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Gene Expression Profile of Early Prostate Cancer Cells

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The thesis of Su-Shin Hao is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my mom for raising me, and my sister for humoring me.

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ABSTRACT OF THE THESIS

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Professor Daniel J. Donoghue, Chair
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Prostate cancer is a complicated disease. The five-year patient survival ranges from nearly 100% for local and regional stage cancers to 28% once the cancer becomes distantly invasive. This suggests that the lethal and non-lethal prostate cancers are likely separate diseases and may possess different early expression profiles. We compared the gene expression of early prostate cancer cells of varying Gleason scores using transcriptome analysis and confirmed the expression of certain overexpressed genes with Western blotting.

Of the approximately 40 differentially expressed genes found in our RNAseq

study, 21 were singled out due to their pronounced (greater than 2.5-fold) over- or under-expression in other cancers. *SERPINB2, PI3, SPRR2D, RRAD, S100P, SLPI, LCN2, EDN1, CLDN4, LAMP3, ANGPTL4, and COL1A2* were upregulated. *SOX2, MELK, CENPF, TOP2A, CDC20, MCM3, DLGAP5, ANLN, and RRM2* were downregulated. The magnitude of gene up- or down-regulation, with the exception of *COL1A2*, were much lower in passaged PrCa.

Lipocalin-2 protein was highly expressed by PrCa 87 and NEp 21 with PrCa 87 expressing four times the amount of NEp 21. Sox-2 protein was expressed highest in PrCa 109. SerpinB2 was expressed the highest in PrCa 87, then PrCa 109, then PrCa 76 while NEp 21 and NEp 83 had negligible amounts of SerpinB2. These results, if repeated in large numbers of prostate cancer short-term cultures, may facilitate the prevention of overtreatment due to uncertainty about lethality of the patient's prostate cancer.

1 Introduction

1.1 Current state of prostate cancer treatment

Prostate cancer is a disease that affects 1 in 6 men in their lifetimes, and is the second leading cause of cancer death in American men with 1 in 38 men dying of prostate cancer [1]. Neither the etiology nor the mechanisms responsible for the initiation or progression of prostate cancer are known. Nor is it clear at the time of prostate cancer diagnosis which patients' cancers will progress, though it is widely assumed that many cases will progress to potentially lethal disease given enough time.

Patients are routinely diagnosed through a blood PSA-level screening which may be followed by a digital rectal exam (DRE) which may be followed up with a biopsy. If cancerous tissue is detected, androgen deprivation therapy (ADT) is conducted via biochemical or surgical routes and most patients will see a regression of the cancer. Prostate cells require androgen to grow, and ADT effectively stops or slows growth of the prostate. Alternatively, surgery (prostatectomy) or radiation therapy may also be recommended. The cancer inevitably returns in a castration resistant (CRPC) form which no longer responds to ADT [2]. Castration resistance is classified as an increase in tumor volume via radiographic tests or rising prostate-specific antigen levels despite low levels of testosterone. Once CRPC progresses to metastasis, the 5-year survival rate is approximately 30% [3].

The prostate specific antigen (PSA) test's ability to determine if the subject has prostate cancer is controversial. A number of noncancerous causes of elevated PSA levels such as riding a bicycle or ejaculation could result in false positives [4]. Interestingly, although the widespread use of the PSA test caused an increase in recorded prostate cancer incidence in 1991, the mortality rate remained relatively the same [5]. The Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial combined several randomized trials of prostate cancer screening. In these trials, the PSA test and DRE were assessed for their ability to prevent prostate cancer deaths. It was found that the group which received screening had a higher incidence of disease than the control men; however, the death rates were the same [1].

This suggested that many men were unnecessarily treated for a disease that would not have affected them in their lifetimes. Healthcare providers have difficulty determining whether or not a patients prostate cancer could be lethal and sometimes treatment is given unnecessarily as most men diagnosed with prostate cancer die of other causes. PSA testing thus leads to overdiagnosis followed by overtreatment followed by lasting harms such as urinary incontinence, bowel control issues, or surgical complications such as death. The initial biopsy itself is not entirely safe, causing similar complications such as pain, urinary incontinence and infection [4].

1.2 Androgen deprivation and the rise of cancer stem cells

My lab and others have hypothesized that rather than a switch from androgen dependent phase (AD) to androgen independent phase(AI), the seeds of potentially lethal, AI prostate cancer cells exist at the very beginning of the disease [6, 7]. Such cells are thought to be very low in number, ranging from 1 in 10^7 to 0.1 % to 1% depending on

the method used, leading to difficult detection and are commonly called cancer stem cells (CSC) due to expression of stem cell markers [8, 9, 10]. The rarity of these CSC would cause early prostate cancers to appear AD since the majority of cells in the tumor mass are differentiated and androgen-reponsive. ADT then selects for AI CSC resulting in CRPC typically within 14 to 20 months of beginning ADT [10, 11].

In pursuit of this hypothesis, my lab has devised methods to grow prostate cancer stem cells (PrCaSC) from clinical samples of early prostate cancers at the time of prostatectomy. While isolation of these PrCaSC from clinical material is difficult due to their scarcity, these primary cells can be propagated in culture for short periods of time and a small number of culture transfers. Early prostate cancer cells were chosen for our experiments because we want to discover the origins of this disease rather than the progression.

These cells have been extensively characterized *in vitro* and *in vivo* [8]. These cells are positive for CD44, CD133, CK5/14, c-kit, integrin $\alpha 2\beta 1$, SSEA, E-cadherin, ALDH7A1, and TERT. They were also capable of generating locally invasive tumors when orthotopically xenografted into SCID mice [8]. The general consensus for the definition of PrCaSC is $CD44^{+}$, $\alpha 2\beta 1^{hi/+}$, and $CD133^{+}$ based on colony forming efficiency, invasive ability, anchorage-independent growth and ability to persist *in vitro* for extended periods of time [9, 12] Interestingly, $PSA^{lo/-}$ PrCaSC were found to be especially tumorigenic *in vivo* [12].

Prostate cancer cells sampled from prostate cancer prostatectomies, which were previously characterized by Fiñones et al in 2013, will be further analyzed in comparison to normal prostate epithelial cells [8]. Using transcriptome analysis, 3 normal and 3 cancer prostate cell cultures from the biopsies, grown for one transfer in culture, were compared and fold differences in mRNA expression were determined. Approximately 30 genes were found to be 15 to 35 fold up- or down-regulated in short-term cultured

cells. The possible roles of these transcriptional changes in the early cancer process were derived from published literature and 4 of these changes were studied further by Western blotting.

I decided to confirm protein expression of lipocalin-2 (*LCN2*), octamer-binding protein 4 (*OCT4*), SRY-box 2 (*SOX2*), and serpinB2 (*SERPINB2*). *LCN2* is linked to angiogenesis, epithelial-mesenchymal transition (EMT), and metastasis [13]. Angiogenesis is very important for a tumor as the existing blood vessels are only meant to support the original, non-tumorous tissue. Upregulated *LCN2* likely serves this need by activating vascular endothelial growth factor (*VEGF*) which enhances angiogenesis. *OCT4* and *SOX2* are strongly associated with stem cells, EMT, and PrCa disease progression through loss of differentiation [14]. Lastly, *SERPINB2*, which is linked to prevention of retinoblastoma protein proteolysis and possibly anti-apoptosis [15, 16, 17].

A comparison of expression levels of proteins between normal prostate epithelial cells (NPr) and early prostate cancer cells (PrCa) may reveal a new biomarker for diagnosis. A new biomarker to facilitate distinguishing between the lethal form of prostate cancer and non-lethal varieties would have significant value in the clinic. Investigations of prostate cancer cell progression, invasion, and metastasis would be served by such stage-specific biomarkers.

2 Materials and Methods

2.1 Obtaining Samples

Cell samples were obtained from patients undergoing prostatectomy and diagnosed with stage I and II prostate cancer. Sections of the prostate cancer were frozen and harvested along with proximal surrounding tissue. The samples were digested by stirring in 150U/mL collagenase I (Sigma-Aldrich) in growth medium at 37°C. The samples were aliquoted and frozen live in 90% FBS and 10% DMSO (Sigma-Aldrich). Samples are stored in liquid nitrogen. Normal prostate epithelial cell cultures were obtained from Lifeline Cell Technology. The names of the cell samples are arbitrarily assigned numbers.

2.2 Cell Culture

Cells were grown in 6-well or 12-well tissue culture plates (Corning) coated with laminin (Sigma-Aldrich). The prostate cell samples were then grown in keratinocyte serum free medium (Gibco) with 40mM L-glutamine (Gibco), 12.5μL gentamycin and 2.5μg/mL ampthotericin 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 $CaCl_2$, and 0.025% BSA (Sigma-Aldrich). Cultures were incubated at 37°C in 10% CO_2, 5% O_2, and medium was changed every other day.

Stem cells were grown in embryonic stem cell (ES) media composed of 500mL Knock-out DMEM, 2.5mL gentamycin, 10mL GlutaMAX (Life Technologies), 5mL Non-essential Amino Acids (Life Technologies), 65mL Serum Replacement, 65mL Plasmanate, 5mL Fungizone. Basic FGF was added shortly before use at 10ng/mL.

Early PrCa were passaged once after initial *in vitro* growth of prostatectomy samples. Passaged PrCa were passaged at least two more times afterwards and left in culture until confluent, typically 2 to 3 weeks longer than early PrCa.

2.3 Protein and RNA Isolation from Trizol Lysate

Media was aspirated from cell culture plates and pre-warmed ATV trypsin (Life Technologies) was added to each well. The wells were incubated at 37°C for 20 minutes. Cells were collected in a centrifuge tube and resuspended in PBS and then in Trizol reagent (Life Technologies). Trizol lysates were stored at -80°C.

Using chloroform, the insoluble fraction was separated and purified into total RNA using Qiagen RNEasy mini kit according to the kit's protocol. The soluble fraction was purified for protein. Protein isolation was done according to the protocol written by Life Technologies [18] with the addition of sonication (15 seconds, 10% Amplitude, 20% Pulse until solubilized) at the end. Isolated protein was stored at -70°C.

2.4 RNAseq

Total RNA quality was ascertained using Agilent Tapestation. Samples with an RNA Integrity Number (RIN) greater than 8 were used with Illumina's TruSeq Stranded mRNA Sample Prep Kit to generate RNA libraries from 1 μg of total RNA for each sample. RNA libraries were multiplexed and sequenced with 50 basepair (bp) single end

reads (SR50) to an average depth of approximately 36 million reads per sample on an Illumina HiSeq2500.

Analysis for early PrCa was accomplished using TopHat and Cufflinks [19, 20].

Analysis for passaged PrCa was done with kallisto and limma. RNAseq fastq files were processed into transcript-level summaries using kallisto, an ultrafast pseudo-alignment algorithm with expectation maximization [21]. The reference transcriptome was the current human GENCODE Release 23 (GRCh38.p3) [22]. Transcript-level summaries were combined into gene-level summaries by adding all transcript counts from the same gene. Gene counts were normalized across samples using DESeq normalization [23], and the gene list was filtered based on mean abundance (across samples), which left about 14,000 detected genes for further analysis. Integrity of the experiment (consistency of replicates and the direction of treatment effects) was assessed globally by principal component analysis using R [24]. Differential expression was assessed with the R package, limma [25] applied to log2-transformed counts. Statistical significance of each test was expressed in terms of local false discovery rate (lfdr) [26] using the limma function eBayes [27]. The list of genes sorted by lfdr was analyzed for over-represented biological processes and pathways using a non-parametric version of Gene Set Enrichment Analysis [28, 29].

2.5 Western Blotting

Protein samples were quantified using the Lowry assay (Biorad). 30μg of protein was loaded and separated by 17.5% SDS-PAGE. The proteins were then transferred to Immobilon-P membrane (Millipore). The membrane was blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline with 0.5% Tween-20 (TBS-T). The membranes were incubated with primary antibodies in 3% BSA in TBS-T at 4°C overnight. After

primary incubations, membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibodies (GE Healthcare). Proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare). Membranes were stripped with stripping buffer (2% SDS, 10nM β-mercaptoethanol, 62.5mM Tris pH 6.8) for 30 minutes at 55°C and reprobed with other primary antibodies. GAPDH was used as the loading control (Genetex).

Table 1: A table of antibodies used to determine if protein levels follow the RNA upregulation observed in the transcriptome analysis. Primary antibodies were incubated at 4°C overnight and secondary antibodies were incubated at 22°C for 1 hour.

Gene	Source	Concentration
GAPDH (control)	Genetex (GTX627408) mouse monoclonal IgG	1:5000
Oct-4	Santa Cruz Biotech (SC-3655099, A-9) mouse monoclonal IgG	1:100
Sox-2	Santa Cruz Biotech (SC-17320, Y-17) goat polyclonal IgG	1:400
Serpin-B2 (PAI-2)	Santa Cruz Biotech (SC-6649, A-19) goat polyclonal IgG	1:250
Lipocalin-2 (NGAL)	R&D (AF1757) goat polyclonal IgG	1:2000

3 Results

3.1 Transcriptome analysis

An RNAseq was performed to determine which genes are upregulated or down-regulated in early prostate cancer cells compared with their expression in normal prostate epithelial cells. In the first sequencing done in 2012 with early PrCa, we found over 20 differentially expressed genes with a q-value of less than 0.005 (Table 2).

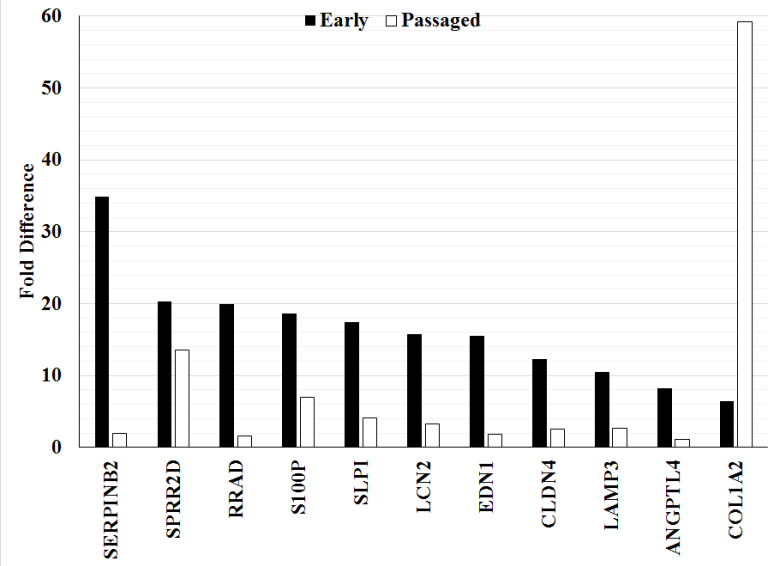

Figure 1: **Overexpressed genes of early and passaged PrCa.** 3 PrCa patient samples were compared to 3 NEp cell cultures with identical growing conditions. Early PrCa RNAseq was analyzed using TopHat and Cufflinks (q <0.005). Passaged PrCa analysis used kallisto and R package, limma.

When comparing early cultured cells and passaged PrCa, all of the overexpressed

genes underwent a drop in expression greater than 2.5 times excluding *SPRR2D* and *COL1A2*. *COL1A2* is a startling 9 times higher in expression in passaged PrCa compared to early PrCa, the opposite direction of the others. SPRR2D's expression in passaged PrCa is uncertain due to a q-value of 0.8 while *COL1A2* was 0.048 (Figure 1). In the case of SPRR2D, PrCa 87 had a transcript count of 17 fold higher than the other two PrCa.

The first RNAseq had 1824 genes with a q-value of less than 0.1 while the second RNAseq had 41. With the exception of COL1A2, the numbers reported for the passaged PrCa are likely false positives (q >0.7). These numbers were still included to display the likelihood of a difference between the two states of PrCa. In all overexpressed genes, except for *RRAD, S100P, and COL1A2*, PrCa 87 had higher expression of that gene than the other PrCa (76, 109) after passaging. In our *in vivo* experiments, PrCa 87 is the only culture we've tested which consistently generated tumors if early cells are used (data not shown). This is also seen in the Western blots (Figure 3).

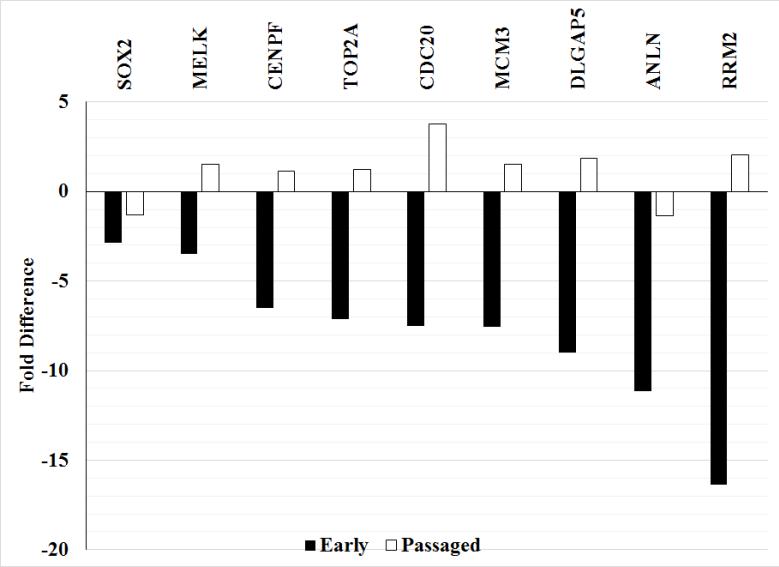

Figure 2: **Underexpressed genes of early and passaged PrCa.** 3 PrCa patient samples were compared to 3 NEp grown in identical conditions. Early PrCa RNAseq was analyzed using TopHat and Cufflinks (q <0.005). Passaged PrCa analysis used kallisto and R package, limma.

Underexpressed genes were also subject to a large difference in expression. While

the results of the second sequencing cannot be entirely accepted due to a high q-value, we can still see that these genes are no longer as downregulated as in early PrCa (Figure 2).

Table 2: **Changes in gene expression between early and passaged prostate cancer cells.** Three PrCa patient samples were compared to three normal prostate epithelial cells. RNAseq data for early PrCa was analyzed using TopHat and Cufflinks. Passaged PrCa analysis used kallisto and R package, limma.

Gene	Early PrCa	Passaged PrCa	Title
SERPINB2	34.9	1.99	Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 2
SPRR2D	20.3	13.49	Small Proline-Rich Protein 2D
RRAD	19.9	1.58	Ras-Related Associated With Diabetes
S100P	18.6	6.96	S100 Calcium Binding Protein P
SLPI	17.40	4.14	Secretory Leukocyte Peptidase Inhibitor
LCN2	15.71	3.25	Lipocalin 2
EDN1	15.49	1.79	Endothelin 1
CLDN4	12.25	2.59	Claudin 4
LAMP3	10.47	2.66	Lysosomal-Associated Membrane Protein 3
ANGPTL4	8.22	1.09	Angiopoietin-Like 4
COL1A2	6.49	59.23	Collagen, Type I, Alpha 2
SOX2	-2.86	-1.29	SRY (Sex Determining Region Y)-Box 2
MELK	-3.46	1.52	Maternal Embryonic Leucine Zipper Kinase
CENPF	-6.50	1.14	Centromere Protein F, 350/400kDa
TOP2A	-7.11	1.23	Topoisomerase (DNA) II Alpha 170kDa
CDC20	-7.52	3.76	Cell Division Cycle 20
MCM3	-7.57	1.54	Minichromosome Maintenance Complex Component 3
DLGAP5	-9.01	1.85	Discs, Large (Drosophila) Homolog-Associated Protein 5
ANLN	-11.16	-1.33	Anillin, Actin Binding Protein
RRM2	-16.38	2.05	Ribonucleotide Reductase M2

3.2 Western blots show upregulated expression of several proteins associated with non-prostate cancers

To confirm the fold changes observed on the RNA level were reflected in the protein level, western blots were generated (Figure 3). For SERPINB2 and more notably LCN2, PrCa 87 had noticeably more protein than the other PrCa and NEp.

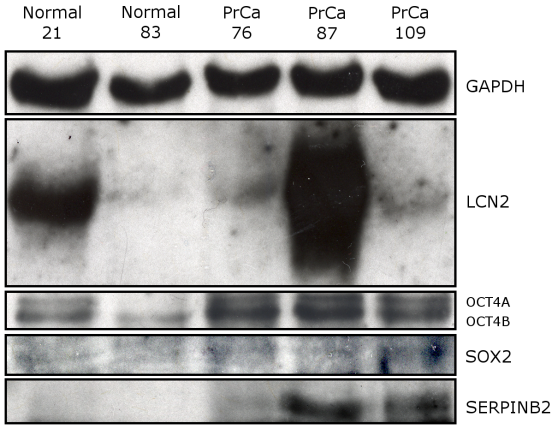

Figure 3: **Protein is expressed more in PrCa.** GAPDH was used as a loading control. Samples were purified from the same lysates as used in the second RNAseq and are passaged PrCa.

We see that NEp 21 expresses a great amount of LCN2 despite being a normal prostate epithelial culture. NEp 21 also has bands for OCT4A and SERPINB2 which NEp 83 does not. SOX2 protein is expressed almost equally between all cultures with higher expression in PrCa 109 despite *SOX2* being downregulated in the RNAseq (Table 2).

4 Discussion

Prostate cancer continues to be an enigmatic disease. Early treatment is important for any cancer; however, non-lethal and lethal prostate cancer often look similar in early stages leading to overtreatment. In efforts to elucidate the beginnings of this unpredictable disease, we have generated and analyzed two RNAseq data sets of RNA isolated from prostate cancer cells and their controls: early PrCa vs. NEp and culture-passaged PrCa vs. NEp. In the early PrCa data set, we found overexpression of the following genes: *SERPINB2, PI3, SPRR2D, S100P, RRAD, SLPI, LCN2, EDN1, CLDN4, LAMP3, ANGPTL4, COL1A2*. *SOX2, MELK, CENPF, TOP2A, CDC20, MCM3, DLGAP5, ANLN, RRM2* were found to be underexpressed compared to NEp. When the same samples were passaged and analyzed once more with RNAseq, we found that many genes which were upregulated in the early PrCa data set were no longer overexpressed in the passaged PrCa data set. The same was true for downregulated genes. We pursued Western blotting to learn if the change in gene expression led to increased protein synthesis only to find inconsistent results. There was no consistent trend between benign and malignant prostate cancer with no obvious trend between the different prostate epithelial cultures for the tested proteins. This study is not of sufficient scale to get a proper feel for the disease but is still necessary.

The genes chosen from the RNAseq data set were selected based on their relationship with cancer in the literature. Several themes were present among the differ-

entially expressed genes. Genes further studied were involved in metastasis/invasion, growth/proliferation, anti-apoptosis, and hypoxia (Table 3). Many of the downregulated genes are involved in the cell cycle and proliferation. Their downregulation in our early PrCa samples is puzzling. A few genes are just as perplexingly overexpressed. SERPINB2, RRAD, and ANGPTL4 are thought to have tumor suppressive functions.

Table 3: **Themes found within the set of overexpressed genes.** (M) metastasis, (P) proliferation, (AA) anti-apoptosis, (H) triggered by hypoxia.

Gene	M	P	AA	H	Cancers
SERPINB2		■	■		Bladder, Breast, Colorectal, Endometrial, Lung, Ovarian, Prostate
RRAD				■	Breast, Esophageal, Lung, Nasopharyngeal, Ovarian
S100P	■				Breast, Colorectal, Gastric, Lung, Ovarian, Pancreatic, Prostate
SLPI					Colon, Gastric, Lung, Ovarian, Prostate
LCN2	■			■	Bladder, Breast, Colorectal, Lung, Ovarian, Pancreatic, Prostate
EDN1		■	■		Breast, Cervical, CNS, Colon, Kidney, Lung, Ovarian, Prostate
CLDN4	■				Breast, Colon, Esophageal, Gastric, Ovarian, Pancreatic, Prostate
LAMP3	■			■	Breast, Cervical
ANGPTL4	■		■	■	Breast, Colorectal
COL1A2	■				Bladder, Colorectal, Head/Neck, Liver

High expression of SERPINB2 has been linked to a good prognosis for breast, small cell lung, and ovarian cancer, but a poor prognosis for colorectal cancer and shorter progression free survival in endometrial cancer [30]. SERPINB2 is known for binding to the urokinase plasminogen activator (uPA) and its receptor (uPAR) which causes internalization and destruction of the receptor thus preventing proteolytic cleavage of plasminogen to plasmin which could degrade the extracellular matrix [17]. RRAD is

believed to inhibit aerobic glycolysis and invasion with low expression being associated with a poor prognosis in hepatocellular carcinoma [31, 32, 33]. ANGPTL4 is thought to inhibit angiogenesis although its effect seems to be context dependent and varies from cancer to cancer [34]. The effects of *ANGPTL4* upregulation can range from extravasation, increased proliferation, and angiogenesis or inhibition of all three depending on the cancer [35].

The difference between the RNAseq results of early and passaged PrCa strongly suggests that PrCa grown *in vitro* for extended periods of time with transfers and passaging are hardly the same cells. The published literature show the downregulated genes in Figure 2 are found upregulated in other works [36, 37, 38, 39, 40, 41]. These genes are overexpressed primarily by PrCa 87 once passaged but not as a primary culture. One possible explanation for this discrepancy in behavior between PrCa samples is that each passage results in the differentiation and loss of CSC and PrCa 87 was found to have the most colonies formed from 50mg of dispersed tissue. PrCa 87 formed 350 colonies, PrCa 76 formed 180, and PrCa 109 formed 40 [8]. The CSC of PrCa 87 may also be more amenable to growth in our medium.

Cancer is taught to be a disease where normal cell cycle-abiding, apoptosis-respecting, immune system-fearing cells accumulate a series of mutations to become the ”cancer cell.” If these specific mutations or overexpressions could be controlled, then we could suppress the cancer. This way of thinking may not be productive since the number of genes aberrantly expressed in prostate cancer and the many interactions between them is enormous. Targeting a small subset of these genes, overexpressed or otherwise, will likely cause the cell to find another path to circumvent those efforts.

Two companies have conducted clinical trials testing their endothelin-A receptor antagonists in hopes of suppressing EDN1 activity. EDN1 has been shown to stimulate cancer growth, be associated with invasive breast cancer, contribute to metastasis, and

stimulate angiogenesis [42]. High expression of EDN1 in a tumor strongly correlated with low disease specific survival [43]. Despite all the signs pointing towards EDN1 as being important in prostate and bladder cancer, all 6 clinical trials designed to suppress prostate cancer by blocking EDN1 signaling were negative [42].

Instead of attempting to target every last cancer stem cell in the body using established therapies (cut, burn and poison) that are often associated with vast collateral damage, one would do better to target the cancer stem cells to lose its cancer phenotype by biologically pushing the cell to differentiate. Differentiation therapy is currently being used successfully to treat acute promyelocytic leukemia (APL). ATRA has recently come into the spotlight as a result of a 2015 publication showing the effects of the drug on the protein, PIN1. Kun Ping Lu and his associates have demonstrated that the use of ATRA on three breast cancer cell lines (MDA-MB-231, SKBR3, T47D) resulted in ablation of 11 oncogenic proteins and proliferation inhibition of many more breast cancer cell lines while having a lesser effect on normal breast cell lines. These authors go on to demonstrate that ATRA would shrink the size of tumors in mice injected with a breast cancer cell line (MDA-MB-231) [44].

Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1 (PIN1) has been shown to activate 32 oncogenic proteins and to inactivate 19 tumor suppressing proteins [44]. PIN1 is therefore a very central regulator in the carcinogenic phenotype through wholesale activation of oncogenes and the suppression of tumor suppressors. As a result of these activities, a system utilizing PIN1 immunohistochemical staining has been developed to determine probability of recurrence in PrCa patients [45, 46]. Out of 17 patients who had PSA recurrence following radical prostatectomy, 88.3% had the highest cytoplasmic and/or nuclear PIN1 score (3, out of 0-3 scale) [46]. In agreement with these findings, PIN1 is upregulated 2.4-fold in our passaged PrCa samples (not shown).

We have reason to suggest, but cannot confirm, PIN1 is overexpressed in our

early PrCa samples as well. The original fastq files are no longer available and we could not reanalyze the data using GENCODE, kallisto, and limma as the passaged PrCa were. Other difficulties included growing primary prostate cancer cells and their tendency to differentiate and senesce with each passage *in vitro* [8]. There was a limited supply of primary PrCa and we depleted a few patient samples before reaching a conclusion. When we resorted to using Trizol lysates containing the passaged PrCa, the purified protein had to be dissolved in a solution with a high molar concentration of urea and SDS; we had to use the Bradford assay instead of the Lowry assay to quantify our protein, and running gels consistently was difficult due to possibly unreliable quantification methods [47]. We were also hoping to correlate the RNAseq data with patient outcomes; unfortunately, the data linking our cells to the patients were lost by our clinical collaborators.

Mutations most probably play a central role in prostate cancer. One interpretation of our results suggests that mutations occur in prostate stem cells rather than in the more differentiated prostate epithelial cells. However, each patient's prostate cancer likely has its own set of prostate stem cell mutations which caused the divergence from its original program (Figure 3). Chasing down mutations for each patient is costly and missing the forest for the trees. ATRA and metformin are two drugs which have been shown to target CSC specifically and make CSC more susceptible to other drugs [44, 48].

Two phase 2 clinical trials for ATRA were performed in 1997 and 1999. Unfortunately, the conclusion was negative [49, 50]. The trials were conducted with patients confirmed to have metastatic CRPC, and only orally prescribed ATRA was used in these trials. Since 1999, it has been found that PIN1 is a key target of ATRA in APL, and liposome-encapsulated ATRA is much more effective at inducing and maintaining remission in APL than oral ATRA [44, 51]. Moreover, prostate cancers often overexpress PIN1; one study shows 45/49 prostate cancers overexpress PIN1 [52]. We have evidence that androgen independent cancer stem cells drive the cancer from the earliest stages of

the disease [8]. Additionally, the frequency of CSC increases with ADT; by the time the patient develops CRPC, the disease may be impossible to contain due to the sheer number of CSC [11]. This argument suggests that a clinical trial utilizing liposomal ATRA in combination with other chemotherapeutic agents as well as the selection of pre-metastatic patients with cancers that express high levels of PIN1 as subjects may yield curative results.

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