RNA-Seq Reveals Transcriptomic Program Associated with Stemness in Taxane Resistant Prostate Cancer

Christina K. Cajigas-Du Ross
RNA-Seq Reveals Transcriptomic Program Associated with Stemness in Taxane Resistant Prostate Cancer

by

Christina K. Cajigas-Du Ross

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology and Molecular Genetics

August 2018
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

________________________________________, Chairperson
Carlos A. Casiano, Professor of Microbiology and Molecular Genetics and Biochemistry

________________________________________
Eileen Brantley, Assistant Professor of Pharmacology and Physiology

________________________________________
Kimberly J. Payne, Associate Professor of Anatomy

________________________________________
Juli Unternaehrer, Assistant Professor of Biochemistry

________________________________________
Charles Wang, Professor of Microbiology and Molecular Genetics
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Carlos Casiano for his support and mentorship throughout my graduate career. Dr. Casiano, thank you for allowing me to think creatively and apply that creativity to my various projects. I would also like thank Drs. Marino and Daisy De Leon who recruited me to Loma Linda University and facilitated a year of research in the Casiano lab before beginning the graduate program.

To the members of my dissertation committee, thank you for your advice, direction, and mentorship. Dr. Payne—I would like to thank you for your support as a mentor, and your advice on life and science have helped me become a better scientist. Your guidance through my comprehensive exams was critical to helping me pass with high scores. Dr. Brantley—thank you for always taking the time to ask about my experiments and offering guidance, support, and career building advice. Dr. Unternaehrer—thank you for always being available to answer questions and provide feedback that strengthened my work; your expertise and guidance were crucial to my work in prostate cancer stem cells. Dr. Wang—thank you for running the RNA-sequencing; the Loma Linda Center for Genomics was very helpful, especially Xin Chen’s contribution to my dissertation project.

To my friends Leslimar Ríos-Colón and Leanne Burnham—your support, friendship, and love throughout these long six years have been crucial to my success in this program. Lesli thank you for training me when I first joined the lab and teaching me how to perform many of the techniques that I utilized in my various projects.

To my husband, Jonathon M. Du Ross—thank you for your constant support, love, facilitating late nights in the lab, and being the best father to our son Clayton.
Throughout this process you always gave me hope and encouragement, helping me to stay positive and remain focused on my goals. Our marriage and family made the move to California the best decision I could have ever made. To my son, Clayton—you are my single greatest achievement, you bring me so much happiness even in the darkest of times and I am so blessed to be your Mom. Finally, thank you to my parents Israel and Sandra Cajigas. Mom and Dad—thank you for your infinite love and support throughout this whole process; I could not have done this without you. To my brother Israel James, you always served as an example of success, and watching your achievements inspired me to work hard and continue my education. And finally, to my sister-in-law Julie, thank you for your friendship, love and constant support.
CONTENTS

Approval Page .................................................................................................................. iii

Acknowledgements .......................................................................................................... iv

List of Figures ..................................................................................................................... ix

List of Tables ...................................................................................................................... xi

List of Abbreviations ......................................................................................................... xiii

Abstract ............................................................................................................................. xv

Chapter

1. Introduction ................................................................................................................... 1

   Prostate Cancer Background and Available Therapies .............................................. 1

      PCa Screening and Diagnosis ................................................................................. 2
      Androgen Receptor and ADT ................................................................................ 5
      Castration-Resistant PCa ...................................................................................... 6
      Glucocorticoid Receptor “Take-over” Pathway ..................................................... 8
      AR Splice Variants Contribute to CRPC ............................................................. 9
      Taxane Chemotherapy ...................................................................................... 10

   Mechanisms of Taxane Chemoresistance ............................................................... 11

      Multidrug Transporters ..................................................................................... 11
      Tubulin Isoforms .............................................................................................. 12
      Survival Pathways and Inflammatory Cytokines .............................................. 13

         STAT1 and STAT3 .................................................................................. 13
         NF-κB ......................................................................................................... 13
         IL-6 and IL-8 ............................................................................................. 14
         CCL2 ........................................................................................................ 15

      Chaperone Proteins ..................................................................................... 15

         Heat Shock Proteins .................................................................................. 15
         Clusterin .................................................................................................. 16

      AR Splice Variants in the Context of DTX-Resistance ..................................... 17
      LEDGF/p75 Promotes DTX-resistance ......................................................... 18
3. Selected Unpublished Data .............................................................. 101
   LEDGF/p75 May Play a Role in Regulating Inflammatory Cytokines
   Contributing to PCa Aggressiveness and Chemoresistance ................. 101
   Migratory Potential of DTX-resistant Compared to DTX-sensitive
   Cells .................................................................................................. 106
   Co-targeting LEDGF/p75 and CLU Individually and Together in
   Combination with DTX to Resensitize DTX-resistant PCa cells to
   Taxane Therapy .............................................................................. 108
   Targeting LEDGF/p75 with SMIs to Resensitize Chemoresistant
   mCRPC Cells to DTX Treatment ...................................................... 114
   Adherent and Tumorsphere PC3-DR and DU145-DR Cells Upregulate
   CSC Markers CD133+ and CD117+ ............................................... 118
   References .................................................................................... 121

4. Overall Discussion ......................................................................... 124
   Conclusions and Future Directions .................................................. 129
   References .................................................................................... 131

5. Methodology .................................................................................. 135
   Cell Culture .................................................................................... 135
   Mycoplasma Testing ....................................................................... 137
   Preparation of Whole Cell Lysates .................................................. 138
   Antibodies ..................................................................................... 139
   Immunoblotting Procedures ........................................................... 140
   RNA Extraction for RNA-sequencing .............................................. 143
   RNA Extraction for Quantitative Real-Time PCR ............................. 144
   Quantitative Real-Time PCR .......................................................... 145
   Tumorsphere Forming Assays ........................................................ 147
   Flow Cytometric Analysis of Stem Cell Markers, ALDH activity and
   Cell Death ....................................................................................... 148
   Detection of Cell Viability by Propidium Iodide Staining ................... 150
   Cell Migration Assay ...................................................................... 151
   MTT Viability Assays ..................................................................... 151
   Statistical Analysis ...................................................................... 153
   Final Remarks ............................................................................... 153
## FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PCa Progression and Treatment Options</td>
<td>4</td>
</tr>
<tr>
<td>2. DTX-resistant PC3 and DU145 Cell Lines Upregulate Known Markers of DTX Resistance</td>
<td>42</td>
</tr>
<tr>
<td>3. Gene Expression Profiling Analysis Reveals Upregulation of CSC-associated Genes</td>
<td>44</td>
</tr>
<tr>
<td>4. Hierarchical Clustering Heat Map of Global Gene Analysis</td>
<td>46</td>
</tr>
<tr>
<td>5. GSEA Generated Heat Map of the Top-ranked 50 Overlap Genes</td>
<td>47</td>
</tr>
<tr>
<td>6. In-house qPCR Validation of the Expression of Selected Top-Ranked Genes from RNA-seq Results</td>
<td>52</td>
</tr>
<tr>
<td>7. Protein Expression Validation of RNA-seq Results in DTX-Sensitive and DTX-Resistant mCRPC cells</td>
<td>54</td>
</tr>
<tr>
<td>8. Expression of Selected Top-Ranked Genes in Clinical PCa Tissues</td>
<td>56</td>
</tr>
<tr>
<td>9. DTX-resistant mCRPC Cells Exhibit a Mesenchymal-like Phenotype Compared to DTX-sensitive Cells</td>
<td>59</td>
</tr>
<tr>
<td>10. DTX-resistant mCRPC Cells Upregulate Markers Associated with CSC-like Characteristics Compared to DTX-sensitive cells</td>
<td>63</td>
</tr>
<tr>
<td>11. Tumorsphere Formation Capacity is Higher in DTX-resistant DU145 Cells Compared to Sensitive Cells</td>
<td>67</td>
</tr>
<tr>
<td>12. DU145-DR Derived Tumorspheres Show Increased Resistance to DTX Compared to DU145-DR Adherent Cells</td>
<td>70</td>
</tr>
<tr>
<td>13. Quality Assessment Metrics for RNA-seq Data</td>
<td>79</td>
</tr>
<tr>
<td>14. Quality Assessment on External RNA Spike-in Controls</td>
<td>80</td>
</tr>
<tr>
<td>15. Gating Strategy for Multicolor Flow Cytometric Analysis of CSC Markers</td>
<td>90</td>
</tr>
<tr>
<td>17. Inflammatory Gene Arrays Reveal Possible Target Genes of LEDGF/p75</td>
<td>104</td>
</tr>
<tr>
<td>18. Preliminary Validation of Inflammatory Array Results</td>
<td>105</td>
</tr>
</tbody>
</table>
19. Representative Scratch-Wound Images with Graphs Showing Percent Wound Recovery ............................................................... 107

20. Knockdown Studies Reveal that CLU and LEDGF/p75 Expression are Independent................................................................. 109

21. SiRNA-mediated Knockdown of LEDGF/p75 and CLU is More Effective in Resensitizing PC3-DR PCa Cells Than Single Knockdown .................. 111

22. MTT Assay Reveals Double Knockdown of LEDGF/p75 and CLU does not Result in More Cell Death than CLU knockdown alone .................. 112

23. Flow Cytometry Reveals No Significant Increase in Cell Death in Double LEDGF/p75 and CLU Knockdown compared to CLU knockdown alone .......... 113

24. Model of LEDGF/p75 Structure Illustrating the IBD Domain .................. 115

25. Top SMIs that Have Cytotoxic Activity in DTX-resistant PCa Cells Alone or in Combination with DTX ......................................................... 117

26. DTX-resistant mCRPC Upregulate Markers of CSC-like Phenotype Compared to DTX-sensitive Cells as Measured by Flow Cytometry .............. 119

27. DU145 and DU145-DR Tumorsphere Cells Upregulate Markers of CSC-phenotype Compared to DTX-sensitive Cells ........................................ 120
### TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Putative prostate CSC Makers Identified in PCa Cell Lines, Animal Xenografts and Human Primary PCa Tissues</td>
<td>22</td>
</tr>
<tr>
<td>2. GSEA Top 25-ranked RNA-seq Upregulated Genes</td>
<td>48</td>
</tr>
<tr>
<td>3. GSEA Top 25-ranked RNA-seq Downregulated Genes</td>
<td>49</td>
</tr>
<tr>
<td>4. Primer Sequences for qPCR</td>
<td>83</td>
</tr>
<tr>
<td>5. Antibodies Used for Detection of EMT and CSC Markers by Flow Cytometry</td>
<td>88</td>
</tr>
<tr>
<td>6. Fluorescence-Minus-One (FMO) Staining Strategy for Detection of CSC and EMT Markers by Flow Cytometry</td>
<td>91</td>
</tr>
</tbody>
</table>
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D-PCA</td>
<td>Three Dimensional Principal Component Analysis</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding Cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding Cassette Sub-Family B Member 1</td>
</tr>
<tr>
<td>ABCC3</td>
<td>ATP binding Cassette Subfamily C member 3</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde Dehydrogenase</td>
</tr>
<tr>
<td>ARV</td>
<td>Androgen Receptor Splice Variant</td>
</tr>
<tr>
<td>ARV7</td>
<td>Androgen Receptor Splice Variant Number 7</td>
</tr>
<tr>
<td>BOP1</td>
<td>Block of proliferation 1</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine Ligand 2</td>
</tr>
<tr>
<td>CLU</td>
<td>Clusterin</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>CTX</td>
<td>Cabazitaxel</td>
</tr>
<tr>
<td>CYC1</td>
<td>Cytochrome c-1</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially Expressed Gene</td>
</tr>
<tr>
<td>DNAJC12</td>
<td>DNAJ Heat Shock Protein Family Member C12</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DTX</td>
<td>Docetaxel</td>
</tr>
<tr>
<td>DPP4</td>
<td>Dipeptidyl pptidase 4</td>
</tr>
</tbody>
</table>
DR  Docetaxel Resistant
ENPP1  Ectonucleotide Phosphodiesterase 1
FABP5  Fatty Acid Binding Protein 5
FMO  Fluorescence-Minus-One
GAPDH  Glyceraldehyde 3-Phosphate Dehydrogenase
GnRH  Gonadotrophin-Releasing Hormone
GR  Glucocorticoid Receptor
GSEA  Gene Set Enrichment Analysis
Hh  Hedgehog Pathway
HSP27  Heat Shock Protein 27
HSP90  Heat Shock Protein 90
HRP  Horseradish Peroxidase
IDT  Integrative DNA Technologies
IkB kinases  IKK
Interleukin 6  IL6
Interleukin 8  IL8
LEDGF/p75  Lends Epithelium Derived Growth Factor of 75 kDa
mCRPC  Metastatic Castration Resistant Prostate Cancer
MRP1  Multidrug Resistance Protein 1
NADHUFAB1  NADH Dehydrogenase 1 Alpha/Beta Subcomplex 1
MYC  V-Myc Myelocytomatosis viral Oncogene Homolog
nCLU  Nuclear Clusterin
NGS  Next Generation Sequencing
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NES</td>
<td>Nestin</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-Specific Antigen</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>sCLU</td>
<td>Secreted Clusterin</td>
</tr>
<tr>
<td>SMI</td>
<td>Small Molecule Inhibitor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transduction Activation Transcription</td>
</tr>
<tr>
<td>STR</td>
<td>Short Tandem Repeat</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor β1</td>
</tr>
<tr>
<td>TGM2</td>
<td>Transglutaminase 2</td>
</tr>
<tr>
<td>TSPAN8</td>
<td>Tetraspanin 8</td>
</tr>
<tr>
<td>RANK-L</td>
<td>Receptor Activator of Nuclear Factor κB ligand</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
</tbody>
</table>
ABSTRACT OF THE DISSERTATION

RNA-Seq Reveals Transcriptomic Program Associated with Stemness in Taxane Resistant Prostate Cancer

by

Christina K. Cajigas-Du Ross

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, August 2018
Dr. Carlos A. Casiano, Chairperson

There is no cure for advanced prostate cancer (PCa), and taxane chemotherapy is the only treatment option once other therapies have failed. However, this is problematic since all patients eventually develop chemoresistance. Emerging treatments for advanced PCa have shown promise at the benchside, but clinical trials have not resulted in newly approved drugs due in part to redundant survival pathways utilized by prostate tumor cells to maintain therapy-resistance. Using RNAsequencing—an innovative approach for quantifying gene expression changes—this dissertation sought to elucidate chemoresistance-associated molecular pathways as a catalyst to develop new therapeutic targets. Results revealed a differential upregulation of stemness-associated genes in PCa cells selected for chemoresistance. In addition, chemoresistant cells formed robust stem cell prostaspheres compared to chemosensitive cells, and expressed other markers of cancer stem cells. Overall, these findings support the hypothesis that PCa chemoresistance is driven by cancer stem cells.
CHAPTER ONE

INTRODUCTION

Prostate Cancer Background and Available Therapies

Prostate cancer (PCa) is currently the most commonly diagnosed cancer and the second leading cause of cancer deaths in American men with 164,690 estimated new cases and 29,430 deaths projected in 2018 [1]. PCa also exhibits a striking racial disparity, as African American men are 1.4 times greater risk of being diagnosed and 2.5 times more likely to die of this disease compared to Non-Hispanic white males [2, 3]. In addition, African American men are diagnosed with PCa at an earlier age and with a more advanced stage compared with Non-Hispanic white men, which may partially explain the significant mortality disparity seen in this population. Although PCa disproportionately and aggressively affects African American men [2, 3], this group is still underrepresented in potentially life-saving clinical trials. Furthermore, this underrepresentation could affect the development of newer cancer treatments since specific molecular targets might be more sensitive to slight differences in genetic variation seen across different ethnic or racial backgrounds [4]. The likelihood of being diagnosed with PCa increases with age; this is problematic considering the increasing aging male population. Therefore, the aging male population and the complexity of addressing the mortality disparity make developing new effective treatments for PCa crucial for tackling this public health dilemma and the subsequent fiscal burden that is expected over the next few decades [2-5].
**PCa Screening and Diagnosis**

PCa is diagnosed in patients through a combination of screening procedures. Digital rectal examination (DRE) is typically performed by a primary care physician. The DRE allows the physician to detect abnormal size or hard nodules of the prostate. The prostate-specific antigen (PSA) blood test is a screening tool used in combination with DRE. PSA is synthesized in the prostate and the concentration in the blood increases with PCa [6]. Circulating serum PSA is considered high or abnormal when detected above 4 ng/ml [7]. While elevated levels of PSA are not always indicative of PCa, it often requires follow-up monitoring or screening, including biopsy.

Once a tissue sample of the prostate is removed for biopsy, a pathologist is able to assign a Gleason score, which is used by the physician to determine aggressiveness and stage of the cancer. The higher the Gleason score, the more likely the PCa will spread rapidly and be aggressive [8]. Gleason scores range from a value of 1-5 with number 1 representing small uniform glands and number 3 representing moderately differentiated cells with infiltration of cells from glands at the margins. A score higher than 3 represents poorly differentiated and anaplastic cells with irregular masses of neoplastic cells and lack of glands [8]. PCa biopsies typically reveal phenotypic heterogeneity of histological subtypes found in the prostate adenocarcinoma cells. They include luminal secretory, neuroendocrine, basal, and luminal epithelial cells [9]. As a result of this heterogeneity, the pathologist may identify the two most common grades and add them to determine the combined Gleason score, which can range from 6-10. A PCa diagnosis typically occurs when the combined Gleason score is at least 6, with scores above 7 indicative of aggressive PCa.
The heterogeneity of this malignancy makes it an enormous challenge to develop therapeutic strategies that will be effective for all PCa patients. At early stages, treatment options including radical prostatectomy and radiation can be curative. But treatment options for advanced-stage diagnosis including androgen deprivation therapy (ADT) and taxane chemotherapy are not as effective since the disease has progressed and is phenotypically aggressive (Figure 1). Therefore, a diagnosis with advanced PCa is problematic for the patient given the lack of curative options.
Figure 1. Prostate Progression and Treatment Options. Stages and treatment options available for PCa. PCa is curable if treated while localized in the prostate. Unfortunately, once tumor cells migrate out of the organ, the disease is no longer curable and treatments are only meant to delay disease progression. ADT is the main treatment option at any state of PCa, especially following biochemical recurrence. Metastatic castration resistant PCa is treated with taxane chemotherapy and secondary ADT. Once a patient is terminal and no longer responding to chemotherapy, palliative care is the only treatment option available.
**Androgen Receptor and ADT**

Men initially diagnosed with PCa are given several treatment options including prostatectomy (prostate removal) or radiation to destroy cancerous cells still remaining in the prostate capsule after removal. Many patients, however, are not cured by these treatments and their cancer returns [10]. This is typically detected by an increased in PSA levels, known as biochemical recurrence. For men diagnosed with advanced PCa, treatment with curative intent is no longer an option, and ADT remains the main therapeutic modality [10, 11]; this is because prostate tumor growth is initially dependent on androgens. Testosterone, secreted primarily by the testes, is the main circulating androgen that is secreted in the blood. When testosterone enters the prostate cells, 90% is converted to a more active hormone dihydrotestosterone (DHT) by the enzyme 5-alpha-reductase. DHT has a 5-fold higher affinity for androgen receptor (AR) than testosterone [10, 11]. AR is a nuclear receptor comprised of an amino-terminal activating domain, a carboxy-terminal ligand-binding domain, and a DNA-binding domain. In its basal state, AR is bound to heat-shock proteins in a conformation that prevents DNA binding.

Androgen binding to AR induces a conformational change that causes dissociation from binding proteins and leads to the formation of AR homodimer complexes that can bind to androgen response elements in the promoter regions of target genes that contribute to cancer progression and aggressiveness [10].

ADT includes any treatment that suppresses androgen activity by decreasing androgen production through medical castration or by using anti-androgens to block AR signaling. The benefit of ADT in PCa treatment is the upregulation of pro-apoptotic genes that are normally repressed by AR activation [12], leading to a temporary reduction
in tumor aggressiveness. Common anti-androgens include cyproterone acetate, flutamide, nilutamide, and bicalutamide, all of which block androgens from binding to AR receptor (antagonists). Medical ADT is most commonly used in combination with long-acting gonadotrophin-releasing hormone (GnRH) agonists that are equally effective in reducing testosterone levels as removal of the testes with less detrimental effects. In addition, prostate tumors express GnRH receptors and GnRH analogs are shown preclinically to have antitumor activity [12].

**Castration-Resistant PCa**

Despite the important clinical benefits seen with ADT including improved survival, less morbidity, and better quality of life, it is not curative. This is because, after an average of 2-3 years of ADT, PCa cells reactivate AR signaling and continue to proliferate despite extremely low levels of circulating testosterone, a stage called castration-resistant prostate cancer (CRPC) [11, 12]. Cells bypass AR targeting through a variety of mechanisms including AR amplification and mutation, co-activator and co-repressor modifications, aberrant activation/post-translational modification, altered steroidogenesis, the presence of AR splice variants, and as discussed below, glucocorticoid receptor bypass [11].

AR amplification and increased AR sensitivity are both utilized by PCa cells to bypass androgen ablation therapy, and both mechanisms are dependent on androgens being present. By amplifying expression of the AR gene, cells have an increased number of AR resulting in increased AR expression and enhanced ligand-occupied receptor content despite low androgen concentrations [11, 12]. This is most likely achieved
through clonal selection of cells that proliferate despite low levels of circulating androgens as evidenced by the fact that tumors were found to have this AR amplification only after androgen ablation [10]. Increased AR sensitivity is another mechanism that results in AR signaling activation despite low androgens. This pathway results in high AR expression, increased stability and enhanced nuclear localization of AR in tumor cells [10]. Finally, PCa cells can also increase local production of androgens by elevating 5-alpha-reductatse activity thereby increasing the rate of conversion of testosterone to DHT [10].

PCa cells can also undergo AR point mutations causing an increase in AR activity in response to low circulating androgens. These mutations can also broaden the ligand pool to which AR responds, causing PCa cells to circumvent normal growth regulation by androgens. These mutations lead to aberrant activation of the androgen signaling axis by decreasing the specifity of ligand binding, and allow the ability for non-androgenic steroids and androgen antagonists to bind to AR and activate transcription of AR target genes [10]. Another pathway by which AR can be activated includes mutations to the many co-activators and co-repressors that function normally to regulate AR activation. Mutations to the co-regulator complexes can improve androgen-stimulated AR activation and also lead to disease progression [10, 11].

Metastatic CRPC (mCRPC) is a lethal stage of PCa that is marked by recurrence of elevated PSA and progression of metastatic lesions [10]. Since 2004, the standard first-line chemotherapeutic agent for mCRPC has been the taxane drug docetaxel (DTX), a microtubule-stabilizing agent that moderately increases overall survival [13, 14]. Eventually, however, chemoresistance occurs in all DTX-treated patients resulting in
continued disease progression ultimately leading to patient death [15]. Research focusing on the mechanisms of DTX-resistance have led to the development of new treatment options for mCRPC to target those pathways implicated. They include the next-generation AR-targeting agents abiraterone acetate and enzalutamide, therapeutic vaccines, and the second generation taxane cabazitaxel [15, 16]. Abiraterone acetate is an inhibitor of cytochrome P-450 17A1, the enzyme crucial for androgen biosynthesis. Unlike GnRH analogs, abiraterone acetate reduces androgen synthesis at the adrenal, prostate, and intratumoral sites. Enzalutamide is a more potent AR-signal inhibition compared to conventional anti-androgens because it inhibits AR nuclear translocation, DNA binding, and co-activator recruitment [10]. Both abiraterone acetate and enzalutamide are administered sequentially or in combination with DTX and unfortunately lead to a modest median of 4-5 month improvement in survival [10, 12]. In 2012, the FDA expanded approval for abiraterone acetate use in patients who have not yet received chemotherapy [12].

**Glucocorticoid Receptor “Take-Over” Pathway**

Despite the initial success reported with abiraterone and enzalutamide treatment, after an average of 6-12 months, response is limited due to acquired resistance. Recent work has highlighted another mechanism by which patients become resistant to anti-androgens, specifically enzalutamide [11, 17]. This pathway involves an increased expression of glucocorticoid receptor (GR) and a bypass of AR-target genes with evidence showing that GR can bind to over half of all AR binding sites [11]. GRs are nuclear receptors very similar in structure to AR. In addition, glucocorticoids initially
have a suppressive effect on PCa and are often given in combination with early CRPC treatments [11]. Because the DNA binding domain of the glucocorticoid receptor is similar to that of AR, its upregulation in patients treated with chemotherapy or ADT may contribute to enzalutamide resistance [11]. GR expression was found to be upregulated in these resistant tumors and implicated in substituting AR to activate a similar but distinguishable set of genes that was determined to be necessary for the maintenance of enzalutamide resistance [17]. These findings were critical in establishing a key mechanism detailing an escape from AR blockade through the selection of cells that are able to drive the expression of AR target genes via an alternative nuclear receptor [17].

**AR Splice Variants Contribute to CRPC**

There is a significant subset of patients receiving abiraterone acetate or enzalutamide who do not respond to either treatment; this is called innate or primary resistance. In addition, the vast majority of patients who do initially respond to these two drugs will eventually develop acquired or secondary resistance. Understanding the mechanisms involved in primary and secondary resistance is an area of recent focus, with the activity of AR splice variants (ARVs) being implicated as a putative resistance mechanism in CRPC [18]. ARVs are truncated versions of wild type AR that are considered constitutively active. The role of ARV expression in clinical CRPC is currently being established, with some groups showing that ARVs are associated with poorer prognosis, and others reporting higher levels of ARV expression in CRPC bone metastasis compared to hormone-sensitive PCa bone metastasis [11].
There are over 20 described ARVs, with the most abundant being the ARV7. ARV7 is clinically relevant, with detection in human clinical samples from patients with CRPC [18]. Secondly, ARV7 is also a constitutively active variant that is capable of stimulating transcription of AR genes in the absence of androgens and cannot be inhibited in vitro or in vivo by drugs that target the AR ligand-binding domain [18]. Thirdly, ARV7’s relevance is supported by the fact that its expression is increased 20-fold in CRPC tissues compared to hormone-sensitive tissues [18]. Therefore, there is evidence for the use of ARV7 as a prognostic marker to either determine poor outcomes in patients with CRPC or as a treatment-selection marker that can provide additional prognostic information[18]. Before the overall contribution of ARV7 can be determined, however, further work needs to be done including testing the clinical utility of ARV7 in the context of other therapies (i.e. taxanes), validating assay performance, and validating the clinical relevance of ARV7 status in predicting response/resistance to treatment in a context-specific manner [11, 18].

**Taxane Chemotherapy**

DTX chemotherapy is the current standard of care for patients diagnosed with CRPC, based on the SWOG 9916 and TAX327 clinical trials, which demonstrated a 3-month survival advantage of DTX over mitoxantrone [11, 14, 19]. DTX is an anti-mitotic chemotherapeutic agent that inhibits mitosis by stabilizing the microtubules and binding to the β subunit, preventing depolymerization and resulting in cell death. With the approval of abiraterone acetate and enzalutamide, DTX is often not the first line therapy of choice [11]. The CHAARTED trial compared DTX and ADT vs. ADT alone in
hormone-naïve Pca patients. This clinical trial demonstrated that DTX can be used as an initial treatment option for hormone-naïve patients since DTX in combination with ADT resulted in a 17-month survival advantage over ADT alone in patients with visceral metastases [11, 20]. The STAMPEDE trial showed similar results to CHAARTED with DTX in combination with ADT resulting in a 10-month survival benefit over ADT alone in men with high-risk locally advanced or metastatic PCa [11, 21]. Despite its effectiveness, all DTX-treated patients eventually develop resistance, and there are numerous mechanisms implicated in this acquired resistance [11, 15].

The taxane drug cabazitaxel (CTX) is a chemotherapeutic agent approved by the Food and Drug Administration (FDA) for use in patients with mCRPC who failed DTX chemotherapy. CTX was developed to have poor affinity for ABCB1 due to the role of this efflux pump in mediating DTX resistance [15, 22]. CTX was found to have a 2.4-month survival benefit compared to mitoxantrone in patients with mCRPC who progressed with DTX treatment [11, 23]. As with DTX, patients treated with CTX also develop resistance and face disease progression [11, 15, 24] Therefore, there is a need for novel combinatorial therapies aimed at killing the tumors and circumventing resistance.

**Mechanisms of Taxane Chemoresistance**

*Multidrug transporters*

The mechanisms contributing to DTX-resistance are well-studied but poorly understood as evidenced by the fact that therapies aimed at targeting these pathways have failed clinically [11]. DTX-resistance has been linked to the activity of multidrug transporters that act as efflux pumps to reduce the intracellular concentrations of
chemotherapeutic agents [24, 25]. Multidrug transporters are membrane proteins belonging to the ATP-binding cassette (ABC) family of transporters. The most notable are ABCB1, MDR protein 1 (MRP1) or ABCC1, and breast cancer resistance protein (BCRP) or ABCG2 [11], all of which are associated with drug resistance [24].

ABCB1 is weakly expressed in normal prostate but its expression increases as disease stage and tumor grade increase. It is also highly expressed in DTX-resistant PCa cells [24-26] and primary cancer cell cultures [24]. MRP1/ABCC1 and BCRP/ABCG2 are both associated with a multi-drug resistant phenotype. Unlike ABCB1, ABCC1 is more readily expressed in DTX-sensitive PCa cell lines, but like ABCB1 its expression also correlates with advanced PCa and high Gleason score [24, 27]. ABCG2 has been shown to have a possible role in PCa progression with expression correlating with worse patient survival. In addition, phosphorylation of ABCG2 by the serine/threonine kinase Pim-1 mediates DTX-resistance in PCa cell lines [28]. Interestingly, ABCG2 is a well established universal and PCa stem cell marker [29], and its expression has been implicated in the innate chemoresistance observed in stem cells [24]. Preclinical data determining the role of multidrug transporters in chemotherapy resistance led to the development of CTX, designed to have low affinity for ABCB1 efflux [15, 24].

**Tubulin Isoforms**

Another mechanism associated with DTX-resistance is the upregulation of class III β-tubulin isoforms which are common in DTX-resistant cancer cell lines and result in less stable microtubules [24]. Elevated βIII-tubulin is correlated with poor response to tubulin-targeting agents and with poor prognosis in many human malignancies. In
addition, targeting of this isoform restores DTX sensitivity [30]. DTX-resistant cells have elevated expression of βIII-tubulin, which is clinically associated with disease progression, tumor aggressiveness, and poor response to taxane therapy [30, 31]. Although this isoform has been well-characterized in DTX-resistant breast cancer, this mechanism of resistance has yet to be confirmed in PCa due to lack of metastatic biopsy samples of taxane-resistant tumors to specifically examine tubulin alterations. Further studies are required to determine the impact of these modifications in clinical decision-making [31].

**Survival Pathways and Inflammatory Cytokines**

**STAT-1 and STAT-3**

The binding of DTX to β-tubulin induces a stress response that activates multiple survival pathways inducing c-JNK, STAT-1 and STAT-3, and NF-κB. STAT (signal transducer and activator of transcription) proteins are transcription factors that regulate gene expression and influence cell growth, differentiation, proliferation, and apoptosis [32]. When PC3 and DU145 mCRPC cells become resistant to DTX, STAT1-dependent clusterin (CLU) expression is upregulated [33]. CLU is an anti-apoptotic protein that is associated with PCa progression and chemoresistance [34]. STAT-3 expression is also implicated in DTX-resistance through the serine-threonine kinase PIM1, contributing to cell survival [35].

**NF-κB**

Inflammation has long been established as a major driver in PCa carcinogenesis
and progression [36], and its role in DTX-resistance has been well-studied with several groups demonstrating inflammatory cytokine upregulation in mCRPC patients treated with DTX. NF-κB is found in the cytoplasm and is inhibited by IκB-α. When IκB kinases are stimulated, IκB-α is degraded and NF-κB is able to translocate to the nucleus and activate transcription of a wide array of genes that code for angiogenic factors, cell adhesion molecules, anti-apoptotic factors, and cytokines [32, 37]. These genes are thereby able to contribute to cell survival, invasion, metastasis, and chemoresistance [32].

**IL-6 and IL-8 Signaling**

Among the many targets of NF-κB are the genes that encode for Interleukin 6 (IL-6) and Interleukin 8 (IL-8), two cytokines that stimulate PCa cell growth in an autocrine and paracrine manner and are involved in the development and progression of the disease [38-41]. Androgen-independent PC3 and DU145 PCa cells have elevated IL-6 and IL-8 production in conditioned media due to NF-κB activity. This same observation does not occur in androgen-sensitive LNCaP PCa cells [38, 40]. PC3 and DU145 cells have higher NF-κB activity and secreted more IL-6, making them more resistant to DTX compared to LNCaP cells [42]. Pharmacological inhibition of NF-κB reduced IL-6 levels and increased the cytotoxic effects of DTX in PC3 and DU145 cells but not LNCaP cells [42]. This finding is supported clinically by a significant association in mCRPC PCa patients between high serum and tumor expression levels of IL-6 and decreased response to DTX treatment [37, 42]. High IL-6 and IL-8 production enhances proliferation and inhibits apoptosis in PC3 and DU145 cells [38, 40]. Through the JAK/STAT pathways, IL-6 and IL-8 are effectors of NF-κB activity in DTX-resistance [38, 40].
CCL2

Chemokines, which function to induce chemotaxis in neighboring cells, have also been implicated in DTX-resistance. Most notable is the chemokine ligand 2 (CCL2), which is expressed in PCa cell lines and primary tumors and is correlated with malignant potential [43, 44]. CCL2 expression in mCRPC cell lines is increased by DTX treatment through the activation of JNK signaling resulting from DTX binding to microtubules [45]. In addition, CCL2 can act in an autocrine manner to promote cell survival and resistance to DTX through stimulation of Erk/MAP kinase and PI3K/AKT signaling pathways [46, 47].

Chaperone Proteins

Heat Shock Proteins

Chaperone proteins have long been implicated in DTX-resistance, with heat shock proteins and CLU being the most notable and extensively studied [32]. Heat Shock Protein 27 (HSP27) and HSP90 are overexpressed in mCRPC cells and their expression increases as cells become DTX-resistant [48, 49]. In addition, targeting of these proteins in cellular models resulted in DTX resensitization [49, 50]. Unfortunately, however, clinical trials targeting HSP90 have yet to improve overall survival [32]. Clinical studies with HSP27 using the second generation antisense drug OGX-427 resulted in a decrease in circulating tumor cells in mCRPC patients [49]. Results from Phase I clinical trials in mCRPC patients using OGX-42 in combination with DTX have yet to be reported.
Clusterin

CLU, a chaperone protein, is upregulated in DTX-resistant PCa cells and is considered a key protein in mediating DTX-resistance in PCa [34]. CLU exists in two isoforms resulting from two distinct transcriptional start sites. They include a truncated nuclear form (nCLU) that promotes CRPC cell death [51], and a secreted form (sCLU) that prevents cell death [52]. nCLU and sCLU are not produced simultaneously, and a shift from nCLU to sCLU production occurs during PCa progression [53]. Therefore, nCLU expression is not detected in prostate tumor cells and sCLU is overexpressed in DTX-resistant cells, with elevated expression levels correlating with DTX resistance [54]. sCLU in vitro knockdown with antisense oligonucleotide increases the sensitivity of resistant PCa cells [55].

As previously mentioned, sCLU expression is induced by DTX through STAT-1 activation in PC3 and DU145 cells. AKT inhibition suppressed CLU expression in DTX-resistant CRPC cells and resensitized them to DTX, showing that CLU expression is dependent on AKT activation of STAT-1 [56]. sCLU confers its anti-apoptotic effects in the cytoplasm of the cell by binding and stabilizing the Ku70-Bax complex preventing the release of Bax to the mitochondria to initiate cytochrome c release and thereby preventing caspase-9 and -3 dependent apoptosis [34, 57].

CLU is also regulated by transforming growth factor β1 (TGF-β1) [58]. TGF-β1 is in turn upregulated by the transcription factors YB-1 and Twist 1 [58]. Through this upregulation, CLU contributes to epithelial-mesenchymal transition (EMT) and metastasis of PCa cells [58]. CLU reduction reduced TGF-β1 induction of N-cadherin and fibronectin, both markers of EMT, thereby inhibiting the migratory and invasive
properties of TGF-β1 [58]. Targeting of CLU in an in vivo model also suppressed metastasis. These findings indicate that CLU is an important mediator of TGF-β1-induced EMT, and CLU may be a promising target for reducing cancer metastasis [58].

**AR Splice Variants in the Context of Taxane Chemoresistance**

PCa is fundamentally AR-driven especially in the context of disease etiology and progression. Because the intraprostatic response of PCa cells to androgens depends on the expression and sensitivity of ARs, ADT has been a mainstay of PCa treatment and typically precedes taxane chemotherapy. As mentioned above, constitutively active ARVs have been shown to be overexpressed in mCRPC and confer resistance to ADT by inhibiting the nuclear translocation of the androgen-AR complex [59-62]. More recently, there has been a shift in focus to the role of AR in DTX-resistance in mCRPC patients with AR mutations. An initial study suggested that the presence of AR splice variants may affect sensitivity to taxane treatment and that tumors predominantly expressing ARV7 would likely be resistant to DTX [63].

Since this initial report, a separate group has coordinated a replication study and their results refute the initial findings regarding ARV7 and DTX-resistance [64]. In addition, a separate group found that detection of ARV7 in circulating tumor cells of mCRPC patients was not associated with taxane-resistance, negating the clinical significance of ARV7 in patients receiving DTX [18]. Considering the inconsistency reported in the literature regarding AR-DTX interactions, studies exploring DTX-resistance using cellular models must take into account the AR status of cell lines used to determine the most clinically relevant scenario for the question being addressed.
**LEDGF/p75 Promotes DTX-Resistance**

The Lens Epithelial Derived Growth Factor protein of 75 kDa (LEDGF/p75) is activated during cellular response to stress. It is a transcription co-activator with oncogenic function [65, 66] that specifically promotes cellular survival against environmental stressors such as oxidative stress, radiation, heat, serum starvation, and cytotoxic drugs [26, 65-70]. LEDGF/p75 has been shown to have a role in PCa and other cancers, contributing to resistance to various cytotoxic drugs, including DTX [26, 68, 70, 71]. DTX-resistant PCa cell lines upregulate transcript and protein expression of LEDGF/75 compared to DTX-sensitive cells [26, 48, 71]. Our group has also demonstrated that siRNA-mediated knockdown of LEDGF/p75 is able to partially resensitize DTX-resistant cells to DTX therapy [26].

**Epithelial to Mesenchymal Transition**

EMT occurs when there is a breakdown of cell-to-cell-to-extracellular matrix adherence to the epithelial lining [72], a process that facilitates cancer metastasis. E-cadherin, a major component of epithelial adherence junction, functions to control EMT [72, 73]. Loss of E-cadherin is the hallmark step that signals the start of EMT [72]. Crucial mesenchymal markers include vimentin, N-cadherin, and E-cadherin transcriptional repressors including SNAI1 (Snail), SNAI2 (slug), TWIST1(Twist), ZEB1, and ZEB2; all of which contribute to enhanced cell mobility [72, 73].

In our studies, Snail, Twist, vimentin, and N-cadherin were found elevated in the DTX-resistant PC3-DR and DU145-DR cells. Snail is a zinc-finger protein that binds to the E-cadherin promoter [72]. Snail is upregulated during enzalutamide resistance and is
highly expressed in metastatic PCa [72]. Snail’s role in facilitating metastasis involves the downregulation of tight junction proteins including zona occludin 1 [72]. Twist is a protein that plays critical roles during tumorigenesis also through the regulation of E-cadherin expression [73]. Twist’s expression is significantly correlated with Gleason score and metastasis. Twist also functions to regulate N-cadherin expression by inducing transcriptional activation [73, 74]. Slug, another transcription factor, is regulated by AR signaling and contributes to the development of CRPC during ADT [73]. This evidence suggest that EMT contributes to drug resistance in PCa.

The link between EMT and cancer stem cells (CSCs) in contributing to drug resistance in CRPC has recently been explored in various cancer types [73-76]. What is emerging from these studies is the hypothesis that characteristics of EMT are closely associated with signatures of CSCs, leading to the drug resistant phenotype. Evidence for this has been observed in breast and pancreatic cancer, where TGFβ, a potent EMT inducer, stimulated a marked increase of cells with a CSC phenotype and marker expression [73]. In addition, a study in breast cancer demonstrated that upregulation of Zeb1, an EMT regulator, was sufficient to switch cells from a non-cancer stem cell phenotype to a CSC status [76]. This link between EMT markers and CSC-like phenotype in DTX-resistance in CRPC is explored in this dissertation.

**Cancer Stem Cells**

PCa resistance to chemotherapy, the primary cause of treatment failure, is driven by the survival of subpopulations of prostate tumor cells that eventually contribute to aggressive disease progression, characterized by metastasis to the bone and vital organs
[77]. In addition, PCa tumors are highly heterogeneous, with many areas containing genetically distinct clones [78], a characteristic that also contributes to therapy resistance and tumor relapse [77]. An emerging explanation for both the cellular heterogeneity and treatment resistance in PCa and other solid tumors is the CSC hypothesis, which states that solid tumors are organized hierarchically with only a small subset of cells capable of tumor-initiating and tumor-propagating capacity [77, 79].

CSCs are defined by their capacity for self-renewal, potential to differentiate into any cell-type within a tumor, and proliferative capacity to drive the establishment of a tumor. The ability to expand or repopulate a bulk tumor is the result of aggressive metastatic activity, and increased resistance to chemotherapy and radiotherapy [79]. The CSC theory establishes that stem-like cells maintain the tumor population. Recent studies suggest that cancer cells can de-differentiate into CSCs under certain tumor microenvironmental conditions [79, 80]. This phenomenon is referred to as plasticity [79, 80].

Chemotherapeutic drugs, like DTX, have cytotoxic effects on the bulk tumor, but due to the presence of CSCs, these effects are temporary since this small cell population employs multiple redundant mechanisms that facilitate cell survival. In addition, the non-proliferative state of CSCs allows them to exist as cellular reserves facilitating an environment where a small population of cells can persist after anti-proliferative treatments repopulate the tumor and/or metastasize [77]. Recently the emerging role of CSCs in the acquisition of PCa chemoresistance has been explored and partially characterized [75, 77, 81, 82]. CSCs employ many of the same resistance mechanisms previously mentioned but at an enhanced level, including increased activity of drug-
efflux pumps, heightened DNA repair efficiency, and increased detoxification enzyme expression; but unlike other cells, they employ quiescence [83]. Quiescence is of particular concern when considering that the mechanism-of-action of many cytotoxic drugs depends on the propensity of cancerous cells to be metabolically active and rapidly dividing.

CSCs are typically identified based on the presence and/or absence of several cell surface markers, the combination of which is specific for the CSC phenotype identified in a particular tumor type. Markers for PCa stem cells include cell surface proteins such as CD44, CD133, CD117, ABC transporters (ABCB1, ABCG2), cytoplasmic proteins such as NES, enhanced aldehyde dehydrogenase (ALDH) activity, and nuclear proteins such as Sox-2, Oct 3/4, and Nanog [84]. Table 1 highlights the validated CSC markers utilized in PCa studies.
Table 1. Putative prostate CSC Makers Identified in PCa Cell Lines, Animal Xenografts and Human Primary PCa Tissues.

<table>
<thead>
<tr>
<th>CSC Marker</th>
<th>Cell Line/Model/Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44/02β1&lt;sup&gt;pos&lt;/sup&gt;/CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Primary tumors</td>
</tr>
<tr>
<td>CD44&lt;sup&gt;+&lt;/sup&gt;</td>
<td>LAPC-4 and LAPC-9 models</td>
</tr>
<tr>
<td>CD44&lt;sup&gt;/02β1&lt;sup&gt;high&lt;/sup&gt;</td>
<td>LAPC-9 model</td>
</tr>
<tr>
<td>CD133&lt;sup&gt;high&lt;/sup&gt;/CD44&lt;sup&gt;high&lt;/sup&gt;</td>
<td>PC-3-MM2 cell line</td>
</tr>
<tr>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;/CD44&lt;sup&gt;+&lt;/sup&gt;</td>
<td>PC-3 and DU145 cell lines</td>
</tr>
<tr>
<td>CD44&lt;sup&gt;+&lt;/sup&gt;/CD24&lt;sup&gt;+&lt;/sup&gt;</td>
<td>LNCaP and DU145 cell lines</td>
</tr>
<tr>
<td>CD44&lt;sup&gt;/ABC&lt;/sup&gt;G2 CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>PC-3, VCAF, LNCaP, 22RV1, and DU145, C4-2B cell lines</td>
</tr>
<tr>
<td>PSA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>LNCaP, LAPC-4 and LAPC-9 cell lines; primary CaP tumors</td>
</tr>
<tr>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Primary tumors</td>
</tr>
<tr>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>LAPC-4, LNCaP and CWR22RV1 cell lines</td>
</tr>
<tr>
<td>ALDH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>PC-3-M-Pro4 and C4-2B cell lines; primary tumors</td>
</tr>
<tr>
<td>ALDH1A1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>PC-3 and LNCaP cell lines</td>
</tr>
<tr>
<td>TRA-1-60&lt;sup&gt;+&lt;/sup&gt;/CD151&lt;sup&gt;+&lt;/sup&gt;/CD166&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Primary tumors</td>
</tr>
<tr>
<td>E-cadherin&lt;sup&gt;+&lt;/sup&gt;</td>
<td>DU145 and PC-3 cell lines</td>
</tr>
<tr>
<td>CD117/ABC1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22RV1 cell line</td>
</tr>
<tr>
<td>OCT4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Primary tumor cells</td>
</tr>
<tr>
<td>ABCG2&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Tumorsphere cells derived from LNCaP, 22RV1, DU145 and PC-3 CaP cell lines</td>
</tr>
</tbody>
</table>

From Ni J et al., 2014 [79]
Therapies Targeting CSC pathways

Current targeting of CSCs focuses on the signaling pathways upregulated in stem cells that are specific to their function. These pathways include Hedgehog, Wnt, Notch, and NFkB pathways. The Hedgehog (Hh) pathway plays an important role in regulating CSCs in PCa by regulating target genes involved in proliferation, survival, and metastasis [85]. This pathway also promotes chemoresistance by increasing transcription of ABCB1 and ABCG2 in PCa [85]. Hh inhibitors have shown promise in vitro with numerous clinical trials currently testing inhibitors for PCa treatment [77].

Wnt signaling contributes to CSC development, making it a promising target. As with the Hh pathway, many therapeutic agents targeting Wnt are under clinical study including monoclonal antibodies against the Wnt cascade and a small molecule inhibitor that has been shown to inhibit PCa tumor growth in vitro [77].

Notch signaling is overactivated in PCa and its silencing is predicted to inhibit tumor growth and differentiation [86, 87]. Many pharmacological agents targeting Notch signaling are undergoing clinical study including siRNAs, and monoclonal antibodies against Notch receptors and/or ligands. GSI, a gamma-secretase inhibitor, is being combined with bicalutamide in patients whose PCa recurs after surgery or radiation [87].

Many challenges exist with targeting CSCs, many of which involve the lack of understanding of the underlying pathways and the genetic alterations that maintain or create CSC-niches in the tumors. For example, targeting the biomarkers that identify prostate CSCs and signaling pathways that sustain this population are potential targets for novel drug development alone or in combination [79]. However, there remains a need to better understand where CSCs originate and how they develop and are sustained. As with
other cancers, prostate CSCs are phenotypically and functionally diverse [79], therefore, understanding the relationship between distinct CSC populations is important to develop strategies to target them.

**Therapies to Circumvent DTX-Resistance**

The main goal of novel therapies for mCRPC are to either bypass DTX-resistance using an alternative/redundant anti-tumoral pathway or to directly inhibit the pathways directly causing resistance. Anti-tumoral approaches worth mentioning include Sipuleucel-T, abiraterone acetate, enzalutamide, and alpharadin. Sipuleucel-T is an immunotherapy approved in 2010 by the FDA. This anti-tumor vaccine resulted in a 4.1 month improvement in median overall survival compared to placebo group [88]. Lastly, the radiopharmaceutical alpharadin radium-223 chloride was found to be effective in patients with CRPC and mCRPC with no differences found with previous DTX usage [89]. Despite the increased survival reported for each new treatment, effectiveness is limited.

The number of therapies available to overcome development of DTX-resistance are far less numerous [32]. Only two are FDA approved for use in CRPC, including CTX and denosumab. As previously mentioned, CTX is a DTX-related taxane that was developed to limit drug efflux and thereby make it more effective [11]. Denosumab is an antibody targeted against the receptor activator of nuclear factor κB ligand (RANK-L). This treatment was developed to inhibit the NFκB survival pathway, which is activated by taxane therapy [32].
Many promising treatments developed to circumvent DTX-resistance pathways have failed to show success in the clinical setting. Of importance is OGX-011 or Custirsen, a next generation oligonucleotide targeting sCLU. Despite initial success in preclinical models and Phase I and II studies, Custirsen in combination with DTX or CTX failed to show any benefit over chemotherapy alone in Phase III studies [90, 91], with the SYNERGY trial reporting 5% adverse effects in the DTX, prednisone, and Custirsen group leading to patient death [91]. Another promising treatment was the IL-6 monoclononal antibody siltuximab (CNTO 328). This Phase II study involved siltuximab treatment in combination with mitoxantrone compared to mitoxantrone alone. Unfortunately, the study did not meet its primary endpoint and resulted in no apparent improvement in survival outcomes [92].

**RNA Sequencing as an Approach to Transcriptome Profiling**

Next generation sequencing (NGS) has revolutionized transcriptomics by making it feasible to analyze expressed genes from any tissue or species without needing to identify known transcripts. This is significant because previous global RNA analysis was restricted only to known splice variants or nucleotide sequences. RNA sequencing (RNA-seq) is a more sensitive technique compared to microarrays, with a longer range and the ability to determine allele-specific expression making it the current preferred method of large-scale RNA studies. Unfortunately, the data produced from these studies is dense and complex, requiring significant time commitments to mine the data sets and extrapolate the results [93].
Hypothesis and Purpose of Dissertation Work

Understanding the molecular mechanisms underlying the acquisition of PCa chemoresistance is critical for developing novel combinatorial therapies for the prevention or reversal of taxane-resistance, which will improve patient survival and ultimately result in a curative option for mCRPC. Several mechanisms involved in the acquisition of DTX-resistance have been identified, including the role of multidrug resistance pumps (e.g. ABCB1), impaired apoptotic pathways (e.g. Bcl-2), cytokine and chemokine induction (e.g. IL-6, CCL2), alterations in microtubule structure and function, NF-kB pathway activation, and upregulation of stress proteins (e.g. Hsp27, clusterin, and LEDGF/p75) [11, 26, 31, 32].

Unfortunately, efforts aimed at targeting or disrupting some of these pathways in the clinical setting have been largely unsuccessful [13]. This is illustrated by the recent failure of phase III clinical trials with Custirsen [90, 91]. Such failures highlight the importance of continued efforts towards discovering new mechanisms and molecular pathways associated with the taxane-resistant phenotype. Given the scarcity of genomic studies examining the transcriptomic programs activated during development of DTX-resistance in mCRPC, we performed RNA-seq on DTX-sensitive and DTX-resistant mCRPC cells in an effort to identify new gene pathways potentially involved in taxane resistance. Due to the inconsistency in the literature regarding AR-DTX interactions [63, 64], we chose not to focus on the role of AR in DTX and avoided the use of AR-positive cell lines. As such, PC3 and DU145 are AR-negative CRPC cell lines which are often used for DTX-resistance studies, and were deliberately chosen as cellular models for the purposes of this study.
We hypothesized that the RNA sequencing will reveal differentially expressed upregulated and downregulated genes when cells transition from DTX-sensitivity to DTX-resistance. The goal of the present study is to identify new therapeutic targets that are critical for the acquisition of DTX-resistance. This will lead to the development of new therapies to halt the progression of advanced PCa, resulting in a cure for this malignancy.
References


71. Mediavilla-Varela, M., et al., Docetaxel-induced prostate cancer cell death involves concomitant activation of caspase and lysosomal pathways and is attenuated by LEDGF/p75. Mol Cancer, 2009. 8: p. 68.


CHAPTER TWO

RNA SEQUENCING REVEALS UPREGULATION OF A TRANSCRIPTOMIC PROGRAM ASSOCIATED WITH STEMNESS IN METASTATIC PROSTATE CANCER CELLS SELECTED FOR TAXANE RESISTANCE

Christina K. Cajigas-Du Ross¹,³, Shannalee R. Martinez¹,³, Leanne Woods-Burnham¹,³, Alfonso M. Durán¹,³, Sourav Roy², Anamika Basu¹, Joshua A. Ramirez¹, Greisha L. Ortiz-Hernández¹,³, Leslimar Ríos-Colón¹,³, Evgeny Chirshev³, Evelyn S. Sanchez-Hernández¹,³, Ubaldo Soto³, Celine Greco⁴,⁵, Claude Boucheix⁴,⁵, Xin Chen⁶, Juli Unturnaehrer³, Charles Wang³,⁶, Carlos A. Casiano¹,³,⁷

The work presented in this chapter has been published.

(Oncotarget. 2018 9(54): 30363-30384 PMID:30100995)

¹Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA; ²Department of Entomology and Institute for Integrative Genome Biology, University of California Riverside, Riverside, CA 92521, USA; ³Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA; ⁴Inserm, UMR-s935, Villejuif, France; ⁵Univ. Paris-Sud11, Université Paris-Saclay, France; ⁶Center for Genomics, Department of Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA; and the ⁷Department of Medicine, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

Address correspondence to Carlos A. Casiano, PhD, Center for Health Disparities and Molecular Medicine, Mortensen Hall 142, 11085 Campus St., Loma Linda University School of Medicine, Loma Linda, CA 92350, USA; Tel: 1-909-558-1000 ex. 42759; Fax 1-909-558-0196; Email: ccasiano@llu.edu

Keywords: prostate cancer, RNA sequencing, targeting docetaxel resistance, cancer stem cells
Abstract

Patients with metastatic castration-resistant prostate cancer (mCRPC) develop resistance to conventional therapies including docetaxel (DTX). Identifying molecular pathways underlying DTX resistance is critical for developing novel combinatorial therapies to prevent or reverse this resistance. To identify transcriptomic signatures associated with acquisition of chemoresistance we profiled gene expression in DTX-sensitive and -resistant mCRPC cells using RNA sequencing (RNA-seq). PC3 and DU145 cells were selected for DTX resistance and this phenotype was validated by immunoblotting using DTX resistance markers (e.g. clusterin, ABCB1/P-gp, and LEDGF/p75). Overlapping genes differentially regulated in the DTX-sensitive and -resistant cells were ranked by Gene Set Enrichment Analysis (GSEA) and validated to correlate transcript with protein expression. GSEA revealed that genes associated with cancer stem cells (CSC) (e.g., NES, TSPAN8, DPPP, DNAJC12, and MYC) were highly ranked and comprised 70% of the top 25 genes differentially upregulated in the DTX-resistant cells. Established markers of epithelial-to-mesenchymal transition (EMT) and CSCs were used to evaluate the stemness of adherent DTX-resistant cells (2D cultures) and tumorspheres (3D cultures). Increased formation and frequency of cells expressing CSC markers were detected in DTX-resistant cells. DU145-DR cells showed a 2-fold increase in tumorsphere formation and increased DTX resistance compared to DU145-DR 2D cultures. These results demonstrate the induction of a transcriptomic program associated with stemness in mCRPC cells selected for DTX resistance, and strengthen the emerging body of evidence implicating CSCs in this process. In addition, they provide
additional candidate genes and molecular pathways for potential therapeutic targeting to overcome DTX resistance.

**Introduction**

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second cause of cancer deaths among American men [1]. For men diagnosed with advanced PCa, treatment with curative intent is no longer an option, and androgen deprivation therapy (ADT) remains the main therapeutic modality [2, 3]. Despite its initial effectiveness at reducing tumor growth, ADT ultimately fails, resulting in metastatic castration-resistant prostate cancer (mCRPC), a lethal stage of the disease that is marked by recurrence of elevated prostate specific antigen (PSA) and progression of metastatic lesions [3, 4]. Since 2004, the standard first-line chemotherapeutic agent for the treatment of mCRPC has been the taxane drug docetaxel (DTX), a microtubule-stabilizing agent that moderately increases overall survival [4, 5]. Eventually, however, chemoresistance occurs in all DTX-treated patients resulting in continued disease progression [6]. In recent years, new treatment options for mCRPC have been developed, including the next-generation androgen receptor-targeting agents abiraterone acetate and enzalutamide, therapeutic vaccines, and the second generation taxane cabazitaxel [6, 7]. Unfortunately, these novel therapeutic agents, which are often administered sequentially or in combination with DTX, only moderately improve overall patient survival due to the development of therapy resistance.

Understanding the molecular mechanisms underlying the acquisition of PCa chemoresistance is critical for developing novel and effective combinatorial therapies for
the prevention or reversal of taxane resistance. Several mechanisms involved in the development of DTX-resistance have been identified, including the increased expression and activity of multidrug resistance pumps (e.g. ABCB1/P-gp/MDR1), impaired apoptotic pathways (e.g. Bcl-2), cytokine and chemokine induction (e.g. IL-6, CCL2), alterations in microtubule structure and function, NF-kB pathway activation, and upregulation of stress survival proteins (e.g. Hsp27, clusterin, and LEDGF/p75) [2, 8, 9]. Unfortunately, efforts aimed at targeting or disrupting some of these pathways in the clinical setting have been largely unsuccessful [4]. This is illustrated by the recent failure of phase III clinical trials with Custirsen, a second generation oligonucleotide administered in combination with DTX designed to disrupt the production of clusterin (CLU), a cytoprotective anti-apoptotic chaperone protein overexpressed in PCa [10, 11]. Such failures highlight the importance of continued efforts towards discovering new mechanisms and molecular pathways associated with the taxane-resistant phenotype.

Chemoresistance, the primary cause of treatment failure, is driven by the survival of subpopulations of prostate tumor cells that eventually contribute to aggressive disease progression, characterized by metastasis to the bone and vital organs [12]. In addition, PCa tumors are highly heterogeneous, with many areas containing genetically distinct clones [13], a characteristic that also contributes to therapy resistance and tumor relapse [12]. An emerging explanation for both the development of resistance and the cellular heterogeneity in PCa and other solid tumors is the cancer stem cell (CSC) hypothesis, which proposes that solid tumors are organized hierarchically with only a minor subset of cells capable of tumor-initiating and tumor-propagating capacity [12, 14].
Recent studies revealed that markers associated with epithelial-to-mesenchymal (EMT) transition and CSCs are elevated in DTX-resistant mCRPC cells [15, 16], and that CSCs derived from immortalized normal prostate epithelial cells showed increased DTX resistance compared to parental adherent cells [17]. Given the scarcity of next generation sequencing (NGS) studies examining the transcriptomic programs activated during development of DTX-resistance in mCRPC, we performed an RNA sequencing (RNA-seq) analysis on DTX-sensitive and DTX-resistant mCRPC cells in an effort to identify gene pathways potentially involved in taxane resistance. GSEA analysis of the overlapping upregulated genes in DTX-resistant PC3-DR and DU145-DR cells revealed an induction of a transcriptomic program associated with stemness as cells transitioned from DTX sensitivity to resistance. To validate this finding, we characterized the CSC phenotype in tumorspheres from DTX-resistant PC3-DR and DU145-DR cells using CSC markers previously validated in prostate tumorspheres. Understanding the role of CSCs in PCa chemoresistance, including the transcriptomic pathways that define their activation and maintenance is critical to identifying new targets for combinatorial therapies aimed at circumventing taxane resistance in mCRPC.

**Results**

**PC3-DR and DU145-DR Cells Upregulate Markers of Taxane Resistance**

We developed PC3-DR and DU145-DR cell lines by selecting and expanding the surviving cells in the presence of incrementally increasing concentrations of DTX until cells could be maintained in 10 nM DTX with minimal cell death [8, 18]. Our group reported recently that these DTX-resistant cell lines are also resistant to paclitaxel and
cabazitaxel, other clinically relevant taxanes [8]. We also demonstrated that these DTX-resistant cells overexpress the stress oncprotein Lens Epithelium Derived Growth Factor of 75 kD (LEDGF/p75), and that depletion of this protein partially resensitized these cells to DTX [8, 18]. In the present study, we confirmed that the DTX-resistant PC3-DR and DU145-DR cell lines used in the RNA-seq analysis and other experiments displayed significant upregulation of proteins previously implicated by our group and others in PCa progression and DTX resistance [8, 9, 19-23] including LEDGF/p75, CLU, and ATP-binding cassette sub-family B member 1 (ABCB1), compared to the sensitive cells (Figure 2A-2C). This validation step was critical prior to initiating our RNA-seq analysis comparing the transcriptome profiles of DTX-sensitive and DTX-resistant PCa cell lines.
Figure 2. DTX-resistant PC3 and DU145 Cell Lines Upregulate Known Markers of DTX Resistance. Upper panel: Western blots of (A) LEDGF/p75, (B) CLU, and (C) ABCB1 showing upregulation of these proteins in DTX-resistant PC3 and DU145 mCRPC cells, compared to the parental, sensitive cells. Bottom panel: quantification of fold change in protein expression (A n=8, B n=5, C n=4 independent experiments). *P<0.05; **P < 0.05; ***P < 0.001. Due to the absence of ABCB1 expression in the parental PC3 and DU145 cells, for quantification purposes we normalized its expression in these cells to an arbitrary value of 0.10. Error bars represent mean standard deviation (SD).
**RNA-seq Analysis Revealed Upregulation of Genes Associated with CSC-like Characteristics**

Principal Component Analysis (PCA) 3D mapping of our RNA-seq data demonstrated that the DTX-sensitive PC3 and DU145 cells were clearly separated from each other based on global transcriptome expression profiles (Figure 3A). However, once these cell lines became DTX-resistant they were clustered together spatially, suggesting an acquired similarity in transcriptomic profiles. Global gene heat map also demonstrated the clustering of the DTX-resistant cell lines based on their transcriptome expression profiles (Figure 4). Our RNA-seq data revealed that of 31,864 total genes detected, 3,754 and 2,552 were differentially upregulated with statistical significance (FDR > 0.05, and fold change [FC] > 2) in the DU145-DR and PC3-DR cells, respectively, compared to their DTX-sensitive counterparts (Figure 3B, 3C). Of these genes, 1,254 overlapped between the PC3-DR and DU145-DR cells. GSEA of the top 25 ranked overlap genes between the DTX-sensitive and DTX-resistant PC3 and DU145 cells revealed a distinct on/off switch of genes, suggesting a pattern of upregulated/downregulated genes associated with the development of DTX-resistance in both cell lines (Figure 3D) (see Figure 5 for top 50 ranked genes). An exhaustive PubMed literature search also revealed that 17 of the top 25 (70%) ranked overlapping genes upregulated in the DTX-resistant cell lines have been shown to be associated with or contribute to a CSC phenotype (Table 2). Top downregulated genes are listed in Table 3.
Figure 3. Gene Expression Profiling Analysis Reveals Upregulation of CSC-associated Genes. (A) Principal component Analysis (PCA) mapping demonstrates clustering of DTX-resistant cell lines based on gene expression profiles. (B) Diagram showing the distribution of statistically significant differentially regulated genes in each cell line, comparing DTX-resistant (DR) to sensitive (S). (C) Diagram demonstrating the overlap or shared genes common to both PC3 and DU145 cells, comparing DR to S. (D) Heatmap of the top ranked genes generated using GSEA analysis on the common overlap genes between both sensitive PC3 and DU145 cells compared to PC3-DR and DU145-DR. Red represents fold upregulation and blue represents fold downregulation. (E) GSEA gene set pathway analysis revealed one pathway to be significantly enriched in the DTX-resistant PC3-DR and DU145-DR cells compared to sensitive PC3 and DU145 cells ($P=0.032$) involving precursor metabolites and energy. A positive value indicates correlation with the sensitive phenotype and negative value indicates correlation with the resistant phenotype.
Figure 4: Hierarchical Clustering Heat Map of Global Gene Analysis for all Cell Lines (PC3, DU145, PC3-DR, and DU145-DR).
Figure 5. GSEA Generated Heat Map of the Top-ranked 50 Overlap Genes. Significantly downregulated (blue) or upregulated (red) between DTX-sensitive PC3 and DU145 compared to DTX-resistant PC3-DR and DU145-DR cells.
Table 2. GSEA Top-ranked RNA-seq Upregulated Genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Title</th>
<th>Rank Score (GSEA)</th>
<th>Log² Fold Change PC3 vs. PC3-DR</th>
<th>Log² Fold Change DU145 vs. DU145-DR</th>
<th>Stem Cell-Associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSPAN8</td>
<td>tetraspanin 8</td>
<td>-4.782</td>
<td>5.357</td>
<td>5.815</td>
<td>YES</td>
</tr>
<tr>
<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
<td>-4.497</td>
<td>3.813</td>
<td>4.727</td>
<td>YES</td>
</tr>
<tr>
<td>DNAJC12</td>
<td>DNAJ (Hsp40) homolog, subfamily C, member 12</td>
<td>-4.466</td>
<td>4.411</td>
<td>6.032</td>
<td>YES</td>
</tr>
<tr>
<td>F2R</td>
<td>coagulation factor II (thrombin) receptor</td>
<td>-4.429</td>
<td>4.289</td>
<td>3.820</td>
<td>YES</td>
</tr>
<tr>
<td>GLB1L2</td>
<td>galactosidase beta 1-like 2</td>
<td>-4.206</td>
<td>3.421</td>
<td>3.670</td>
<td>YES</td>
</tr>
<tr>
<td>PLD6</td>
<td>phospholipase D family member 6</td>
<td>-4.194</td>
<td>3.537</td>
<td>3.529</td>
<td>YES</td>
</tr>
<tr>
<td>DPP4</td>
<td>dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)</td>
<td>-4.135</td>
<td>3.890</td>
<td>4.956</td>
<td>YES</td>
</tr>
<tr>
<td>FAM102B</td>
<td>family with sequence similarity 102, member B</td>
<td>-3.967</td>
<td>3.196</td>
<td>3.083</td>
<td>YES</td>
</tr>
<tr>
<td>ST6GAL1</td>
<td>ST6 beta-galactosamid alpha-2,3-sialyltransferase 1</td>
<td>-3.909</td>
<td>2.925</td>
<td>1.439</td>
<td>YES</td>
</tr>
<tr>
<td>CD55</td>
<td>CD55 molecule, decay accelerating factor for complement (Cromer blood group)</td>
<td>-3.908</td>
<td>2.652</td>
<td>3.861</td>
<td>YES</td>
</tr>
<tr>
<td>HSF1</td>
<td>heat shock transcription factor 1</td>
<td>-3.885</td>
<td>3.083</td>
<td>3.990</td>
<td>YES</td>
</tr>
<tr>
<td>NES</td>
<td>nestin</td>
<td>-3.806</td>
<td>3.477</td>
<td>5.980</td>
<td>YES</td>
</tr>
<tr>
<td>ZNF503</td>
<td>zinc finger protein 503</td>
<td>-3.725</td>
<td>2.409</td>
<td>3.432</td>
<td>YES</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
<td>-3.685</td>
<td>2.779</td>
<td>2.695</td>
<td>YES</td>
</tr>
<tr>
<td>PMP22</td>
<td>peripheral myelin protein 22</td>
<td>-3.641</td>
<td>2.798</td>
<td>2.597</td>
<td>YES</td>
</tr>
<tr>
<td>WDR25</td>
<td>WD repeat domain 25</td>
<td>-3.633</td>
<td>2.690</td>
<td>2.673</td>
<td>-</td>
</tr>
<tr>
<td>CCDC50</td>
<td>coiled-coil domain containing 50</td>
<td>-3.622</td>
<td>2.355</td>
<td>2.989</td>
<td>-</td>
</tr>
<tr>
<td>FABP5</td>
<td>fatty acid binding protein 5 (psoriasis-associated)</td>
<td>-3.591</td>
<td>2.538</td>
<td>4.071</td>
<td>YES</td>
</tr>
<tr>
<td>FLVCR2</td>
<td>feline leukemia virus subgroup C receptor 2</td>
<td>-3.586</td>
<td>2.460</td>
<td>3.167</td>
<td>-</td>
</tr>
<tr>
<td>SMAGP</td>
<td>small cell adhesion glycoprotein</td>
<td>-3.579</td>
<td>2.538</td>
<td>3.345</td>
<td>-</td>
</tr>
<tr>
<td>MROH1</td>
<td>maestro heat-like repeat family member 1</td>
<td>-3.548</td>
<td>2.535</td>
<td>3.679</td>
<td>-</td>
</tr>
<tr>
<td>MAF1</td>
<td>MAF1 homolog (S. cerevisiae)</td>
<td>-3.501</td>
<td>2.470</td>
<td>3.158</td>
<td>-</td>
</tr>
<tr>
<td>CLGN</td>
<td>calmegin</td>
<td>-3.455</td>
<td>2.784</td>
<td>2.488</td>
<td>YES</td>
</tr>
<tr>
<td>KLF4</td>
<td>kruppel-like factor 4 (gut)</td>
<td>-3.452</td>
<td>2.271</td>
<td>2.657</td>
<td>YES</td>
</tr>
<tr>
<td>ACOX2</td>
<td>acyl-coenzyme A oxidase 2, branched chain</td>
<td>-3.437</td>
<td>2.552</td>
<td>2.371</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. GSEA Top 25-ranked RNA-seq Downregulated Genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Title</th>
<th>Rank Score (GSEA)</th>
<th>Log2 Fold Change PC3 vs. PC3-DR</th>
<th>Log2 Fold Change DU145 vs. DU145-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRT7</td>
<td>keratin 7</td>
<td>4.702</td>
<td>-7.497</td>
<td>-7.843</td>
</tr>
<tr>
<td>HLA-C</td>
<td>major histocompatibility complex, class I, C</td>
<td>4.500</td>
<td>-5.337</td>
<td>-4.225</td>
</tr>
<tr>
<td>LAMA3</td>
<td>laminin, alpha 3</td>
<td>4.494</td>
<td>-4.056</td>
<td>-4.376</td>
</tr>
<tr>
<td>PLA2G16</td>
<td>phospholipase A2 Group XVI</td>
<td>4.332</td>
<td>-3.905</td>
<td>-4.489</td>
</tr>
<tr>
<td>ERBB2</td>
<td>v-erb-b2 erythroleukemia viral oncogene homolog 2, neuroglioblastoma derived oncogene homolog (avian)</td>
<td>4.267</td>
<td>-3.339</td>
<td>-3.632</td>
</tr>
<tr>
<td>AIFM2</td>
<td>apoptosis inducing factor, mitochondria associated 2</td>
<td>4.193</td>
<td>-4.127</td>
<td>-3.373</td>
</tr>
<tr>
<td>C1ORF116</td>
<td>chromosome 1 open reading frame 116</td>
<td>4.157</td>
<td>-3.317</td>
<td>-3.529</td>
</tr>
<tr>
<td>PSMB8</td>
<td>proteasome subunit beta type, 8 (large multifunctional peptidase 7)</td>
<td>4.133</td>
<td>-6.159</td>
<td>-3.699</td>
</tr>
<tr>
<td>JUP</td>
<td>junction plakoglobin</td>
<td>4.100</td>
<td>-4.461</td>
<td>-3.666</td>
</tr>
<tr>
<td>FURIN</td>
<td>furin (paired basic amino acid cleaving enzyme)</td>
<td>4.059</td>
<td>-3.102</td>
<td>-3.391</td>
</tr>
<tr>
<td>ALS2CL</td>
<td>ALS2 C-terminal like</td>
<td>4.032</td>
<td>-3.975</td>
<td>-3.194</td>
</tr>
<tr>
<td>LAM83</td>
<td>laminin, beta 3</td>
<td>3.984</td>
<td>-3.587</td>
<td>-4.206</td>
</tr>
<tr>
<td>PKIG</td>
<td>protein kinase (cAMP-dependent, catalytic inhibitor gamma)</td>
<td>3.963</td>
<td>-3.214</td>
<td>-2.976</td>
</tr>
<tr>
<td>SEMA3C</td>
<td>sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C</td>
<td>3.939</td>
<td>-2.957</td>
<td>-3.166</td>
</tr>
<tr>
<td>MROH6</td>
<td>maestro heat-like repeat family member 6</td>
<td>3.906</td>
<td>-4.171</td>
<td>-2.881</td>
</tr>
<tr>
<td>TNS3</td>
<td>tensin 3</td>
<td>3.858</td>
<td>-2.992</td>
<td>-2.889</td>
</tr>
<tr>
<td>LY6E</td>
<td>lymphocyte antigen 6 complex, locus E</td>
<td>3.834</td>
<td>-3.344</td>
<td>-2.945</td>
</tr>
<tr>
<td>SSH3</td>
<td>slingshot homolog 3 (Drosophila)</td>
<td>3.801</td>
<td>-2.733</td>
<td>-2.984</td>
</tr>
<tr>
<td>PTPRF</td>
<td>protein tyrosine phosphatase, receptor type, F</td>
<td>3.765</td>
<td>-2.463</td>
<td>-3.473</td>
</tr>
<tr>
<td>WNT7B</td>
<td>wingless-type MMTV integration site family, member 7B</td>
<td>3.686</td>
<td>-2.694</td>
<td>-4.630</td>
</tr>
<tr>
<td>PTK28</td>
<td>PTK2B protein tyrosine kinase 2 beta</td>
<td>3.632</td>
<td>-3.658</td>
<td>-3.552</td>
</tr>
<tr>
<td>CAPN5</td>
<td>calpain 5</td>
<td>3.614</td>
<td>-2.852</td>
<td>-2.380</td>
</tr>
<tr>
<td>SLC27A3</td>
<td>solute carrier family 27 (fatty acid transporter), member 3</td>
<td>3.592</td>
<td>-3.285</td>
<td>-2.310</td>
</tr>
<tr>
<td>MICAL2</td>
<td>microtubule associated monooxygenase, calponin and LIM domain containing 2</td>
<td>3.590</td>
<td>-2.301</td>
<td>-2.910</td>
</tr>
<tr>
<td>GCA</td>
<td>grancalcin, EF-hand calcium binding protein</td>
<td>3.575</td>
<td>-3.679</td>
<td>-2.472</td>
</tr>
</tbody>
</table>


Gene Set Enrichment Analysis (GSEA) also identified the gene set “GO_Gene Generation of Precursor Metabolites and Energy” as the only significantly enriched pathway in the DTX-resistant PC3-DR and DU145-DR cells ($P = 0.032$) (Figure 3E). This analysis yielded 8 genes ($\text{NADUFAF2, ENPP1, NDUFAB1, NDUFA8, PFKM, GNPDA1, CYC1, MYC}$) that were positive for core enrichment in this gene set. Of these genes, ectonucleotide phosphodiesterase 1 ($\text{ENPP1}$), cytochrome c-1 ($\text{CYC1}$), NADH dehydrogenase 1 alpha/beta subcomplex 1 ($\text{NADUFAB1}$), and v-myc myelocytomatosis viral oncogene homolog ($\text{MYC}$) have been associated with stem cell maintenance, phenotype acquisition, or reprogramming [24-29], suggesting that upregulation of specific genes involved in metabolism may contribute to an enrichment of cells with CSC-like characteristics (Figure 3E). Taken together, the RNA-seq analysis of transcript expression in DTX-sensitive vs. DTX-resistant PCa cell lines provides evidence for the acquisition of a transcriptomic program associated with stemness as a mechanism contributing to the development of DTX-resistance.

**Validation of Transcript and Protein Expression of Selected Genes in DTX-resistant Cells Confirmed RNA-seq Results**

To confirm the RNA-seq data, we performed in-house qPCR validation on selected genes that showed robust upregulation in both PC3-DR and DU145-DR cells, compared to the sensitive, parental cell lines. The selection of specific genes for validation was determined by two criteria: the GSEA ranked gene order (Table 2 and Table 3), and exhaustive literature searches implicating these genes in cancer, PCa, therapy resistance, DTX resistance, stem cells, CSCs, or EMT. For our in-house
validation of RNA-seq data, new RNA samples were extracted from a different set of DTX-sensitive and DTX-resistant cells than those used for the RNA-seq analysis. Consistent with the RNA-seq results, transcript expression of dipeptidyl peptidase 4 (DPP4), tetraspanin 8 (TSPAN8), nestin (NES), DNAJ heat shock protein family member C12 (DNAJC12), fatty acid binding protein 5 (FABP5), and block of proliferation 1 (BOP1) were upregulated in PC3-DR and DU145-DR cells compared to the corresponding sensitive cell lines (Figure 6). As an internal control for in-house validation, we also chose two genes found robustly downregulated in the RNA-seq results, transglutaminase 2 (TGM2) and ATP-binding cassette subfamily C member 3 (ABCC3). Transcript expression of both genes was robustly downregulated in PC3-DR and DU145-DR cells compared to the sensitive cell lines, further confirming the RNA-seq results (Figure 6). The magnitude of fold-increase observed for each of these genes was more robust in DU145-DR cells than in PC3-DR cells, suggesting cell-type dependent differences in gene expression during the acquisition of resistance to DTX. Despite these differences, $P$ values were consistently < 0.01 for each of the selected genes in both DTX-resistant cell lines.
Figure 6. In-house qPCR Validation of the Expression of Selected Top-ranked Genes from RNA-seq Results in DTX-Sensitive and DTX–Resistant mCRPC cells. qPCR validation for selected genes in (A) PC3 vs. PC3-DR and (B) DU145 vs. DU145-DR cells. White bars represent parental PC3 or DU145 and colored bars represent PC3-DR or DU145-DR. *P < 0.05; **P < 0.05; ***P < 0.001. All RNA samples were analyzed in at least three independent experiments using at least three biological replicates per experiment. Error bars represent mean ± SD.
After validation of the transcript expression of selected genes in the DTX-resistant PC3-DR and DU145-DR cells, we sought to confirm corresponding protein upregulation in these cells compared to their sensitive counterparts by immunoblotting using specific antibodies. Significant upregulation of DPP4, TSPAN8, NES, DNAJC12, FABP5, and BOP1 was observed in the PC3-DR and DU145-DR cells, consistent with the qPCR and RNA-seq results (Figure 7A-7F). Also consistent with the RNA-seq and qPCR results, the protein expression of TGM2 was downregulated in the DTX-resistant cells (Figure 7G).
Figure 7. Protein Expression Validation of RNA-seq Results in DTX-sensitive and DTX-resistant mCRPC Cells. Representative Western blot images and protein fold change quantification are shown for (A) DPP4 (n=3), (B) TSPAN8 (n=4), (C) NES (n=6), (D) DNAJC12 (n=4), (E) FABP5 (n=7), (F) BOP1 (n=4), and (G) TGM2 (n=4). *P< 0.05; **P< 0.05; ***P< 0.001. All proteins were analyzed in at least three independent experiments. Error bars represent mean ± SD.
Analysis of Cancer Gene Microarray Datasets Reveals Consistent Upregulation of DNAJC12, FABP5, and BOP1 in PCa Tissues

After confirming that transcript and protein expression of selected genes reflected the upregulation observed in the RNA-seq analysis, we sought to examine the expression of these genes in human PCa tissues. Transcript expression of the selected genes in PCa tissues, compared to normal prostate tissue, was analyzed using 16 PCa gene expression microarray datasets from the Oncomine database. All 16 datasets had data for FABP5, whereas data for DPP4 and TSPAN8 were available in 15 datasets, and data for BOP1, DNAJC12 and NES were available in 14, 10 and 8 datasets, respectively.

Of the selected genes, DNAJC12, FABP5, and BOP1 were the most consistently upregulated in prostate tumors compared to normal prostate tissues in the dataset collection (Figure 8A-8C), with significant upregulation of DNAJC12 in 6 of the 14 datasets (Figure 8A), FABP5 in 14 of the 16 data sets (Figure 8B), and BOP1 in 7 of the 14 datasets (Figure 8C). DPP4 and TSPAN8 transcripts were significantly upregulated only in 4 of the 14 datasets (Figure 8D-8E). Interestingly, significant upregulation of NES transcript was detected only in 1 of the 8 datasets (Figure 8F). The magnitude of the fold-increase observed for the individual genes was modest, with only FABP5 showing over 2-fold increase in multiple datasets. However, the P values were <0.01 for most of the DNAJC12, FABP5, and BOP1 datasets, indicating that upregulation of these transcripts is highly significant in PCa tissues compared to normal tissues. On the other hand, NES transcripts were significantly downregulated in 4 of the 8 datasets, whereas DPP4 and TSPAN8 transcripts displayed significant downregulation in 1 and 2 out of 15 datasets, respectively.
**Figure 8.** Expression of Selected Top-Ranked Genes in Clinical PCa Tissues. Fold change between transcript expression levels of selected top ranked genes (from RNA-seq analysis) in prostate tumors versus normal prostate tissues as derived from cancer gene microarray datasets in the Oncomine database. Individual dataset names appear in the legend box at the right. $P$ values for the differences in gene expression between PCa and normal prostate tissues were obtained from Oncomine. The number of samples in each dataset is different, therefore higher fold change does not always correspond to statistical significance. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. 
**PC3-DR and DU145-DR Cells Upregulate Markers Associated with EMT and CSCs**

The observation that highly ranked genes (GSEA) in the RNA-seq results were associated with CSC development or are known markers of CSCs (e.g. *NES, DPP4, TSPAN8*), led us to assess the expression of established EMT and CSC markers in our DTX-resistant cell lines. Microscopic assessment of DTX-resistant cells revealed a mesenchymal phenotype with clearly defined edges and the classical spindle-shaped morphology, compared to the flattened, polygonal-shaped sensitive PC3 and DU145 (Figure 9A). Using multicolor flow cytometry, we analyzed the following populations in both DTX-sensitive and DTX-resistant cells: E-cadherin positive and N-cadherin positive (Figure 9B, 9C), as well as CD44+ and CD44+/CD24- (Figure 10A, 10B). Consistent with their mesenchymal phenotype, DTX-resistant cells showed significantly reduced E-cadherin expression compared to DTX-sensitive cells, concomitant with an increase in N-cadherin expression, as determined by flow cytometry (Figure 9B, 9C) and immunoblotting (Figure 9D, left two panels). Notably, loss or downregulation of E-cadherin is associated with poor prognosis in PCa [16]. We also observed that loss of E-cadherin in the DTX-resistant cell lines was coupled with upregulation of Vimentin (Figure 9D, center panel) and transcription factors Snail and Twist (Figure 9D, center and right two panels).

Vimentin, a well-established marker of EMT [30], was robustly and significantly upregulated in the PC3-DR cells compared to sensitive PC3 but its upregulation did not reach statistical significance in DU145-DR cells compared to sensitive DU145. Both Snail and Twist are known to repress E-cadherin expression, with Twist having a dual role in contributing to the upregulation of N-cadherin expression [15, 16, 30]. Taken
together, these findings support growing evidence implicating EMT in PCa DTX-resistance [15, 16, 31].
A) 

Sensitivity and resistance in PC3 and DU145 cell lines:

- PC3 Sensitive
- PC3 Resistant
- DU145 Sensitive
- DU145 Resistant

B) 

Bar graphs showing the number of E-Cadherin and N-Cadherin positive cells in PC3 and PC3-DR, as well as DU145 and DU145-DR.

C) 

Flow cytometry analysis of E-Cadherin (ECad) and N-Cadherin (NCad) in PC3 and DU145 cell lines:

- PC3, ECad
- PC3, NCad
- DU145, ECad
- DU145, NCad

D) 

Expression analysis of E-Cadherin (ECad), N-Cadherin (NCad), Vimentin, Snail, and Twist in PC3 and DU145 cell lines:

- Western blot analysis for ECad and N-Cad
- Quantitative analysis of fold change for ECad, NCad, Vimentin, Snail, and Twist
Figure 9. DTX-resistant mCRPC Cells Exhibit a Mesenchymal-like Phenotype Compared to DTX-sensitive Cells. (A) Differences in morphology between DTX-sensitive and DTX-resistant PC3 and DU145 cells visualized by Hoffman modulation contrast microscopy (scale bar set at 40 µm). (B) Percent of live PC3 and DU145 cells (DTX sensitive and -resistant) that stained positive for E-cadherin and N-cadherin as determined by flow cytometry (n=3 biological replicates) *P< 0.05, **P< 0.01, ***P< 0.001. (C). Representative flow charts of bar graph data showing downregulation of E-cadherin and upregulation of N-cadherin in the DTX-resistant cell lines. (D) Representative Western blots showing expression (upper panels) and quantification (lower panels) of E-cadherin (n=3), N-cadherin (n=3), Vimentin (n=3), Snail (n=3), and Twist (n=3). *P< 0.05, **P< 0.01, ***P< 0.001. Error bars represent mean ± SEM.
In addition to these findings, we observed that the DTX-resistant cell populations displayed a higher frequency of cells expressing established CSC markers (Figure 10). CD44, one of these markers, is a multifunctional class I transmembrane glycoprotein that is highly expressed in most cancer types, where it contributes to tumor progression [32]. While we observed a significant proportion of PC3-DR cells with CD44+ expression compared to sensitive cells, there was no significant increase in the frequency of CD44+ cells in the DU145-DR population (Figure 10A, left two panels). However, because CD44 is expressed in almost all normal and cancer cells, specifically in normal prostate and PCa cells, there is a reported discrepancy and ambiguity regarding the functional aspects of this marker in prostate CSC maintenance [32]. This discrepancy is supported by our observation that sensitive DU145 cells showed high CD44+ expression (Figure 10A, 10B, left panels), and has been circumvented by using CD44 in combination with other markers to detect CSC subsets in PCa [32-36].

To better refine our detection of the putative CSC population in DTX-resistant cells, we used the well-validated combination of CD44+/CD24- [32, 35, 36]. CD24 is a luminal cell surface protein that contributes to metastasis and functions in cell-cell and cell-matrix interactions [32, 33, 36]. Because prostate CSCs arise from the basal cell compartment, the CD44+/CD24- marker combination is commonly used to identify these cells [32, 33]. We observed that both PC3-DR and DU145-DR cells contained substantial CD44+/CD24- subpopulations compared to the sensitive PC3 and DU145 cells (Figure 10A, 10B, right two panels).

Elevated aldehyde dehydrogenase (ALDH) activity is also emerging as a functional marker of a CSC-like phenotype because of its importance for CSC
maintenance, signaling, and drug resistance [37]. To further confirm the acquisition of CSC-like characteristics in the DTX-resistant cells, we measured by flow cytometry the frequency of Aldefluor+ cells in our DTX-resistance cells compared to sensitive cells. Both PC3-DR and DU145-DR showed robust increase of ALDH activity compared to their sensitive counterparts (Figure 10C, 10D).
Figure 10. DTX-resistant mCRPC Cells Upregulate Markers Associated with CSC-like Characteristics Compared to DTX-sensitive Cells. (A) Percent of CD44+ and CD44+/CD24- cells for PC3 vs PC3-DR and DU145 vs DU145-DR, with (B) representative flow cytometry plots showing compensation windows used in the FMO analysis for each marker. Flow data is represented as frequency of live cells determined by annexin-V staining. (C) PC3-DR and (D) DU145-DR cells have a significantly greater percentage of ALDH+ cells compared with sensitive PC3 and DU145 cells as determined by aldefluor assay (+DEAB control used for gating). Representative flow plots are shown together with bar graphs. All flow measurements were acquired from at least 3 independent experiments conducted separately. **P< 0.01, ***P< 0.001. Error bars represent mean ± SEM.
**Increased Tumorsphere Formation Capacity and DTX-resistance in DU145-DR Cells**

Upon confirmation of increased frequency of cells expressing EMT and CSC markers in the adherent (2D) DTX-resistant cell cultures, we sought to examine and compare tumorspheres (3D cultures) formed by sensitive and resistant DU145 cells. Tumorsphere formation is a widely used functional approach for enriching CSC populations, especially when specific surface CSC markers are not well defined or change with tumor heterogeneity [38-40]. We chose to focus these studies on the DU145 cell line because its DTX-sensitive cells formed large numbers of tumorspheres, consistent with previous reports that this cell line has a robust ability to form spheres even in the absence of external growth factors or drugs [34]. We observed that under tumorsphere-forming conditions, DU145-DR cells showed a 2.3-fold increase in tumorspheres compared to sensitive DU145 cells, as evidenced by phase contrast (4X) microscopic examination (Figure 11A, 11B). DU145-DR tumorspheres were loosely clustered, tethered together in grape-like clusters to form large aggregates (Figure 11C). This morphology was a stark difference from the tightly compact tumorspheres of sensitive DU145 cells.

Consistent with our analysis of adherent DTX-resistant cells (2D), flow cytometry analysis of DTX-resistant tumorspheres (3D) revealed a decreased frequency of E-cadherin expressing cells concomitant with increased frequency of N-cadherin expressing cells in the DU145-DR 3D cultures compared to DU145 3D cultures, (Figure 11D, 11E, left two panels). In addition, we detected increased frequencies of CD44+ and CD44+/CD24- populations in DU145-DR 3D compared to DU145 3D cultures (Figure 11D, 11E, right two panels). Furthermore, consistent with the 2D data, DU145-DR tumorspheres
had a significantly higher number of Aldefluor+ cells than DU145 tumorspheres (Figure 11F).
**A**

CD44+ Cells

- PC3
- PC3-DR
- DU145
- DU145-DR

**B**

Flow cytometry histograms for CD44+ and CD24+ cells in PC3, PC3-DR, DU145, and DU145-DR.

**C**

ALDH+ cells

- PC3 + DEAB control
- PC3 + DEAB Test

**D**

ALDH+ cells

- DU145 + DEAB control
- DU145 + DEAB Test

- DU145-DR + DEAB control
- DU145-DR + DEAB Test
Figure 11. Tumorsphere Formation Capacity is Higher in DTX-resistant DU145 Cells Compared to Sensitive Cells. (A) Phase contrast microscopy images of DU145 and DU145-DR tumorspheres (3D) with (B) quantification of tumorsphere percentage using Image J software. (C) Tumorsphere morphology visualized using Hoffman modulation contrast microscopy (scale bar set at 40 µm). (D) Percent of live cells positive for the cell surface markers E-cadherin and N-cadherin, and CSC markers CD44+/ CD24-, CD44+/ CD24-, with (E) representative flow cytometry plots. (F) Representative flow cytometry plots showing increased percentage of ALDH+ cells in DU145-DR tumorspheres as determined by aldefluor assay with bar graphs. All flow measurements were acquired from at least 3 independent experiments conducted separately. *P<0.05, **P<0.01, ***P<0.001. Error bars represent mean ± SEM.
Other groups have demonstrated that tumorspheres derived from DU145 3D cells are more resistant to DTX treatment compared to DU145 2D cells [40, 41]. To further investigate the link between CSCs and DTX-resistance, we sought to determine if our DU145-DR tumorspheres were more resistant to DTX compared to DU145-DR 2D cells after exposure to increasing concentrations of DTX for 72 hours. Using propidium iodide (PI) staining of dead cells followed by flow cytometric analysis, we found that DU145-DR 3D tumorspheres were significantly more resistant to 10 nM DTX, the maintenance dose of DTX-resistant cell lines compared to the DU145-DR 2D cells grown in monolayer (Figure 12A, 12B). There were no statistical differences, however, at other lower or higher doses (Figure 12A), or at 24 or 48-hour time points (data not shown).
Figure 12. DU145-DR Derived Tumorspheres Show Increased Resistance to DTX Compared to DU145-DR Adherent Cells. (A) DU145-DR adherent and tumorsphere cells treated with increasing concentrations of DTX (nM range). (B) DU145-DR 3D tumorspheres were more resistant to 10 nM DTX than the adherent DU145-DR 2D cells. All samples were normalized to untreated controls and to DU145-DR 3D percent viability. All measurements were acquired from at least 3 independent experiments with 3 biological replicates each. *P< 0.05. Error bars represent mean SEM.
Discussion

There is a critical need for new drugs targeting non-traditional molecular targets that could be used alone or in combination with current agents for the treatment of therapy-resistant mCRPC. The present study used an RNA-seq approach to define transcriptomic signatures associated with DTX-resistance with the ultimate goal of identifying potentially novel therapeutic targets for overcoming this resistance. For these studies, we chose androgen-refractory PC3 and DU145 cells, which are widely used as cellular models that emulate late-stage mCRPC disease. While sensitive to DTX-treatment, these cell lines become resistant to the clinically relevant taxanes DTX, cabazitaxel, and paclitaxel upon incremental exposure to DTX and selection of surviving cells [8]. Resistance to both DTX and cabazitaxel is inevitable in mCRPC patients undergoing chemotherapy [2], but the mechanisms underlying this resistance remain to be clearly established.

PCa is fundamentally AR-driven especially in the context of disease initiation and progression. Because the intraprostatic response of PCa cells to androgens depends on the expression and sensitivity of AR, ADT has been a mainstay of PCa treatment and typically precedes taxane chemotherapy, although data from the recent “STAMPEDE” clinical trial showed improved patient survival when long-term primary ADT was combined with abiraterone acetate or DTX [42]. Constitutively active AR splice variants have been shown to be overexpressed in mCRPC and confer resistance to ADT by inhibiting the nuclear translocation of the androgen-AR complex [43-46]. A recent study suggested that AR splice variants may also affect sensitivity to taxanes and that tumors predominantly expressing the ARv7 variant, associated with ADT resistance, would also
be likely be resistant to DTX [47]. However, an independent group was unable to replicate these results under similar experimental conditions [48]. Furthermore, another group found that detection of ARv7 in circulating tumor cells of mCRPC patients was not associated with taxane-resistance and that certain patients with ARv7-positive status at baseline converted to ARv7-negative status during the course of taxane therapy, adding uncertainty to the clinical significance of this variant in patients receiving taxanes [49]. These discrepancies also contributed to our decision to focus on the AR-negative cell lines PC3 and DU145 for the present study.

Our RNA-seq analysis revealed over 1,200 genes that were differentially regulated in both the PC3-DR and DU145-DR cell lines. We focused on this set of overlap genes because differences in their expression are more likely to reflect transcriptomic changes induced by long-term DTX treatment regardless of the PCa cell type (e.g., PC3-bone metastasis vs. DU145-brain metastasis). Differentially expressed genes within this pool of overlap genes could potentially be exploited as therapeutic targets in heterogeneous metastatic prostate tumors that have acquired taxane resistance. GSEA of our RNA-seq data revealed several top ranked genes from the overlap dataset that an exhaustive PubMed literature review determined as being associated with tumor aggressiveness, chemoresistance, or CSC phenotype. Of note, GSEA yielded only one significant pathway enriched in the DTX-resistant cell lines compared to sensitive cells that yielded 8 genes positive for core enrichment. Of these, 4 genes (ENPP1, CYC1, NADHUFAB1, and MYC) are associated with stem cell maintenance, acquisition or reprogramming [24-29], suggesting that in PC3 and DU145, DTX-resistance may be driven and maintained by the acquisition of CSC-like characteristics. Determining
metabolic differences between DTX-sensitive and -resistant mCRPC cells will be imperative in future follow-up studies.

Using qPCR and immunoblotting, we validated several of the top upregulated genes in the DTX-resistant cells. These included genes associated with PCa aggressiveness, such as FABP5 and BOP1, as well as genes implicated in CSC function such as DPP4, TSPAN8, DNAJC12, and NES. FABP5 is an intracellular lipid-binding protein that is emerging as a critical regulator of PCa cell proliferation and putative marker of aggressive PCa [50-53]. The robust FABP5 transcript and protein upregulation observed in the DTX-resistant cells suggest that this protein could be a promising target for the treatment of chemoresistant mCRPC. Another gene highly ranked in the GSEA was BOP1, an integral component of the ribosomal RNA processing machinery that contributes to colorectal tumorigenesis through promotion of cell migration and invasion [54, 55]. Interestingly, the BOP1 gene is located in chromosome 8q24, a genomic region associated with PCa aggressiveness [54] that also encompasses MYC [56], one of the top upregulated genes in the DTX-resistant mCRPC cells revealed by our RNA-seq analysis.

An emerging stem cell marker, DPP4 (CD26) was also robustly upregulated in the DTX-resistant cells. DPP4 is a transmembrane glycoprotein that functions as an exopeptidase to promote cell migration through MMP-9, and contributes to the upregulation of CD44 [57]. This protein is upregulated in many cancers and associated with colon CSCs derived from DTX-resistant cells, which form larger and more tumorspheres [58-61]. The robust upregulation in protein expression observed in PC3-DR and DU145-DR suggest that DPP4 might be a prostate CSC marker that identifies a chemoresistant phenotype. The robust transcript and protein upregulation of TSPAN8
(TM4SF3) in the DTX-resistant cells also suggest a role for this protein in PCa chemoresistance. TSPAN8, promotes cell-to-cell communication by regulating integrins and other cell surface proteins [62], and its expression has been correlated with metastasis and worse prognosis in colon cancer where it contributes to cell motility through a complex with E-cadherin [63]. TSPAN8 is also considered a pancreatic CSC marker [64]. Genome splicing-sensitive microarray analysis revealed upregulation of TSPAN8 and DPP4 in DU145 tumorspheres compared to adherent DU145 2D cells [65].

DNAJC12, also known as Hsp40, has been implicated in cancer but its role in tumorigenesis is not clearly defined [66, 67]. The DNAJ family of proteins are considered regulators of CSC function [68], and DNAJC12 transcript expression was found to be upregulated in breast CSCs compared to adherent breast cancer cells [69]. NES, a cytoskeletal intermediate filament protein, has been associated with increased migration in PCa cells [70], and increased NES expression correlated with high tumor grade, invasive phenotype, and predictor of poor response to therapy [71]. Consistent with our observation that NES is robustly upregulated in DTX-resistant mCRPC cells with CSC-like characteristics, NES expression was previously reported in PCa tumorspheres that showed increased chemoresistance to paclitaxel [72], and was associated with a mesenchymal phenotype [73].

Oncomine data analysis comparing transcript expression of DPP4, TSPAN8, and NES between prostate tumor tissues and normal tissues revealed inconsistent upregulation of these genes in the different datasets. An explanation for this could be that the prostate tumors used to generate most of these gene expression datasets were not derived from advanced or chemoresistant disease. Alternatively, gene expression changes
found in DTX-resistant cells occur in only a small subset of cells, most likely those with stemness properties. Since tumors contain varying proportions of cells with and without stemness properties, it will be difficult to consistently detect global gene expression changes in CSCs present in PCa tissues since they comprise a minority of the population. A limitation of the Oncomine database is the assessment of gene expression in normal vs. PCa tissues without extensive clinical data (type of treatment, tumor stage, etc.) for several of the datasets. Therefore, to further validate the expression of selected genes of interest in clinically relevant tissues, it will be important in future studies to obtain mCRPC biospecimens with annotated clinical data from patients with and without taxane treatment, and that responded to or failed the treatment. We recognize, however, the intrinsic difficulties in obtaining such biospecimens.

The beneficial effects of chemotherapeutic drugs like DTX are hindered by the development of chemoresistance. Emerging evidence demonstrates that a small population of CSCs present within the tumors possesses multiple redundant mechanisms that facilitate tumor cell survival in the presence of therapeutic agents [12, 14, 17]. In addition, the relatively non-proliferative state of CSCs makes this small population of cells intrinsically resistant to conventional chemotherapies, most of which target rapidly dividing cells. This resistant population comprises a tumor cell reserve that persists even after anti-proliferative treatments and repopulates the tumor in metastatic sites [12]. The emerging role of CSCs in the acquisition of PCa chemoresistance [12, 15, 17, 74], and the observation that highly-ranked genes in our RNA-seq analysis were associated with a CSC-like phenotype or genetic program, led us to characterize this population in DTX-sensitive and -resistant mCRPC PC3 and DU145 cells. Putative CSCs are typically
identified based on the presence and/or absence of several cell surface markers, the combination of which is specific for the CSC-like phenotype identified in a particular tumor type [71]. Our observation that the PC3-DR and DU145-DR cell cultures were enriched with cell populations expressing several of these CSC markers, including significantly elevated ALDH activity compared to DTX-sensitive parental cells, is consistent with the acquisition of CSC-like characteristics. Furthermore, our finding that DU145-DR cells have an enhanced capacity to form tumorspheres (3D) and increased ALDH activity compared to DU145 tumorspheres, is an indicator of the increased CSC-like characteristics of the resistance cells. In addition, our DU145-DR tumorspheres showed increased resistance to 10 nM DTX, a clinically relevant dose, compared to adherent DU145-DR cells (2D), suggesting that a CSC-like phenotype contributes to enhanced DTX resistance. An accurate assessment of the increased tumorigenic potential of DTX-resistant cells with CSC-like characteristics would be more effectively achieved through \textit{in vivo} studies with animal models using enriched CSC populations acquired by cell sorting.

Targeting CSCs is a promising approach to circumvent tumor chemoresistance [14, 17]. Current strategies focus on targeting signaling pathways upregulated in stem cells that are specific to their function, including the Hedgehog, Wnt, Notch, and NFkB pathways [12]. The present RNA-seq study provides additional candidate genes and molecular pathways for potential therapeutic targeting, and contributes to the emerging body of evidence linking CSCs to PCa chemoresistance. Future pre-clinical studies will focus on establishing mechanistic roles of specific genes identified in our RNA-seq analysis in the maintenance of prostate CSCs and driving taxane resistance, validating
their expression in clinical biospecimens derived from PCa patients that failed taxane therapy, and investigating their potential as therapeutic targets. It will also be important to further define PCa cell-type dependent differences in the expression of CSC and chemoresistance-associated genes, as our RNA-seq analysis demonstrated that PC3-DR and DU145-DR cells have differentially regulated genes that are unique to each of these cell lines. This would be critical for tackling the high heterogeneity that characterizes prostate tumors.

**Materials and Methods**

*Cell Lines, Antibodies, and Cell Culture*

The metastatic PCa cell lines PC3 and DU145 were purchased from the American Type Culture Collection (ATCC, Cat# ATCC-CRL-1435 and ATCC-HTB-81, respectively). Cells were cultured as recommended by the supplier in RPMI medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and gentamicin. Cells were maintained in a humidified incubator with 5% CO₂ at 37°C. DTX-resistant (DR) PC3 and DU145 were developed as described previously [18]. Briefly, PC3 and DU145 cells were cultured in media containing 1 nM DTX (LC Laboratories Cat# D-1000) and surviving cells were passaged four times before increasing the concentration of DTX. This was repeated until resistant cells could be maintained with minimal cell death in the presence of 11 nM DTX.

Short tandem repeat (STR) profiling is a recommended and validated method for authentication of human cell lines and tissues [75]. The importance of cell line authentication is highlighted by the NIH initiative for rigor and reproducibility in
scientific research [76], and is particularly important for scientific studies such as the present one that use established cancer cell lines for pre-clinical mechanistic studies. We utilized the STR service provided by ATCC (Cat# ATCC 135-XV) to authenticate the PC3 and DU145 cell lines used in this study. Both cell lines matched their respective database profiles. The DTX-resistant PC3-DR and DU145-DR cell lines were derived from these validated PC3 and DU145 parental cell lines.

**RNA Isolation and RNA-seq Library Preparation and Sequencing**

Total RNA was extracted from DTX-sensitive and -resistant PC3 and DU145 cells using the miRNeasy Mini Kit (Qiagen Cat# 217004). RNA-seq library construction and sequencing was performed at the Loma Linda University School of Medicine Center for Genomics. RNA-seq library was constructed using the TruSeq Stranded mRNA Low Sample Preparation protocol (Illumina; Cat# RS-1229004DOC). Two µg of total RNA were used as input. Each RNA sample was spiked with 1:100 ERCC RNA spike-in control mix 1 (Life Technologies, Cat# 4456740) prior to the first step of the protocol. All the recommended controls were used during subsequent steps including an End Repair Control, A-Tailing Control, and Ligation Control. The RNA-seq libraries were quantified using Qubit 3.0, and the quality of RNA-seq libraries was checked on Agilent TapeStation. All RNA-seq libraries were sequenced on Illumina HiSeq 4000 at the Loma Linda University Center for Genomics, with 150 bpx2, Paired-End. Quality control was confirmed (Figure 13 and 14).
Figure 13. Quality Assessment Metrics for RNA-seq Data. Box plots representing interquartile range and median of (A) GC content (%) and (B) the Phred quality score distribution over all reads across all 12 samples in each base.
Figure 14. Quality Assessment on External RNA Spike-in Controls. (A) Percentage of reads mapped to genomic regions including exon, intron, and intergenic region. (B) Plot of log₂(FPKM) of ERCCs detected from samples spiked with ERCC Mix1 vs log₂(spike-in concentrations).
**RNA-seq Data Analysis**

For mRNA-seq data visualization and analysis, we utilized pipelines that integrated the QC (FastQC, ShortRead), trimming process (trimmomatic), alignment (Tophat2), reads quantification (cufflinks), and differentially expressed gene (DEG) analysis (cuffdiff) as described previously [77]. Briefly, the RNA-seq raw fastq data were first trimmed using Trimmomatic (V0.35). The trimmed reads were aligned to the human reference genome (NCBI GRCh38) with TopHat V2.1.1 with default parameter settings. The aligned bam files were then processed using Cufflinks V2.2.1 for gene quantification. Reads were then mapped to ERCC transcripts and quantified using TopHat V2.1.1 and Cufflinks V2.1.1 with default parameter settings. Genes with FPKM $\geq 1$ in all samples were used for DEG analysis. Differentially expressed genes (DEGs) were identified by Cuffdiff with FDR $> 0.05$, and fold change (FC) $> 2$.

Hierarchical clustering heat map and PCA of global genes for all cell lines were performed with “R” program (http://cran.r-project.org/) [78] and Partek Genomics Suite 6.6, respectively. GSEA (v3.0, Broad Institute) [79, 80], was performed to compare parental DTX-sensitive PC3 and DU145 with DTX-resistant DU145-DR and PC3-DR. Gene sets were obtained from published gene signatures in the Molecular Signatures Database v1.0 (MSigDB). Analysis was run with 1,000 permutations and a classic statistic. Normalized enrichment score and $p$-values were measured to find enrichments with statistical significance ($p<0.05$).
**Quantitative Reverse Transcription PCR (RT-qPCR)**

For confirmation of RNA-seq results, we selected specific genes for independent in-house validation of their differential regulation in DTX-sensitive vs. -resistant cell lines. Briefly, total RNA was extracted from cell lines using the RNAProtect reagent (Qiagen Cat# 76526) and the RNasy plus mini kit (Qiagen Cat# 74134). RNA (0.5 µg) was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad Cat# 1708891). Primer sequences for gene validation were commercially synthesized by Integrative DNA Technologies (IDT) (see Table 4). Quantitative polymerase chain reaction (qPCR) was performed on the MyiQ real-time PCR and CFX96 Touch Real-Time PCR (Bio-Rad) detection system using iQSYBR Green Supermix (Bio-Rad Cat# 170-8882) according to the manufacturer’s instructions. The cycling conditions were 95°C for 15 min, 95°C for 15s, 60°C for 60s for 35 cycles, followed by melt analysis from 60 to 95°C. Expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Samples were analyzed in at least four independent biological replicates performed experimentally in triplicates.
**Table 4.** Primer Sequences for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5’ to 3’)</th>
<th>Reverse Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPP4</td>
<td>CTCCAGAAAGACAACCTTGACCATTACAGAA</td>
<td>TCACTCATCATCTTGACAGTGCACTTTTAG</td>
</tr>
<tr>
<td>TSPAN8</td>
<td>TTGGCTTCTGTACCTGCTCTTTTTCACTTTCCCCTGTGG</td>
<td></td>
</tr>
<tr>
<td>NES</td>
<td>CTCCAAGAATGGAGGCTGTAGGAA CTCTA</td>
<td>CCTATGAGATGGAGCAGGCAAGA</td>
</tr>
<tr>
<td>DNAJC12</td>
<td>CAGACAAGCATCCTGAAAACCC</td>
<td>TCGCCAGTGGTCATAGCGGGC</td>
</tr>
<tr>
<td>FABP5</td>
<td>ACCCTGGGAGAGAAGGTTTGAGAAGA</td>
<td>TGTAAGGTTCAGACAGTCTGAGTTTT</td>
</tr>
<tr>
<td>BOP1</td>
<td>CCATGCCGAGTCTTTACAACCCACC</td>
<td>AGCAAACACGGCATCATCCATGACC</td>
</tr>
<tr>
<td>ABCC3</td>
<td>CTGTGCACACAGAAAAACCCCG</td>
<td>GGACACCCAGGACCATCTTTG</td>
</tr>
<tr>
<td>TGM2</td>
<td>TAAGAGATGCTGTTGGAG</td>
<td>CGACCCCTGGTAGATAAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGAGATCCCTCACCAGAAATCAA</td>
<td>TTCACACCCATGACGAACAT</td>
</tr>
</tbody>
</table>
**Immunoblotting Procedures**

Whole cell lysates were prepared and the protein concentration in the lysates was determined using the BioRad DC Protein Assay Kit (Cat# 5000112) to ensure equal loading of proteins per lane. Bands were separated by SDS-PAGE (NuPAGE 4-12%, Thermo-Fisher Scientific) followed by transfer to polyvinyl difluoride membrane (Millipore). Membranes were blocked with either 5% dry milk solution or 5% bovine serum albumin both prepared in TBS-T buffer (20 mM Tris-HCL, pH 7.6, 140 mM NaCl, 0.2% Tween 20) and probed with the following primary antibodies: Rabbit anti-LEDGF/p75 (1:1000, Bethyl Laboratories Cat# A300-848A), mouse anti-clusterin alpha chain (1:1000, Millipore Cat# 05-354), rabbit anti-MDR1/ABCB1 (1:1000 Cell Signaling Cat# 13342), rabbit anti-DPP4/CD26 (1:3000, Millipore Cat# MABF752), mouse anti-Nestin (1:1000 Millipore Cat# MAB5326), rabbit anti-DNAJC12 (1:500, Novus Cat# NBP1-57718), rabbit anti-FABP5 (1:5000; a kind gift from Marino De Leon, Loma Linda University, Loma Linda, CA), mouse anti-Snail (1:1000, Cell Signaling Cat# 3895S), mouse anti-Twist (1:200, Santa Cruz Cat# sc-81417), rat anti-Vimentin (1:8000, R&D Systems Cat# MAB2105-SP), rabbit anti-TGM2 (1:1000, Cell Signaling Cat# 3557), rabbit anti-BOP1 (1:1000, Bethyl Cat# A302-148A-M-1), mouse anti-E-cadherin (1:500, BD Biosciences Cat# 610182), or mouse anti-N-cadherin (1:200, Abcam Cat# ab12221). The mouse anti-TSPAN8 primary antibody was from Celine Greco and Claude Boucheix (1:2000) [81].

Following several washes with TBS-T, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Cell Signaling Cat# 7076 and 7074, respectively; goat anti rat,
Santa Cruz Cat# sc-2032). HRP-β-actin was utilized as a loading control (Cell Signaling Cat# 5125). After 2-hour incubation with secondary antibodies, the membranes were washed several times with TBS-T, and protein bands were detected by enhanced chemiluminescence (Thermo Fisher Scientific, Cat# 34580). Bands were quantified using Image J software (National Institutes of Health) and normalized to β-actin control. Samples were analyzed in at least 3 independent experiments using at least 3 biological replicates.

Bioinformatics Analysis of Oncomine Cancer Gene Microarray Database

For analysis of mRNA expression of genes of interest in PCa and normal prostate tissues, we selected 16 datasets from the Oncomine database (Compendia Biosciences; Ann Arbor, MI; www.oncomine.org). These datasets, derived from gene microarray analyses of PCa and normal prostate tissues, provide fold-change data for gene expression with \( P \) values calculated by Oncomine using Student’s \( t \)-tests. The Grasso dataset included 35 castration-resistant metastatic PCa, 59 localized PCa, and 28 benign prostate tissue specimens while the Varambally dataset included 6 hormone-refractory metastatic PCa samples in addition to 7 localized PCa, and 6 normal prostate samples. This allowed us to compare the transcript expression between these 3 categories of tissues in our genes of interest.

Tumorsphere Forming Assays

Cells were cultured in 6-well non-tissue culture treated plates at a density of 25,000 cells/ml, and suspended in F12K/RPMI supplemented with 1% knockout serum
replacement (Fisher Scientific Cat# 10828028), 20 ng/ml human EGF (Millipore Sigma Cat# E9644), 10 ng/ml human bFGF (PeproTech Cat# 100-18B), 0.1% of albumin solution 35% in PBS (Sigma Cat# 091M8416), 1% Pen-Strep, 0.1% insulin (Millipore Sigma Cat# 10516), and 0.1% selenium (Millipore Sigma Cat# 229865). After 24 hours the floating cells were collected and cultured in separate plates in the medium described above. Cells were left for 14 days adding or replacing medium as necessary to maintain growth. Images of cells were taken after at least 14 days post-plating using an Olympus IX70 microscope with phase contrast and Hoffman modulation contrast and equipped with a SPOT RT3 imaging system. Using phase contrast 4X images and Image J software, tumorsphere formation was quantified as percent area in at least four independent experiments.

Flow Cytometric Analysis of Stem Cell Markers, ALDH Activity, and Cell Death

Adherent PC3-DR and DU145-DR cells were cultured in monolayer to 80-90% confluency prior to collection for multicolor flow cytometric analysis of putative CSC markers. Cells were washed with PBS and harvested using a solution containing 0.25% Trypsin and 2.21mM EDTA (Corning Cat# 25-053-CI), followed by incubation in fresh fully supplemented RPMI medium containing 10% FBS for 30 minutes to allow for N-Cadherin and E-Cadherin recycling following enzymatic cleavage. In parallel, tumorspheres derived from PC3-DR or DU145-DR cells were collected and dissociated using 0.25% Trypsin/2.21mM EDTA solution, followed by neutralization with fresh fully-supplemented medium. Following the 30-minute recovery period, cells were then labeled with antibodies against CD44, CD24, N-Cadherin, E-Cadherin, or annexin-V for
15 minutes at room temperature (see Table 5 for antibody specifications). Cells were washed and resuspended in annexin-V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl$_2$; pH 7.4) and analyzed immediately on a MACSQuant Analyzer 10 equipped with violet, blue, and red lasers (Miltenyi Biotec). Post-acquisition data analysis was performed using FlowJo version 10.08.1 (BD).
Table 5. Antibodies Used for Detection of EMT and CSC Markers by Flow Cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Manufacturer Information</th>
<th>Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>V450</td>
<td>BD Biosciences Cat# 561292 Clone: G44-26</td>
<td>Violet</td>
</tr>
<tr>
<td>Annexin-V</td>
<td>FITC</td>
<td>Life Technologies Cat# V13242</td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>PE</td>
<td>BD Biosciences Cat# 555428</td>
<td></td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>PE-Vio770</td>
<td>BD Biosciences Cat# 56345 Clone: 8C11</td>
<td>Blue</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>PE-Vio770</td>
<td>Miltenyi Biotec Cat# 130-099-142</td>
<td></td>
</tr>
</tbody>
</table>
ALDH activity was detected using Aldefluor assay kit purchased from Stem Cell Technologies (Cat# 01700) and performed according to manufacturer’s instructions. Briefly, 2D and 3D cells were prepared and harvested as described above. 400,000 cells were resuspended in 200 µl of aldefluor buffer and 2 µl of aldefluor reagent to form the “test” sample (both provided). 200 µl of that text mix were then immediately transferred to another microcentrifuge tube containing 2 µl of DEAB reagent (provided) to inactivate the aldefluor reagent and become the “control” sample. Both the control and test sample were incubated for 45 minutes at 37°C. Samples were then centrifuged and resuspended in aldefluor buffer to be analyzed immediately on the MACSQuant Analyzer. Post-acquisition data analysis was performed using FlowJo version 10.08.1 (BD) with gates being drawn on the control DEAB+ samples for each cell line 2D and 3D.

Initial gates for intact cells using FSC-A/SSC-A light scatter and doublet discrimination using FSC-H/FSC-A profiles. Single-stained samples were used to define compensation matrices. Following compensation, dead cells were excluded based on annexin-V positivity and only live cells were assessed for putative CSC marker expression. Gate placements were defined using Fluorescence-Minus-One (FMO) controls using SSC-A versus marker of interest (Figure 15 for gating strategy and Table 5 for staining strategy for FMO detection). Data are presented as percent of live cells staining positive for each designated marker and are representative of at least 3 independent experiments.
Figure 15. Gating Strategy for Multicolor Flow Cytometric Analysis of CSC Markers in Cells Grown in Adherent (2D) or Non-adherent (3D) Conditions. Following compensation, gates were set for SSC-A versus marker expression based on Fluorescence-Minus-One (FMO) controls (inset).
Table 6. Fluorescence-Minus-One (FMO) Staining Strategy for Detection of CSC and EMT Markers by flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CD44</th>
<th>Annexin-V</th>
<th>CD24</th>
<th>N-Cadherin</th>
<th>E-Cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD44 Only</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Annexin-V Only</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD24 Only</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-Cadherin Only</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E-Cadherin Only</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FMO-CD44</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FMO-AV</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FMO-CD24</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FMO-N-Cadherin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FMO-E-Cadherin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Full Stain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Detection of Cell Viability by Propidium Iodide Staining

PC3 and DU145 cells, DTX-sensitive or -resistant, were seeded in 6-well cluster plates at 1.25x10^5 cells per well and allowed to adhere for 24 hours. Separately, PC3-DR and DU145-DR cells were seeded in non-adherent conditions in 6-well cluster plates at 5 x10^4 cells per well. Cells were then treated with increasing concentrations of DTX (0.1, 1, 10, 100, 1000 nM) for 72 hours, followed by PI staining using the Dead Cell Apoptosis Kit for flow cytometry (Life Technologies, Cat# V13242) according to the manufacturer’s instructions. Briefly, adherent cells were detached from culture using 0.25% Trypsin/2.21mM EDTA solution for 30 seconds, followed by neutralization using complete RPMI medium containing 10% FBS. Cells grown in non-adherent conditions were collected from culture medium and dissociated using 0.25% Trypsin/2.21mM EDTA solution for 30 seconds, followed by neutralization using complete medium containing 10% FBS. Cells were washed with PBS, suspended in annexin-V binding buffer and stained with PI (1µg/ mL final concentration) for 15 minutes at room temperature in the dark, then immediately analyzed on a MACSQuant Analyzer. Following exclusion of debris and doublet events, single-stained samples were used to define compensation matrices and experimental gates. Data are presented as percentage of cells staining negative for PI (percent viability) (see Figure 16 for gating strategy).
Figure 16. Gating Strategy for Flow Cytometric Analysis of Cell Death. Propidium iodide (PI) staining was analyzed in DU145 and DU145-DR cells grown in adherent or non-adherent conditions following 72 hours of exposure to DTX. Gates for PI were set using unstained controls on pooled samples (right inset).
**Statistical Analysis**

Statistical analysis and graph generation was performed using GraphPad Prism version 6.0c for Mac OSX (GraphPad Software, La Jolla, California USA, www.graphpad.com). Fold change differences in both qPCR, immunoblotting, Oncomine data, and tumorsphere percent area were analyzed using Student’s t-test. Results were considered significant at $P < 0.05$. One-Way ANOVA was used for the analysis of results from PI staining experiments comparing percent viability in the resistant 2D (adherent) compared to resistant 3D (tumorspheres) cultures.

**Acknowledgements**

We thank the Loma Linda University Center for Genomics and Dr. Wang’s group for the RNA-seq library constructions, sequencing and bioinformatics support. We also thank the members of the CHDMM and Casiano Laboratory who contributed to this work by providing technical or conceptual advice.
References


CHAPTER THREE
SELECTED UNPUBLISHED DATA

LEDGF/p75 May Play a Role in Regulating Inflammatory Cytokines Contributing to PCa Aggressiveness and Chemoresistance

Lens Epithelial Derived Growth Factor (LEDGF/p75) is an emerging stress oncoprotein implicated in the transcriptional regulation of survival proteins, contributing to cancer aggressiveness and chemoresistance [1-6]. Work done in our lab demonstrated that LEDGF/p75 is upregulated in clinical prostate tumors and contributes to taxane-resistance in PCa cells [7-9]. Furthermore, targeting of this protein with RNA interference partially resensitizes DTX-resistant cells to docetaxel [10]. The role of inflammation in PCa initiation and progression has long been established [11], with inflammatory cytokines such as IL-6 and IL6-R being implicated in PCa aggressiveness, chemoresistance, and increased serum levels of IL6 in PCa being associated with poor prognosis [11-14]. In addition, overexpression of LEDGF/p75 has been shown to induce IL-6 in human keratinocytes. We hypothesize that LEDGF/p75 plays a role in regulating inflammatory cytokines thereby contributing to PCa aggressiveness and chemoresistance.

To identify inflammatory cytokines regulated by LEDGF/p75 in PCa cells, we monitored changes in inflammatory gene expression using quantitative real time PCR pathway specific gene arrays, focusing on the RT² Profiler Human PCR Inflammatory Response and Autoimmunity Array (QIAGEN). Transient knockdown was done on PC3 cells, PC3-DR and DU145-DR cells. RNA was extracted 48-hours post siRNA
transfection. RNA was purified, cDNA was made and used to run the inflammatory arrays comparing each knockdown to the appropriate scrambled control.

LEDGF/p75 knockdown in DTX sensitive PC3 cells was associated with downregulation of IL-6 and IL-6R transcripts (Figure 17A& B). Consistent with this, Western blotting analysis showed increased cellular levels of IL-6 and IL-6R in response to LEDGF/p75 overexpression. These studies were repeated using both DTX-sensitive and resistant DU145 PCa cells. Our RT-PCR results also indicate that in the context of chemoresistance, depletion of LEDGF/p75 may play a role in the downregulation of inflammatory genes such as CCL5, CXCL2, IL-6, NF-kB, TIRAP, and TLR4 (Figure 17B). The most robust gene downregulation in response to LEDGF/p75 depletion in chemoresistant PCa cells was that of IL-6 (Figure 17B).

To further validate these preliminary results, it was important to use in-house primers for the determined genes of interest and immunoblotting to demonstrate upregulation in DTX-resistant cells and downregulation with knockdown of LEDGF/p75. Preliminary RT-qPCR data demonstrated upregulation of IL-6 in PCR-DR and DU145-DR cells (which overexpress endogenous LEDGF/p75) compared to parental PC3 and DU145 cells (Figure 18A& B). In addition, immunoblotting revealed increased expression of IL-6R in DTX-resistant and LEDGF/p75-overexpressing PC3 cells, and increased protein expression of IL-6 in DU145-DR cells (Figure 18C). While the upregulation of IL-6 protein in the PC3-DR and DU145-DR cells was consistent with the RT-PCR array results from LEDGF/p75-depleted cells, the IL-6R upregulation observed in these DTX-resistant cells was in stark contrast with that observed in LEDGF/p75-depleted cells (Fig. 17B). Furthermore, we were not able to show by immunoblotting the
downregulation of IL-6 protein in cells depleted of LEDGF/p75 (data not shown),
suggesting that either LEDGF/p75 does not regulate directly this inflammatory cytokine,
or the IL-6 protein levels in the DR cells are very stable and not totally dependent on transcript levels.
Figure 17. Inflammatory Gene Arrays Reveal Possible Target Genes of LEDGF/p75. A. siRNA-mediated knockdown of LEDGF/p75 in a panel of PCa cell lines. B. RT² Profiler RT-PCR inflammatory gene array results showing common candidate gene up- or down-regulated in DTX-sensitive and resistant PCa cell lines in response to LEDGF/p75 knockdown. C. The model suggests that when PCa cells transition from chemosensitive to chemoresistant the stress protein LEDGF/p75 is upregulated and may contribute to the upregulation of specific inflammatory genes. *Significant (p<0.05) Upregulation in Red and Downregulation in Blue.
Figure 18. Preliminary Validation of Inflammatory Array Results. A. RT-PCR data showing upregulation of IL-6 in the PC3-DR and DU145-DR PCa cell lines compared to the parental PC3 and DU145 cells. B. RT-PCR data showing upregulation of IL-6R in the DTX-resistant PC3 and DU145 PCa cell lines compared to the parental PC3 and DU145 cells. C. Western blot data showing that increased expression of IL-6 and IL-6R protein correlates with increased LEDGF/p75 expression in PC3-DR, DU145-DR, and LEDGF/p75 overexpression PCa cell lines compared to parental PCa cells.
Further development of these preliminary studies will help elucidate mechanisms by which LEDGF/p75-induced stress and inflammatory genes contribute to chemotherapy resistance in PCa (Figure 17). The potential role of LEDGF/p75 in co-regulating inflammatory cytokines also has important implications for understanding the role of LEDGF/p75 in healthy individuals and autoimmune disease. This is critical given recent observations that LEDGF/p75 is the target of autoantibodies in subsets of healthy individuals and patients with miscellaneous inflammatory or autoimmune conditions [15].

**Migratory Potential of DTX-resistant Cells Compared to DTX-sensitive Cells**

The ability for cancer cells to migrate and invade surrounding tissue is an indicator of their aggressiveness. Using techniques such as migration assays we are able to determine the effects of a drug or targeting of a gene on a cell line’s migratory potential. There is inconsistency in the literature on the effects of DTX-resistance on cell migration. Although DTX-resistant cells are more aggressive and have a mesenchymal-like phenotype we observed in scratch/wound assays reduced migration in DTX-resistant PC3-DR and DU145-DR cells compared to DTX-sensitive PC3 and DU145 cells (Figure 19). As shown in Chapter 2, when PC3 and DU145 cells become resistant to DTX they upregulate markers of EMT and CSC-like phenotype. As such, they migrate differently as individual cells, a phenomenon termed single-cell migration, which causes cells to detach individually and reattach at the center of the scratch/wound, versus migrating as a group of cells to close the wound [16].
Figure 19. Representative Scratch-wound Images with Graphs Showing Percent Wound Recovery. Results show that the DTX-resistant PCa cells (A, PC3-DR and B, DU145-DR) have less percent wound recovery compared to the DTX-sensitive cells (A, PC3 and B, DU145). Results were statistically significant (p<0.0001).
Co-targeting LEDGF/p75 and CLU Individually and Together in Combination with DTX to Resensitize DTX-resistant PCa Cells to Taxane Therapy

Current trends in cancer treatment emphasize combinatorial therapies targeting multiple redundant, but distinct, cell signaling pathways to more effectively sensitize cells to standard therapies [17]. Clusterin (CLU) is an anti-apoptotic protein that protects cells from stressors such as chemotherapy and androgen deprivation. It is upregulated in several cancers and is well defined as a key contributor to PCa resistance to taxane drugs [18-21]. Like LEDGF/p75, CLU is highly upregulated in the mCRPC cell lines PC3 and DU145 after progressive treatment with DTX, leading to induction of chemoresistance [18, 19, 21]. Downregulation of CLU also results in sensitization to DTX cytotoxicity in these chemoresistant cells [18]. Thus, LEDGF/p75 and CLU appear to play similar, if not redundant, roles in promoting taxane resistance in PCa.

Both LEDGF/p75 and CLU are stress survival proteins that are overexpressed in DTX-resistant PC3 and DU145 cell lines, and promote taxane resistance. Therefore, we first determined if their expression was interdependent. Western blot analysis showed that in PC3 cells with stable LEDGF/p75 overexpression, CLU was not overexpressed, compared to vector control, suggesting that overexpression of CLU in these cells may be independent of LEDGF/p75 overexpression (Figure 20A). In addition, siRNA-mediated knockdown of LEDGF/p75 in PC3 cells with ectopic overexpression of LEDGF/p75, and in DTX-sensitive and –resistant PC3 and DU145 cells did not result in downregulation of CLU expression (Figure 20B), again supporting the view that CLU expression is independent of LEDGF/p75 expression.
Figure 20. Knockdown Studies Reveal that CLU and LEDGF/p75 Expression are Independent. Both proteins are upregulated in DTX resistant (DR) cells (A) Knockdown of LEDGF/p75 in DTX-sensitive or resistant cells does not result in downregulation of CLU (B), whereas knockdown of CLU does not result in consistent downregulation of LEDGF/p75 (C).
Western blot analysis of protein lysates from DTX-sensitive and DTX-resistant PC3 and DU145 cell lines with CLU knockdown showed a slight downregulation of LEDGF/p75, which was not consistent in all cell lines (Figure 20C). This can be explained by the fact that CLU knockdown by itself causes apoptotic cell death, leading to caspase-mediated LEDGF/p75 degradation [22], which could explain the slight downregulation of this protein observed in Figure 20C.

Given that LEDGF/p75 and CLU exhibit redundant roles in promoting DTX resistance, through two distinct mechanisms (inhibition of caspase-independent cell death vs caspase-dependent cell death, respectively) [5, 18, 19], we sought to co-target these proteins to more effectively re-sensitize chemoresistant PCa cells to taxane therapy. We performed single or double siRNA-mediated knockdowns of CLU and LEDGF/p75 in the DTX-resistant PC3-DR and DU145-DR for 48 hours in the presence of 10 nM DTX (maintenance dose of DTX) and analyzed the morphology of the cells via Hofmann Modulation Contrast microscopy. As expected, double knockdown of LEDGF/p75 and CLU resulted in greater cell death than single knockdown of each protein (Figure 21). To quantify levels of cell death, cell viability was determined by MTT assay up to 96 hours (Figure 22A& B), and by Annexin/PI staining using flow cytometry (Figure 23A& B). Using both assays to assess cell viability after single and double knockdown of CLU and LEDGF/p75 in the presence of DTX, our results showed no difference in cell viability between the double knockdown and CLU knockdown, suggesting that the effects observed in the double knockdown are due largely to the effects of silencing CLU.
Figure 21. SiRNA-mediated Double Knockdown of LEDGF/p75 and CLU is More Effective in Resensitizing PC3-DR PCa cells to DTX Than Knocking Down Each Protein Alone. (A) Knockdown was verified by immunoblotting. (B) Double knockdown shows increased cell death compared to single knockdown of CLU or LEDGF/p75.
Figure 2. MTT Assay Reveals Double Knockdown of LEDGF/p75 and CLU Does Not Result in More Cell Death than CLU Knockdown Alone. MTT assay was done 48, 72, and 96 hours post knockdown. Experiment was repeated three times in triplicates with no significance found between si-CLU knockdown and siLEDGF/p75-siCLU double knockdown.
Figure 23. Flow Cytometry Reveals No Significant Increase in Cell Death in the Double LEDGF/p75 and CLU Knockdown Compared to Clusterin knockdown alone. AV/PI staining was done 72 hours post knockdown in the presence of 10 nM DTX. L3 refers to the si-LEDGF/p75 knockdown. This experiment was repeated two times.
Targeting LEDGF/p75 with SMIs to Resensitize Chemoresistant mCRPC Cells to DTX Treatment

In addition to its role in cancer, LEDGF/p75 also facilitates HIV-1 integration into transcriptionally active sites in host chromatin by interacting with the HIV-integrase (HIV-IN) [23]. Novel small molecule inhibitors (SMIs) targeting the HIV-IN binding domain (IBD) of LEDGF/p75, located within its C-terminus, have been developed [24] (Figure 24).
Figure 24. Model of LEDGF/p75 Structure Illustrating the IBD Domain. The IBD domain, a site crucial in transcriptional and stress survival activity and the predicted site of SMI binding.
These SMIs disrupt the LEDGF/p75-HIV-IN interaction, likely by binding to this C-terminal IBD, which is essential for HIV integration [23]. The transcriptional and stress survival functions of LEDGF/p75 also depend on the structural and functional integrity of its C-terminus [1, 22]. Therefore, we hypothesized that repositioning these HIV-based SMIs is a promising strategy to overcome mCRPC chemoresistance. SMIs were obtained from Enamine LLC designed by Sanchez, et al [24] DTX-resistant PC3 and DU145 cells were seeded in 96 well plates with and without DTX. 24 hours later different concentrations (0.1, 1, 10, 100 and 100 nM) of each SMI #14 (Cat# T5756746), 46 (Cat # T5863733), 71(Cat# T5251403), 91(Cat# T5755298), and 118 (Cat# T05189618) from Enamine LLC (Figure 25A) with and without DTX were added to each well in triplicates. After 72 hours MTT assay was performed. Preliminary data suggested that select candidate LEDGF/p75 SMIs #14, 46, 71, and 91 promote selective chemoresistance to 10 nM DTX in DTX-resistant PC3-DR and DU145-DR cells (Figure 26B& C). Concentration of 10 nM for each SMI is shown because that is where the change is first noticed and subsequent concentrations were similar (data not shown). Future studies will require determining the cytotoxic activity of these selected SMIs in the presence of various concentrations of DTX, establishing that these SMIs bind to LEDGF/p75, and that they disrupt LEDGF/p75 interactions with other proteins and transcriptional activity.
**Figure 25.** Top SMIs (# 14, 46, 71, 91, 118) that have Cytotoxic Activity in DTX-resistant PCa Cells Alone (gray bars) or in Combination with DTX. A. Chemical structure of inhibitors were designed by Sanchez, *et al.*, 2013. B& C. % Viability determined using MTT assay. Experiment shown is done with 10 nM SMI concentration and 10 nM DTX treatment is shown above. Experiment done two times. Gray bars represent SMI inhibitor treatment only, green or orange designates SMI treatment with DTX.
Adherent and Tumorsphere PC3-DR and DU145-DR Cells Upregulate CSC Markers CD133+ and CD117+

To better define the CSC population in the DTX-resistant cell lines, we performed flow cytometric analysis using the marker combinations of CD44+/CD24- [25-27], and CD44+/CD24-/CD133+ or CD44+/CD24-/CD117+ [26, 27]. CD133 expression is associated with tumor progression, self-renewal capacity, and metastasis colonization and growth [26]. CD117, also known as c-kit is associated with tumor progression, metastasis, and resistance to therapy [26]. We observed that PC3-DR cells showed a statistically significant increase of CD44+/ CD24- / CD133+ cells. DU145-DR cells also showed an increase of this subset, although not significant. Both PC3-DR and DU145-DR displayed a significant increase of CD44+/ CD24- / CD117+ cell subpopulations compared to the parental, drug-sensitive PC3 and DU145 cells (Figure 26).

In addition, we detected increased frequencies of CD44+, CD44+/ CD24- and CD44+/ CD24-/ CD133+ populations in DU145-DR 3D compared to DU145 3D tumorspheres (Figure 27A). Although we also observed an increase in the CD44+/CD24- /CD117+ population in DU145-DR 3D tumorspheres, this finding did not reach statistical significance (Figure 27B).
Figure 26. DTX-resistant mCRPC Cells Upregulate Markers of CSC-like Phenotype Compared to DTX-sensitive Cells, as Measured by Flow Cytometry. (A) CD44+/CD24+ and CD44+/CD24−, CD44+/CD24−/CD133+, and (B) CD44+/CD24−/CD117+. All flow measurements were acquired from at least 3 independent experiments, *P<0.05, **P<0.01. Error bars represent mean ± SD.
Figure 27. DU145 and DU145-DR Tumorsphere Cells Upregulate Markers of CSC-like Phenotype Compared to DTX-sensitive Cells, as Measured by Flow Cytometry. (A) CD44+/ CD24−, CD44+/ CD24−, CD44+/ CD24−/ CD133+, and (B) CD44+/ CD24−/ CD117+. All flow measurements were acquired from at least 3 independent experiments, *P<0.05, **P<0.01. Error bars represent mean ± SD.
References


CHAPTER FOUR
OVERALL DISCUSSION

Despite recent advances in the treatment of advanced PCa including Sipuleucel-T, abiraterone acetate, enzalutamide and CTX [1, 2], resistance to therapy occurs, resulting in patient death within three years of diagnosis [3-6]. DTX is the current gold standard for chemotherapeutic treatment of castration resistant tumors. Unfortunately, most patients receiving DTX experience taxane chemoresistance and disease progression within seven months of starting treatment [1, 2, 7]. Newer therapeutic agents such as CTX (also a taxane derivative) have demonstrated some overall survival benefit of slightly over a year [8]. Therefore, there is an unmet need for new therapeutic drugs used alone or in combination with current chemotherapeutic agents for the treatment of mCRPC. The goal of this study was to utilize RNA-seq to define molecular signatures associated with DTX-resistance in metastatic PCa cells in order to identify novel therapeutic targets for overcoming this resistance.

Examining the RNA-seq data, we observed that of the 33,118 total genes examined, 3,754 genes in the DU145-DR cell line had a significant fold change difference compared to the parental DTX-sensitive DU145 cell line, and 2,552 genes in the PC3-DR cell line had a significant fold change difference compared to the PC3 cell line. We chose to focus our studies on the 1,254 genes that were shared or overlapped between both PC3-DR and DU145-DR cells because these particular differences in gene expression are common to both DTX- resistant cell lines and likely result from long-term DTX treatment. GSEA analysis of the RNA-seq data revealed top ranked genes from the overlap data set, with many of these genes been associated with tumor aggressiveness,
chemoresistance, or a CSC-like phenotype. We selected some of the top upregulated genes in the DTX-resistant cells, including DPP4, TSPAN8, DNAJC12, and NES for validation using qPCR and immunoblotting. In addition, we validated FABP5 and BOP1, genes associated with PCa aggressiveness.

FABP5 is an intracellular lipid-binding protein that is emerging as a critical regulator of PCa cell proliferation [9, 10], and targeting FABP5 decreased malignant progression of CRPC cells in vivo [11]. Recent studies also suggest that FABP5 is a possible marker for aggressive PCa with FABP5 expression associated with high Gleason score [12]. Oncomine analysis demonstrates an upregulation of FABP5 expression in prostate tumors vs. normal, providing further evidence for the role of FABP5 in PCa. Considering the contribution of FABP5 to an aggressive phenotype in combination with our results demonstrating a robust transcript and protein fold upregulation in both PC3-DR and DU145-DR cells, FABP5 may be a promising target for the treatment of chemoresistant mCRPC.

BOP1 is an integral component of the ribosomal RNA processing machinery and a vital component of colorectal tumorigenesis through the promotion of cell migration and invasion. BOP1’s role in cell migration was demonstrated by the observation that its loss contributed to a regression of EMT and an increase in E-cadherin expression, suggesting it may be an upstream inducer of EMT [13, 14]. With our observation of increased EMT phenotype in the DTX-resistant cell lines, these results suggest that BOP1 may play a similar role in PCa by contributing to upregulation of EMT in PC3-DR and DU145-DR cells. In addition, the Oncomine analysis revealed an upregulation of
BOP1 transcript expression in prostate tumor vs. normal, and BOP1 transcript and protein expression was upregulated in DTX-resistant cell lines.

DPP4, also known as CD26, is a transmembrane glycoprotein that functions as an exopeptidase that can inactivate incretins, chemokines, and promotes cell migration through the upregulation of MMP-9 [15]. DPP4 is upregulated in many cancers including lung, T-cell lymphomas, thyroid, and prostate [16, 17], and plays a role in a CSC phenotype in colon cancer [18-20]. In addition, DPP4 contributes to upregulation of CD44, another CSC marker [15]. Furthermore, CD26+ colon CSCs derived from DTX-resistant colon cancer cells were found to form larger and more abundant colonospheres [18]. Consistent with the RNA-seq data, DPP4 transcript and protein fold expression was upregulated in the PC3-DR and DU145-DR cells compared to the sensitive PC3 and DU145 cells. This suggests that DPP4 may be a possible prostate CSC marker that identifies a chemoresistant phenotype.

TSPAN8, also known as Co-029 and TM4SF3, is a member of the tetraspanin family consisting of membrane proteins important in direct and indirect communication between cells by regulating integrins and other cell surface proteins [21]. Also robustly upregulated in the RNA-seq analysis (sensitive vs. resistant), this protein is positively correlated with metastasis and worse prognosis in esophageal [22], liver [23], and colon cancers, where it has been shown to be involved in cell adhesion and motility through a complex formed with E-cadherin [24]. In addition, TSPAN8 is considered a CSC marker in pancreatic cancer [25], and whole genome splicing-sensitive microarray analysis in PCa cells revealed an upregulation of DPP4 and TSPAN8 in DU145 CSC tumorsphere (3D) population compared to adherent DU145 cells (2D) [26]. This provides evidence
for the potential role of TSPAN8 and DPP4 upregulation contributing to a CSC phenotype. As with DPP4, TSPAN8 transcript and protein fold expression was upregulated in the resistant PC3-DR and DU145-DR cells.

DNAJC12, also known as Hsp40, is a member of the heat shock protein family and acts as a co-chaperone that can modulate the function of protein complexes. Little is known about the role of this protein in cancer but it may contribute to tumorigenesis [27, 28]. Oncomine data revealed consistent upregulation of DNAJC12 in PCa versus normal tissue. We report a possible role of DNAJC12 in PCa chemoresistance by demonstrating a transcript and protein upregulation of this protein. In addition, the DNAJ family of proteins have a suggested role as key regulators of CSC function, making them an attractive target for drug design [29]. DNAJC12 transcript expression has been shown upregulated in breast cancer stem cells compared to normal cells [30].

NES is a cytoskeletal intermediate filament protein that was first identified as a marker of neuroepithelial stem/progenitor cells in the brain and is associated with increasing migration in PCa cells and their expression is induced by androgen deprivation [31]. In many tumor types, increased NES expression is correlated with high tumor grade and is suggested to be an indicator of an invasive phenotype in breast, ovarian, gastrointestinal tumors, and melanoma, where NES is also a predictive marker of poor response to therapy [32]. NES was also found in PCa cells and tumospheres expressing stem cell markers including Nanog, Oct4, Sox-2, and CD133, and these tumorspheres were found to be more chemoresistant [33]. Similar to our studies, NES was also found to be increased in hepatocellular carcinoma drug resistant cell lines, and was associated with a mesenchymal phenotype [34]. In breast cancer, NES was found to promote
proliferation, survival, and migration of breast CSCs by enhancing the Wnt/β-catenin activation, a mechanism that may be at play in other cancer types [35].

Chemotherapeutics, like DTX, have temporary cytotoxic effects on the bulk tumor, due to the small population of CSCs with multiple redundant mechanisms that facilitate cell survival. In addition, the non-proliferative state of CSCs make them cellular reserves that allows for a small population of cells to persist even after anti-proliferative treatments and repopulate the tumor or metastasize [36]. With the identification of the emerging role of CSCs in the acquisition of PCa chemoresistance [6, 36-38], and with the observation that many highly-ranked genes were associated with a CSC phenotype or programming, we chose to characterize this population in DTX-resistant PC3-DR and DU145-DR cells and DTX-sensitive PC3 and DU145 cells to determine if CSC frequency was increased in the DTX-resistant cells.

CSCs are typically identified based on the presence and/or absence of several cell surface markers, the combination of which is specific for the CSC-like phenotype identified in a particular tumor type [32]. The observation that the PC3-DR and DU145-DR (2D) adherent cells upregulate CSC markers is consistent with the CSC hypothesis, where therapy resistance develops in part to a small population of cells that are inherently resistant and remain dormant during therapy treatment. When the majority of the bulk tumor is eradicated, this CSC-like population remains and is able to repopulate the area with cells that are resistant to treatment and upregulate proteins important in pathways maintaining and promoting metastasis [36]. In addition, the mesenchymal phenotype displayed by these resistant cells drives this CSC-like phenotype contributing to aggressive disease progression.
The link between EMT and CSC is largely unexplored, but recent evidence suggests that upregulation of EMT proteins and pathways results in a CSC-like phenotype including self-renewal and therapy resistance [39]. We report here that PC3-DR and DU145-DR cells have a higher frequency of cells expressing CSC-like markers, and higher ALDH+ activity. In addition, the DU145-DR 3D cells formed larger and more abundant tumorspheres compared to the DTX-sensitive DU145 3D cells, and these tumorspheres were more resistant to DTX showing increased viability with 10 nM DTX treatment.

DTX is currently the gold standard treatment for mCRPC and its use ultimately results in therapy resistance. This important work reveals genes that are upregulated upon DTX treatment and acquisition of resistance, and that most likely contribute to the resistance phenotype. Therefore, targeting the pathways involving these genes, including those responsible for CSC development, in combination with DTX is a promising strategy to circumventing chemoresistance.

Conclusions and Future Directions

Current targeting of CSCs focuses on the signaling pathways upregulated in stem cells that are specific to their function. These pathways include Hh, Wnt, Notch, and NFkB pathways [36], and targeting them has not resulted in any treatments for chemoresistant patients. This study contributes to the growing body of evidence linking CSCs to PCa chemoresistance [40, 41] and provides a set of genes for possible targeting that are implicated in contributing to this CSC phenotype. Many of the other genes highly ranked by GSEA analysis have roles in cancer and PCa as well as undefined functions,
suggesting that although CSCs or CSC-associated genes are promising targets for new treatments, there are many other pathways and mechanisms that are still largely undefined in this data set, including the role of FABP5 and BOP1 in chemoresistance. It will also be important to explore the cell type-dependent differences in PC3 and DU145 cells that exist between the resistant phenotypes of these cell lines, evident by the genes not overlapped or shared between them.

RNA-seq studies provide the framework for research aimed at elucidating new mechanisms and pathways associated with a DTX-resistant phenotype in order to discover novel targets for future PCa treatments. Future studies will involve targeting the top-ranked genes to determine their function in CSC acquisition or DTX-resistance. Targeting can be done using siRNAs, pharmacological inhibitors, SMIs or antibodies as is the case with TSPAN8 [42]. Promising candidates will have readily available inhibitors or validated antibodies that are used in other cancer types. The goal is to show that inhibition of this protein or pathway associated with the expression of these proteins, will lead to a disruption in tumorsphere formation, or resensitization of DTX-resistant cells. In vivo models will be crucial to determining the effect inhibition of these proteins on tumor volume, metastasis, and/or chemoresistance. Furthermore, future studies should also focus on validating our RNA-seq data in CSCs derived from PCa patients that developed taxane resistance. This will help us identify and prioritize more precisely candidate genes for therapeutic targeting.
References


CHAPTER FIVE

METHODOLOGY

The contents described in this chapter are for the purpose of instructing current and future laboratory members on the key methodologies utilized in this project. This section will provide key details and nuisances that are crucial to reproduce the experiments presented in this dissertation. Furthermore, these methods may assist in troubleshooting common setbacks that may occur when repeating these techniques for follow-up studies.

Cell Culture

The PC3 and DU145 cell lines used in this study were purchased from ATCC; please refer to the “ATCC Culture Methods” section on their website for specific protocols and required growing conditions. These cell lines were also authenticated through ATCC’s STR profiling service. This authentication step should be done before any major publication when using a cell line that was purchased a long time ago (no record remains) or came from another lab. Two additional cell lines were used, PC3-DR and DU145-DR, which were derived from PC3 and DU145 and passaged in the presence of docetaxel (DTX) chemotherapy to be drug resistant (DR). The protocol for generation these DTX-resistant cell lines is in the dissertation of Leslimar Ríos-Colón entitled “Targeting LEDGF/p75 to Sensitive Chemoresistant Prostate Cancer Cells to Taxanes” in the “Methodology” chapter. For this study, PC3, PC3-DR, DU145, and DU145-DR cells were thawed from frozen stocks obtained from the liquid nitrogen storage tank. Each frozen vial was prepared in freezing medium (Fetal Bovine Serum [FBS] supplemented
with 10% DMSO) and stored in a box labeled “Du Ross” in the large liquid nitrogen storage tank. T25 flasks filled with 5 mL of RPMI-1640 cell culture medium were pre-warmed in the incubator for at least 10 minutes prior to adding the cells. RPMI-1640 (500 mL) medium consisted of 10% FBS, 100 µl of gentamycin and 1 ml of normocin to prevent bacterial and fungal contamination. Frozen vials were thawed in the 37°C water bath (without submerging to prevent contamination), added immediately to pre-warmed T25 flask with 5 mL of medium, and placed in the incubator. Incubation conditions were 95% air, 5% carbon dioxide, at 37°C. Cells typically attach within 24 hours, at which point the medium should be changed to remove the DMSO present in the freezing medium.

Once cells were confluent, which could take between 24-72 hours depending on the cell density at the time of culture passaging (often referred to as splitting), they were expanded to a T75 flask. When cells are not confluent enough to passage (80-100%), medium should be changed every 48-72 hours. Be careful not to split cells at anything less than 60-70% confluent, since this will result in slower proliferation over time. Also be careful not to ignore cells for more than 72 hours, since this will result in stress pathways being upregulated due to serum starvation caused by over confluency and may alter the “normal” behavior of your cell line. If cells are left too long without splitting, they may start undergoing cell death so do not use them for experiments and thaw out a new vial, otherwise you may not get consistent results.

The PC3-DR and DU145-DR cells require the addition of 10 nM DTX to the medium each time the medium is changed, either during splitting or routine medium change. 10 mM DTX stocks are kept in the -20°C freezer and one stock is typically kept
in the freezer located in the cell culture room. Dilution steps are as follows: add 1 µl of 10 mM DTX to 99 µl of RMPI complete medium to make a 100 µM stock and label “A”; then take 10 µl of A and add to 90 µl of RMPI medium to make a 10 µM stock and label “B”; finally take 50 µl of B and add to 950 µl of RMPI to make a 500 nM stock and label “C”. Add 200 µl of C to a T75 flask with 10 mL RPMI media to get the final 10 nM DTX concentration required. Following these dilution steps will lead to more consistent DTX concentrations versus adding 1 µl of DTX to 1000 µl of RMPI and adding 10 µl of this to the T75 flask.

**Mycoplasma Testing**

It is crucial to test cell lines for mycoplasma contamination once thawed or at least every 6 months and use normocin antibiotics in the medium to prevent contamination. Some lab members do not use normocin in their medium, so their cells should be quarantined and kept in a separate incubator away from other cells in the lab. This is important to ensure that mycoplasma contamination does not occur, which is very difficult to eliminate. Any contaminated cell lines that cannot be easily acquired again will need to be treated with normocin for at least 6-8 months to rid of contamination. For this reason, normocin should be added to the media used for every cell line grown in the lab. Exceptions should be reviewed by the PI so that appropriate risk assessment can be made. In addition, cell lines that were not grown in normocin should not be frozen and used by the general lab, only by the individual working with them. If they are thawed for distribution to lab members, they need to be tested immediately and grown in medium containing normocin for maintenance and prevention of contamination. There is currently
no evidence that normocin usage will alter cell function or disrupt any selection process, with the exception of protocols that call for antibiotic-free media. Mycoplasma testing is done using the MycoAlert PLUS Mycoplasma Detection Kit from Lonza (Cat# LT07-701) following manufacturers’ instructions. Normocin is purchased from Invivogen (Cat# ant-nr-1).

**Preparation of Whole Cell Lysates**

To collect cells from flasks, medium was aspirated and the walls of the flask were rinsed with 2-4 mL of sterile PBS. The PBS was aspirated and 1 mL trypsin was added to the flask, which was placed in the incubator for a few minutes. Note that some cell lines, especially the parental PC3 and DU145 require more time in trypsin than the DTX-resistant cells, which detach from the flask relatively easily. If cells do not appear to be detaching, a gentle tap from the outside may be enough to detach the cells. Be careful not to leave trypsin on for too long or tap the flask too vigorously, some cell lines, DU145 in particular, are sensitive to vigorous tapping and may grow slowly or in clumps as a result.

Cells were collected in 10 ml plastic tissue culture tubes and stored short-term on ice. Cells were then spun for 4 minutes at 6,000 RPM (centrifuging speed may vary depending on cell line) in a centrifuge, and the supernatant was discarded. Pellets were transferred to a 1 ml microcentrifuge tube and washed two times with PBS to remove any residual trypsin and media. This step is crucial to removing albumin, a highly abundant protein in cell culture media that may interfere with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedures. The cell pellet was dissolved in Laemmli sample buffer containing a protease inhibitor.
cocktail (LSB:CPI). The amount of buffer added depended on the size of the pellet. It is important not to add too much buffer, which could dilute the protein concentration and make it difficult to load enough protein for immunoblotting. Conversely, adding too little LSB:CPI may result in adding very small volumes of the sample to wells in the gels, which may decrease loading consistency. This is because pipetting any volume under 1 µl reduces accuracy. Typically, 150-200 µl of LSB-CPI was added for a pellet derived from one confluent T75 flask and 30-100 µl for a pellet derived from one confluent well in a 6-well plate. Lysates may be stored in -80°C at this point, or they may be sonicated immediately. The lysates were sonicated to ensure proper disruption of cell membranes and chromatin and passed multiple times through a Hamilton syringe. The lysate was boiled in the 100°C heat block for 10 minutes. Lysates are stored permanently in the -80°C.

**Antibodies**

The commercial antibodies used in this study were as follows: Rabbit anti-LEDGF/p75 (1:1000, Bethyl Laboratories Cat# A300-848A), mouse anti-Clusterin alpha chain (1:1000, Millipore Cat# 05-354), rabbit anti-MDR1/ABCB1 (1:1000 Cell Signaling Cat# 13342), rabbit anti-DPP4/CD26 (1:3000, Millipore Cat# MABF752), mouse anti-Nestin (1:1000 Millipore Cat# MAB5326), rabbit anti-DNAJC12 (1:500, Novus Cat# NBP1-57718), rabbit anti-FABP5 (1:5000; a kind gift from Marino De Leon, Loma Linda University, Loma Linda, CA), mouse anti-Snail (1:1000, Cell Signaling Cat# 3895S), mouse anti-Twist (1:200, Santa Cruz Cat# sc-81417), rat anti-Vimentin (1:8000, R&D Systems Cat# MAB2105-SP), rabbit anti-TGM2 (1:1000, Cell Signaling Cat#
3557), rabbit anti-BOP1 (1:1000, Bethyl Cat# A302-148A-M-1), mouse anti-E-cadherin (1:500, BD Biosciences Cat# 610182), or mouse anti-N-cadherin (1:200, Abcam Cat# ab12221). The mouse anti-TSPAN8 primary antibody was a kind gift from Celine Greco and Claude Boucheix (1:2000).

**Immunoblotting Procedures**

Immunoblotting was performed as follows: equal amounts of protein from whole cell lysates were loaded into individual wells of 4-12% polyacrylamide gradient gels (SDS-PAGE, NuPAGE, Thermo Fisher Scientific). Protein concentrations were determined utilizing the DC protein assay kit from BioRad following the manufacturers’ instructions. To compare changes in protein expression, especially slight changes, only 5-10 µg should be loaded onto wells to avoid saturating the chemiluminescence signal. Typically, 15-20 µg is ideal for most antibodies, but titer or sensitivity of each antibody should be considered. Proteins were separated by SDS-PAGE and transferred onto polyvinyl difluoride membranes (PVDF) (Millipore) in a NuPAGE electrophoresis system from Thermo Fisher Scientific.

Protein sample preparation included 5 µl of NuPAGE LDS sample buffer (4X), 2 µl of NuPAGE reducing agent (10X), and cell lysate. Water was added to make a final loading volume of 20 µl. Protein separation was done in MOPS-SDS running buffer for 60 minutes at 175 volts. Protein transfer to PVDF membranes was done in diluted 1X Transfer Buffer (20X Transfer Buffer purchased from Invitrogen) with 100 µl of antioxidant added. To confirm complete protein transfer, membranes were rocked slowly at room temperature in Ponceau S stain for 10-20 minutes depending on the protein
concentration loaded. The membrane was washed gently in double distilled water until the amount of protein loaded in each well was clearly visible. Images were captured by cell phone camera, printed, and documented in the lab notebook. Membranes were then blocked in 5% milk prepared in TBS-T buffer (20 mM Tris-HCL, pH 7.6, 140 mM NaCl, 0.2% Tween 20) for 1-2 hours. Some antibodies required blocking with 5% BSA prepared in TBS-T. Blocking with either milk or BSA was done at room temperature on the rocker. All membranes used in these studies were then probed with primary antibody overnight in the cold room rocker. Not all primary antibodies require overnight incubation since some antibodies can be exposed to membranes for 2-4 hrs with excellent results. Thus, each new primary antibody must be optimized for milk vs BSA blocking buffer, overnight vs 2-4 hr incubation with membranes, appropriate dilution, and cell line-dependent target protein expression.

If multiple antibodies were used on the same membrane they would either be added on different days after developing the first one, or the membrane was cut based on molecular weight regions and each strip individually probed with the corresponding antibody targeting a protein migrating in a specific region. Alternatively one can probe the membrane simultaneously with two same species antibodies [rabbit, human, mouse] recognizing proteins in distant regions of the membrane (e.g 75 kD and 25 kD proteins) to prevent non-specific interactions or interference caused by mixing different species antibodies. This however, has never worked well in my experience for the antibody used in these studies and resulted in “smeared” and “fuzzy” bands. It is important to note that the amount of Tween-20 added to the 1X TBS may vary depending on the procedure. We used 0.2% Tween-20 for every experiment in this study, but other preferred
concentrations include 0.1-0.15% Tween-20 depending on the antibody. Using too much Tween-20 may wash away the antibody from the membrane or prevent tight antibody binding to target protein; using too little, however, may require extensive washing to get a clean, background-free membrane, which could also result in lower protein signal detection. For new antibodies, this may also need to be optimized.

Antibody dilution is different depending on the specific antibody and the expression levels of its target protein in different cell lines or tissue types. When using a new antibody, it is important to optimize the immunoblotting protocol to determine the appropriate dilution and protein concentration for each cell line. Following the manufacturer suggestions typically may not work but these can be used as a starting point to determine a dilution range for optimizing. When optimizing new antibodies, more concentrated dilutions (ranging 1:100-1:500) should be tested against increasing protein concentrations (5-30 ug) to determine optimal concentration. Antibodies should also be optimized for reactivity in 5% milk or BSA, unless either of these is specifically recommended in the manufacturer’s product specification sheet.

The membrane with primary antibody was washed three times with TBS-T on the rocker at room temperature, changing TBS-T every 10 minutes. After this, secondary HRP-conjugated antibody was added for 1-2 hours depending on the primary antibody and amount of protein loaded. Membranes were washed as before and enhanced chemiluminescence (ECL) was used to detect immunoreactivity. ECL was added to membranes in a wet chamber for 4 minutes, after which the membranes were transferred to autoradiography cassettes and exposed to autoradiography film for different time
period to get a range of exposure times that provide an accurate representation of the immunoreactivity.

Images were quantified using ImageJ software and statistical analysis of protein fold change, relative to beta-actin or tubulin loading controls, was done using PRSIM software. It is important to get overexposed and underexposed blots in addition to moderate exposure because this allows for an accurate interpretation of the strength of the immunoreactivity. In addition, getting the optimal exposure for both the protein band of interest and the actin or tubulin becomes crucial when bands are quantified using ImageJ. Bands that are too overexposed are difficult to quantify accurately since they are out of the linear signal range (i.e. the chemoluminescent signal is saturated). We used exposure times that allowed for clear visualization of spaces between adjacent bands in a gel and that showed undersaturated bands.

**RNA Extraction for RNA-sequencing**

It is important to ensure that RNA samples to be used for RNAseq studies are of the highest quality. When working with RNA, it is crucial to clean with ethanol and RNase Eliminator the workbench and any other surface or object that will come in contact with the RNA including pipettes, chemical bottles, centrifuge handles, etc. Apply the RNase eliminator directly to a clean paper towel after using ethanol. Do not add it directly to a surface or object, it is very dense and forms suds that take a long time to dry. Total RNA was extracted from prostate cancer cells using the miRNeasy Plus Mini Kit (Qiagen, Cat# 217004) per manufacturers’ protocol for “Purification of Total RNA from Animal Cells” found in the miRNeasy Mini Handbook (Qiagen website:}
Chloroform and 100% ethanol are not provided in the kit and must be purchased separately. Do not use the 190 proof ethanol used for cell culture, it is important to obtain 100% ethanol and be mindful that if the bottle is opened frequently, it may not retain the purity necessary for this protocol. Briefly, the protocol encompasses sample preparation that involved lysing of the cellular membranes, binding of RNA to the silica filter, washing of contaminants, and elution of pure RNA.

“Step 1b” and “Step 2” from the protocol were utilized for disruption of cells grown on monolayer. For Step 7, the centrifuge in the cold room was utilized for the 15-minute centrifugation and separation. In addition, all optional steps in the protocol to increase yield were followed (Steps 11 and 14). For Step 16, to increase RNA yield, 30 µl of RNase-free water was used to elude the RNA into the collection tube twice. This provided for maximum efficiency of the elution filter. Collected RNA was immediately put on ice in a clearly labeled tube and given to the LLU Genomics core for storage in their -80 freezer prior to RNA sequencing.

**RNA Extraction for Quantitative Real-Time PCR**

As with RNA extraction for RNAseq samples, the workspace must be cleaned thoroughly with ethanol and RNase Eliminator. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Cat# 74134) per manufacturers’ instructions for “Extraction from Animal cells” (Qiagen website: https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en). Briefly, cell samples are first lysed and then homogenized as
indicated in the instructions, adding ethanol to provide ideal RNA binding conditions. Lysates are then loaded into the RNeasy Mini spin column where RNA binds to the silica membrane and all contaminants are washed away using the buffers provided in the kit. Pure, concentrated RNA is eluded in RNase-free water (provided).

The first step requires RNAprotect Cell Reagent that can be purchased separately. For a T75 flask, 1 ml or RNAprotect was added, and for one well in a 6-well plate 500 µl were added. For homogenizing the lysate, “Step 5b” was done using a 1 mL syringe and a 25-gauge needle. As with the miRNeasy kit, all optional steps were followed. For “Step 14”, 30 µl of RNase-free water were used to elude the RNA into the collection tube twice. Before starting the procedure, 70% ethanol must be prepared and RLTPlus (2 mL) must be made using the RLT buffer provided and the beta mercaptoethanol (20 µL) stored in the 4°C fridge. Be sure to inspect the kit before use, and add the necessary components to each buffer. The lids of each bottle will specify what must be added and will allow for a check mark to be written if this step was done previously.

After extraction, RNA was quantified using the NanoDrop spectrophotometer. Be sure to look for A260/280 ratios of around 2.0, indicating that the sample is pure RNA. A low A260/280 ratio (below 1.8) is indicative of contamination with phenol, TRIzol, or other aromatic compounds that are used in the extraction process and may appear in the sample if the washing steps were not complete. Thus, the A260/280 ratio should always be as close to 2.0 for RNA, and around 1.8 for “pure” DNA.

Quantitative Real-Time PCR

The iScript cDNA synthesis kit (BioRad, Cat# 1708891) was used to reverse
transcribe RNA (0.5µg) into cDNA following manufacturers’ instructions. Use the concentration determined by the NanoDrop to calculate the RNA concentration required. Briefly, buffer mix was prepared for all reactions, adding in the following order (per reaction): 4 µl iScript Reaction Mix, 0.5 µg RNA template, 1 µl iScript Reverse transcriptase, and nuclease-free water to attain a final volume of 20 µl. Reaction was mixed thoroughly by pipetting up and down and added to a 96-well qPCR plate. The plate was sealed with adhesive film and placed in the thermal cycler with the following conditions: 5 mins at 25°C, 20 mins at 46°C, 1 min at 95°C, and hold at 4°C. cDNA is very stable and can be stored in the -20 freezer. In addition, from this point on, ethanol is sufficient for cleaning the workspace.

qPCR was done using the MyiQ real-time PCR detection system using iQ SYBR Green Supermix (BioRad, Cat# 1708882) following the manufacturer’s directions. Primer sequences are listed in Chapter 2, Table 4. Primers were commercially synthesized by Integrated DNA Technologies (IDT) and prepared adding 8 µl of the reverse and forward primers in 64 µl of nuclease-free water. For one reaction, 12.5 µl of SYBR green master mix, 1 µl of primer mix, and 10.5 µl of nuclease-free water were added together and placed on ice. Then, 1 µl of cDNA was added to the 96-well qPCR plate and 24 µl of the reaction mix was added to the corresponding well. Each primer had a different reaction mix. The cycling conditions were 95°C for 15 min, 95°C for 15s, 60°C for 60s for 35 cycles, followed by melt analysis from 60 to 95°C. Expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Samples were analyzed in at least four independent biological replicates performed in triplicates. Depending on the cell lines used and treatments being compared,
it may be necessary to optimize housekeeping genes since certain treatments may cause changes in transcript expression. For example, treating cells with dexamethasone or DTX may alter β-actin mRNA expression in some cell lines. Ensuring stable housekeeping gene expression between the control and test sample is crucial to getting reliable mRNA fold changes.

**Tumorsphere Forming Assays**

PC3, PC3-DR, DU145, and DU145-DR cells were cultured in 6-well non-tissue culture treated plates at a density of 25,000 cells/ml, and suspended in F12K/RPMI medium supplemented with 1% knockout serum replacement (Fisher Scientific Cat# 10828028), 20 ng/ml human EGF (Millipore Sigma Cat# E9644), 10 ng/ml human bFGF (PeproTech Cat# 100-18B), 0.1% of albumin solution (35% in PBS) (Sigma Cat# 091M8416), 1% Pen-Strep, 0.1% insulin (Millipore Sigma Cat# 10516), and 0.1% selenium (Millipore Sigma Cat# 229865). 50 mL of F12K medium (500 mL bottle) and 50 mL of RPMI medium (500 mL bottle) were removed inside the sterile hood and the EGF, FGF, and insulin were added outside the hood and filtered with a 10 mL syringe filter and added to the sterile media bottles. After 24 hours, the medium along with the floating cells were re-plated into a new 6-well non-tissue culture treated plate to remove any attached cells. Cells were left for 14 days and medium was added as needed. To visualize tumorsphere formation, images of cells were taken after 14 days post-plating using an Olympus 1X70 Microscope with phase contrast and Hofmann Modulation Contrast (HMC), equipped with a SPOT RT3 imaging system. Images were taken of multiple fields using 4X objective for quantification of % tumorsphere by area.
Flow Cytometric Analysis of Stem Cell Markers, ALDH activity, and Cell Death

Adherent PC3, PC3-DR, DU145, and DU145-DR cells were cultured in monolayer as previously described. Cells were washed with PBS and harvested using trypsin, followed by incubation in fresh fully supplemented complete RPMI medium for 30 minutes to allow for N-Cadherin and E-Cadherin recycling following enzymatic cleavage. Tumorspheres derived from PC3-DR and DU145-DR cells were collected in a 15 ml tube and dissociated using 500 µl-1000 µl of trypsin pipetting up and down vigorously for 2-5 minutes. Complete RPMI medium was added to the tube for recovery.

After a 30-minute recovery period, 1.0x 10^5 cells were plated in a 96 well plate skipping every other well on each side and labeled with antibodies against CD44 (BD Biosciences, Cat# 561292), CD24 (BD Biosciences, Cat# V13242), CD133 (Miltenyi, Cat# 130-098-142), CD117 (Miltenyi, Cat# 130-099-327), N-Cadherin (BD Biosciences, Cat# 555428), E-Cadherin (Miltenyi Biotec, Cat# 130-099-129) or annexin-V for 15 minutes at room temperature. Cells were washed and resuspended in annexin binding buffer and analyzed immediate on the MACSQuant Analyzer 10 equipped with violet, blue, and red lasers (Miltenyi Biotec). Post-acquisition data analysis was performed using FlowJo version 10.08.1 (BD).

Data analysis started with gating for intact cells by comparing forward scatter (FSC-A) to side scatter (SSC-A) and then doublet discrimination using FSC-H/FSC-A profiles. Doublet discrimination is important because it ensures that only single cells are counted and not two cells clumped together with an artificially enhanced signal. Single-stained samples were used to compensate negative matrices. Dead cells were then
compensated based on annexin-V positivity and only live cells were assessed for putative CSC marker expression.

ALDH+ activity was detected using an Aldefluor assay kit purchased from Stem Cell Technologies (Cat# 01700) following manufacturer’s instructions. 2D (monolayer) and 3D (tumorsphere) cells were prepared and harvested as described above in a microcentrifuge tube. 4.0x10⁵ cells were resuspended in 200 µl of aldefluor buffer (provided by the kit) and 2 µl of aldefluor reagent (provided) were added to form the “test” sample. 200 µl of the text mix were then immediately transferred to another microcentrifuge tube containing 2 µl of DEAB reagent (provided) to inactivate the aldefluor reagent and become the “control” sample. To get an accurate control reagent it is important to transfer the 200 µl to the DEAB reagent after less than 5-10 seconds because this stops the reaction and allows for an accurate baseline control. Both the control and test sample were incubated for 45 minutes at 37°C (in CO2 incubator). Samples were then centrifuged and resuspended in aldefluor buffer into round bottom glass cuvettes to be analyzed immediately on the MACSQuant Analyzer. Data analysis was done on FlowJo version 10.08 (BD) with gates drawn on the control DEAB+ samples for the negative gate and the positive gate was drawn on the sample predicted to have the most ALDH+ activity (PC3-DR, DU145-DR). This means that each cell line tested will have a different positive and negative gate.

Running the MACSQuant requires training and guidance from the Flow Cytometry Core, managed by Dr. Kimberly Payne’s Laboratory. Before using the machine be sure to get help from a lab member that has extensive experience running it to ensure it is set up properly and cleaned before and after use. The antibodies used in this
experiment were previously titrated by running a pilot with various concentrations (low and high) of antibody to determine which dilution falls within the voltage range. Therefore, it is not necessary to adjust the voltage of the antibody channels. It is necessary, however, to adjust the voltage for SSC/FSC to ensure that the cells are within the view screen. This is crucial for accurate analysis and is done using the Unstained sample. For ALDH analysis, it is necessary to adjust the voltage of the aldefluor gate using the control and test sample with the highest predicted ALDH+ activity to ensure that the cells appear within the view screen window.

Detection of Cell Viability by Propidium Iodide staining

PC3, DU145, PC3-DR, and DU145-DR cells were seeded in 6-well plates at $1.25 \times 10^5$ cells per well and allowed to adhere for 24 hours. Separately, PC3-DR and DU145-DR cells were seeded in non-adherent conditions in 6-well plates at $5 \times 10^4$ cells per well and treated with 10 nM DTX for 72 hours, followed by PI staining using the Dead Cell Apoptosis Kit for flow cytometry (Life Technologies, Cat# V13242). Adherent cells were detached from culture flasks or dishes using trypsin for 30 seconds, followed by neutralization using complete RPMI medium containing 10% FBS. Cells grown in non-adherent conditions were collected from culture medium and dissociated also using trypsin. Cells were washed with PBS, suspended in annexin-V binding buffer and stained with propidium iodide (PI) (1µg/ mL final concentration) for 15 minutes at room temperature in the dark, then immediately analyzed on a MACSQuant Analyzer. Analysis included identification of intact cells (exclusion of debris), doublet discrimination, and
compensation using single stained cells for negative and positive gates. Data is then presented as cells staining negative for PI (percent viability).

**Cell Migration Assay**

To evaluate migratory potential/response of PC3 and DU145 compared to their chemoresistant counterparts a scratch wound-healing assay was performed. Cells were seeded and grown to 100% confluency in 6-well plates in complete RPMI medium (with DTX added for the resistant cell lines). Three wound areas were generated per well by scratching three lines across the confluent monolayer cell surface with a 200 µl pipette tip. Be careful not to scratch too hard since this may damage the surface of the flask, preventing cells from migrating into the wound. Images of the wound areas were taken at 0, 24, and 48 hours using the Olympus 1X70 inverted microscope equipped with SPOT RT3 Imaging System using a 4X phase contrast or HMC objective. To determine the exact spot where the 0-hour image was taken for the subsequent time points, a circle was drawn on the plate lid. Percent wound recovery was determined using Image J software measuring 6 randomly drawn lines across the wound at each time point used.

**MTT Viability Assay**

PCa cells were seeded in 96-well plates at densities of 5x10^4 or 8x10^4 cells per well and allowed to grow for 24 hours. Cells were then treated with and without 10 nM DTX (in triplicates) in the presence or absence of LEDGF/p75 small molecule inhibitors (SMIs) for 48, 72 and 96 hours. In other experiments, single or double knockdowns of LEDGF/p75 or CLU were performed in PCa cells as described in Chapter 3. Cell
morphology was visualized in control and treated cells using the inverted Olympus IX70 microscope equipped with HMC and imaging system.

To assess cell viability, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich) was performed. This assay indirectly measures the activity of NAD(P)H dehydrogenases, which reduce the yellow MTT substrate into its insoluble formazan, which has a purple/dark blue color in viable cells. The activity of these dehydrogenases often correlates with cell viability and decreases during cell death. It can be also slowed down in cells that are not rapidly proliferating or are metabolically compromised, which could be wrongly interpreted as cell death. The MTT substrate was prepared in dPBS (1 mg/1 mL) and 25 µl was added to each well and placed in the incubator. After a max 2-hour incubation, the plate was spun down (to collect and accurately count all floating cells, which are typically derived from mitotic or dead cells). This centrifugation step is critical when treating cells with DTX and other taxane drugs, which arrest cells in mitosis leading to their detachment from the bottom surface of the plates without causing immediate death. After centrifugation the MTT/medium supernatant was pipetted off the wells (do not aspirate), 150 µl of DMSO were then added to each well, and the plate was placed on the shaker to dissolve the crystals into a colored solution. Absorbance of this solution was measured at 460 and 490 nm using a spectrophotometer. Values were normalized to the absorbance obtained for the untreated control cells. Given that the MTT reagent is light sensitive most of the steps were conducted in the dark.
Statistical Analysis

GraphPad Prism 6 was used for statistical analysis for these studies. Differences between each group (expression, viability, etc.) were analyzed using unpaired Student’s t-test. ANOVA was utilized when comparing multiple groups and P values below 0.05 were considered statistically significant (\( P < 0.05; \ \star \ P < 0.01; \ \star \star \ P < 0.001 \)).

Final Remarks

Additional protocols for experiments not shown in this dissertation, and specific details pertaining to each experiment can be found in the Christina Cajigas-Du Ross’ laboratory notebooks and electronic files stored in the Casiano Laboratory at Loma Linda University Center for Health Disparities and Molecular Medicine (Mortensen Hall rooms 102-103).