gene fusion is associated with low Gleason scores and not with high-grade morphological features." Modern Pathology 23 (2010): 1325-1333.
- (31) Bastus N, Boyd L, Mao X, Stankiewicz E, Kudahetti SC, Tim R, Oliver D, Berney DM, and Lu Y-J. "Androgen-Induced TMPRSS2:ERG Fusion in Nonmalignant Prostate Epithelial Cells." Cancer Research 70.23 (2010):9544-9548.
- (32) Nelles JL, Hu WY, Prins G, "Estrogen Action and Prostate Cancer." Expert Review of Endocrinology and Metabolism 6.3 (2011): 437-451.
- (33) Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA, and Lee PWK. "Aldehyde Dehydrogenase Activity of Breast Cancer Stem Cells is Primarily Due To Isoform ALDH3A1 and Its Expression Is Predictive of Metastasis." Stem Cells 29.1 (2011): 32-45.

- (34) Iczkowski KA, Pantazis CG, McGregor DH, Wu Y, and Tawfik OW. "Telomerase Reverse Transcriptase Subunit Immunoreactivity: A Marker for High-Grade Prostate Carcinoma." Cancer 95 (2002):2487-2493.
- (35) Tricoli JV, Schoenfeldt M, Conley BA. "Detection of Prostate Cancer and Predicting Progression: Current and Future Diagnostic Markers." Clinical Cancer Research 10 (2004): 3943-3953.
- (36) Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan S-H, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, Patel V, Vahey M, Gutkind JS, Sreenath T, Petrovics G, Sesterhenn IA, McLeod DG, and Srivastava. "TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation." Oncogene 27 (2008): 5348-5353.
- (37) Lattouf JB, Saad F. "Gleason score on biopsy: is it reliable for predicting the final grade on pathology?" BJU Int. 90.7 (2002): 694-699.
- (38) Qin J, Liu X, Laffin B, Chen X, Choy G, Jeter CR, Calhoun-Davis T, Li H, Palapattu GS, Pang S, Lin K, Huand K, Ivanov I, Li W, Suraneni MV, and Tang DG. "The PSA^{-lo} Prostate Cancer Cell Population Harbors Self-Renewing Long-Term Tumor-Propagating Cells that Resist Castration." Cell Stem Cell 10 (2010): 556-569.