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14. ABSTRACT

The overall hypothesis is that expression of the dipeptidase DPP4 is downregulated in prostate cancer (PCa) as a mechanism of resistance to androgen deprivation therapy (ADT). My overall objective is to demonstrate that DPP4 downregulation is a mechanism of ADT-resistance and PCa progression and to identify the specific pro-survival growth factor/cytokine targeted by DPP4 for degradation and its associated signaling cascade. Aim 1 will assess the effect of DPP4 downregulation and overexpression on the sensitivity of PCa xenografts to castration. Aim 2 will identify the pro-survival growth factor/cytokine targeted by DPP4 for degradation and the downstream signaling cascades effected. Aim 3 will extend the significance of DPP4 downregulation into primary PCa and CRPC clinical specimens and assess the interaction of DPP4 inhibition with ADT.

15. SUBJECT TERMS

DPP4, CD26, prostate cancer, castration-resistance, androgen deprivation therapy, growth factor, cytokine

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1. INTRODUCTION:

When men develop prostate cancer (PCa) that spreads or metastasizes to other parts of their body, the first- and second-line treatments used by doctors attempt to block the effects of the androgen hormones testosterone and dihydrotestosterone (DHT) on prostate cancer cells. This type of therapy is called androgen deprivation therapy or ADT because it deprives the prostate cancer cells of these important androgens. Testosterone and DHT bind to the androgen receptor (AR) within cancer cells and stimulate the growth and progression of prostate cancer. Gonadotropin-releasing hormone agonists (GnRH-a gonists), abiraterone, and enzalutamide are three drugs commonly used to block the pro-cancer hormone signaling that occurs through testosterone and DHT. GnRH agonists inhibit the testicular production of androgen hormones. Abiraterone inhibits an enzyme called CYP17A1, which decreases the levels of testosterone and DHT. Enzalutamide blocks the ability of testosterone and DHT to bind to androgen receptor (AR) and stimulate prostate cancer cells. These drugs are initially effective at stopping prostate cancer progression, but in nearly all men the cancer eventually becomes resistant. The subject of this research project is to determine how downregulation of the gene DPP4, and its protein product mediates prostate cancer resistance to ADT and how DPP4 inhibitors used to treat Type II diabetes influence prostate cancer progression. Over the past 2 years I have shown that DPP4 downregulation is tightly associated with PCa progression in preclinical models and in clinical biopsy materials. In the VCaP xenograft model and in the majority of clinical cases, as PCa becomes resistant to ADT, AR signaling is restored. DPP4 is similar to PSA in that it is an AR-stimulated gene. However, in the resistant setting, while PSA expression is restored, DPP4 expression is not. This suggests that the continued downregulation of DPP4 might have functional significance in PCa survival, especially since DPP4 is known to degrade various pro-survival growth factors and cytokines. Of greater significance, I have also shown that inhibitors of DPP4 enzyme activity decrease the effectiveness of ADT. My overall hypothesis is that DPP4 expression is downregulated in PCa progression in order to increase local concentrations of prosurvival growth factors/cytokines to overcome androgen deprivation. As it will be difficult to identify therapies capable of increasing DPP4 protein expression within PCa cells, over the past 2 years of pandemic-restricted work, I have developed cell lines that have doxycycline-inducible overexpression and knockdown of DPP4 protein. The next important step in this work will be to use these cell lines in xenograft models to identify the pro-survival growth factor that is degraded by DPP4 and the kinase signaling cascade the growth factor activates to promote ADT resistance. This will allow us to target the growth factor and its associated receptor/kinase cascade directly to block ADT resistance. Unfortunately, the coronavirus pandemic has negatively impacted this xenograft work due to animal facility issues and supply shortages.

2. KEYWORDS:

DPP4, CD26, prostate cancer, castration-resistance, androgen deprivation therapy, growth factor, cytokine

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1: Assess the functional significance of DPP4 downregulation in PCa xenograft setting.
Milestone: Production of stable cell lines capable of inducible knockdown and overexpression of DPP4 (Month 4) – 100% Complete
Milestone: Establishment of VCaP xenografts, PC-BID-1, and LuCaP-35 PDXs, and VCaP xenografts with stable inducible nonsense and DPP4 shRNA and stable inducible overexpression of DDP4 (Month 10-11) – 90% Complete
Milestone: Assess the functional significance of DPP4 downregulation in the PCa xenograft setting (Month 15) – 50% Complete

Goal 2: Determine the signaling cascades effected by DPP4 downregulation/inhibition and the corresponding growth factors/cytokines targeted by DPP4 that are responsible for ADT resistance.
Milestone: Identification of signaling cascades effected by DPP4 downregulation/inhibition (Month 20) – 50% Complete

Goal 3: Determine the clinical significance of DPP4 expression and concurrent ADT/DPP4 inhibitor treatment on PCa progression. (Month 20) – 60% Complete

What was accomplished under these goals?

1. **Major Activities:** major focus over year 1of the award was to extend the preliminary results described in the grant application for the xenograft model systems regarding the interaction of DPP4 inhibitors with castration, develop stable cell lines with inducible shRNA and cDNA to DPP4, and analysis of DPP4 expression in clinical material with clinicopathological correlation. In order to extend the xenograft results, studies were performed in additional xenograft and PDX models on the interaction of sitagliptin treatment with castration on the development of castration-resistance. The development of a VCaP cell line stably expressing inducible shRNA to DPP4 was also completed. Finally, DPP4 immunohistochemistry (IHC) was optimized for FFPE clinical materials and DPP4 expression levels was analyzed in primary PCa, neoadjuvant-treated PCa, and metastatic CRPC. These results were published in manuscript form and are listed later in this report.

   The major focus of year 2 and the no-cost extension year 3 of the award has been the establishment of VCaP xenograft tumors that have doxycycline-inducible overexpression of either an shRNA against DPP4 or a cDNA for DPP4. Once established these xenografts were to be used to conduct experiments to assess the effects of DPP4 knockdown and overexpression on the efficacy of castration. In addition they were also to be used to determine which kinase signaling cascades are most clearly affected by the alteration of DPP4 expression in the xenograft setting using mass-spec phosphoproteome analysis of VCaP xenografts following overexpression of DPP4 in the castrate setting and knockdown of DPP4 in the intact setting. The dox-inducible VCaP cell lines stably expressing the cDNA against DPP4 were successfully established during year 2. The dox-inducible VCaP-shDPP4 cells were tested in xenografts in February 2020, immediately prior to the start of the coronavirus pandemic. Their response to doxycycline was good and the construct has been found to effectively knockdown DPP4 protein expression. Twenty of these VCaP-shDPP4 xenografts were started in order to test the effect of DPP4 knockdown on castration-sensitivity at the beginning of March 2020. However, the xenografts matured in early April 2020 during the initial stages of the coronavirus shutdown at our hospital and all the mice had to be euthanized by the beginning of May due to tumor size. The coronavirus shutdown has also delayed the use of the dox-inducible VCaP DPP4 cDNA overexpressing cell line in the xenograft setting.

The impact of the coronavirus pandemic on xenograft studies at our institution has been dramatic. Our animal facility has experienced significant labor issues that have resulted in restrictions on investigator experiments. Supplies of immunocompromised mice and matrigel have been in extremely short supply and we have experienced incredibly long backorders. These negative impacts of the coronavirus pandemic will be described in further detail later in this report.

2. **Specific Objectives:** My overall objective is to demonstrate the functional significance of DPP4 downregulation in mediating resistance to ADT in CRPC and identifying the mechanism by which this resistance occurs. My specific objectives include demonstrating the functional significance of DPP4 downregulation in preclinical models, identifying the specific growth factor/cytokine and associated signaling cascade that is upregulated in response to DPP4 downregulation, and correlating DPP4 expression/inhibition with PCa progression.

3. **Significant Results and Key Outcomes:**

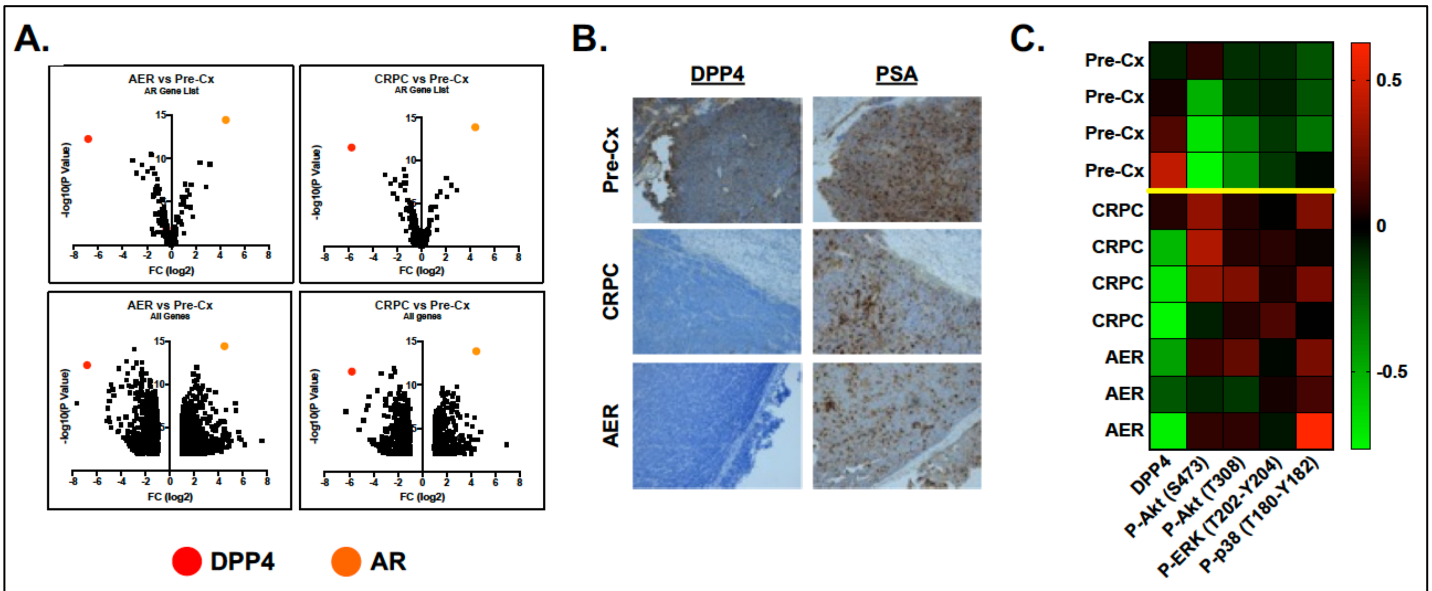
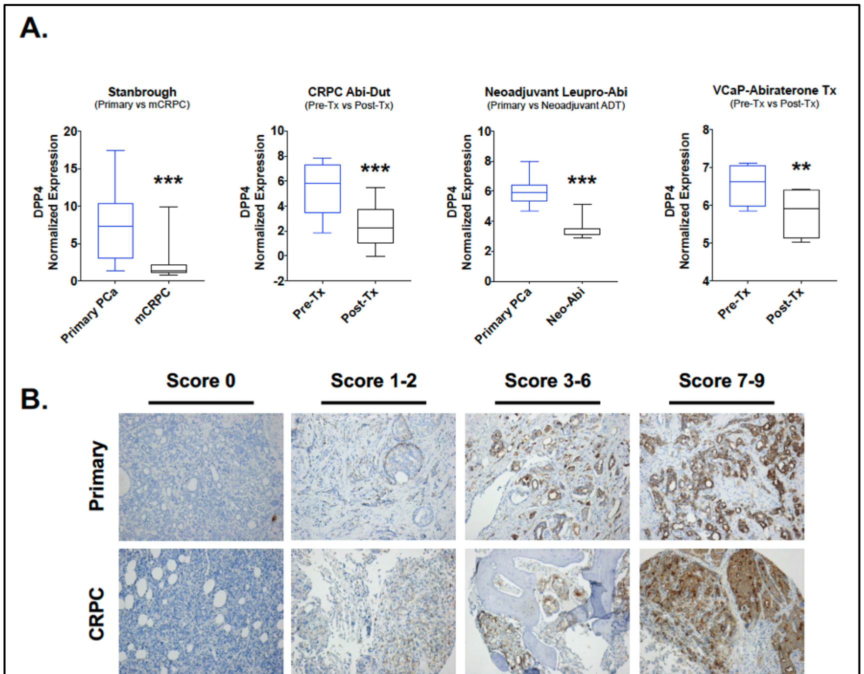


Figure 1 – DPP4 expression in AER VCaP xenograft serial biopsies. AER VCaP tumors were serial biopsied prior to cx (Pre-Cx), at tumor relapse (CRPC), and when tumors exceeded 2000mm³ (Abi/Enza resistant = AER). Initial cohort composed of 22 mice. **A)** Volcano plots of RNA sequencing data depicting AR target genes (267 AR gene signature from Mendiratta et al. PMID: 19289629) and all significantly differentially expressed genes (lower) in the AER vs. Pre-Cx and CRPC vs. Pre-Cx comparisons. The dots corresponding to *DPP4* and *AR* in each figure are highlighted. **B)** Representative images of DPP4 and PSA immunohistochemistry in serial biopsies of VCaP xenografts. **C)** RPPA data from the serial biopsies of 4 separate VCaP xenografts.

DPP4 is downregulated in PCa progression both in preclinical models and clinical specimens. *DPP4* is an AR-stimulated gene and would be expected to decrease with initial ADT, but similar to other AR-stimulated genes such as PSA, its expression should be restored with castration resistance. However, as outlined in preliminary data for this award, this does not occur and *DPP4* expression remains downregulated. To further assess DPP4 downregulation in PCa progression, I submitted serial biopsies of VCaP xenografts prior to castration (Pre-Cx), at castration-resistance (CRPC), and at resistance to combined abiraterone and enzalutamide therapy (Abi/Enza Resistant) for RNA sequencing. The results of this sequencing confirmed the decreased expression of *DPP4* as VCaP xenografts progress. Interestingly, *DPP4* was not only one of the most decreased AR-stimulated transcripts, but was also one of the most decreased of all transcripts **(Figure 1A)**. These decreases were confirmed at the protein level by immunohistochemistry (IHC) and reverse phase protein array (RPPA) **(Figure 1B-C)**. Extending these results into several clinical RNAseq data sets from our group, I found *DPP4* expression levels to be decreased in castration-resistant samples compared to matched treatment-naïve controls **(Figure 2A)**. Moreover, IHC on tissue microarrays (TMAs) showed a striking decrease in DPP4 protein levels in CRPC (Avg. Immunoscore = 1.25) when compared to primary PCa (Avg. Immunoscore = 6.49) **(Figure 2B)**.

These results establish a clear correlation between the downregulation of DPP4 and PCa progression. Extending the preliminary results found in the VCaP xenograft to clinical specimens is a key outcome. Further, showing that expression of the AR-regulated *DPP4* remains low when AR signaling is restored in CRPC is a significant finding. It suggests that the continued downregulation of DPP4 might have functional significance in PCa survival, and hints at the possibility of an altered AR cistrome in castration-resistance with functional consequences.



	Sample #	DPP4 Score 0	DPP4 Score 1-2	DPP4 Score 3-6	DPP4 Score 7-9	Avg. Score	Mann-Whitney U test
Primary	35	2	1	17	15	6.49	
CRPC	85	59	12	10	4	1.25	P = <0.0001

Figure 2 – DPP4 expression is decreased in ADT-resistant clininal specimens. **A)** RNA expression levels of *DPP4* obtained through Affymetrix microarray (Stanbrough) or RNA sequencing (CRPC Abi-Dut, Neoadjuvant Leupro-Abi, and VCaP Abiraterone-Tx) of material from several clinical and preclinical studies comparing Pre-ADT samples to resistant Post-ADT samples.*** = P<0.0001, ** = <0.001, limma for RNA sequencing studies and two-tailed student's t-test for microarray study. Abi – Abiraterone, Dut – Dutasteride, Leupro – Leuprolide. **B)** Representative images of DPP4 immunohistochemistry and DPP4 immunoscoring from a series of hormone-naïve primary prostate cancer (Primary) and CRPC clinical sections.

<u>Inhibition of DPP4 enzyme activity decreases the effectiveness of ADT.</u> DPP4 is a dipeptidase that is known to degrade many different growth factors and cytokines, including some that have been shown to promote prostate cancer survival/proliferation (IGF-1, NPY, SDF1-α). My hypothesis is that the decreases in DPP4 protein and associated decreases in DPP4 enzyme activity observed during castration-resistance result in increases in local concentrations of pro-survival growth factors that allow PCa to survive ADT. Given this hypothesis, inhibitors of DPP4 enzyme activity such as sitagliptin should mimic DPP4 downregulation and decrease the effectiveness of ADT. Preliminary studies treating VCaP xenografts that undergo castration with sitaglipitin supported this hypothesis. These results have now been extended into a genomically different PCa cell line (LNCaP), and more significantly into a hormone-naïve patient derived xenograft (PDX), BID-PC-1 **(Figure 3)**. In particular, the BID-PC-1 PDX is extremely sensitive to castration and can be "rescued" by DPP4 inhibitor treatment. These results are a key outcome as they show that the effects of DPP4 inhibition are penetrant across PCa with different genomic backgrounds, VCaP (AR amplification, TMPRSS2:ERG fusion), LNCaP (PTEN deficient), and BID-PC-1 (hormone-naïve, BRCA2 deficient). As we obtain additional PCa PDX models, I will continue to test the effects of DPP4 inhibition. DPP4 inhibitor treatment in these xenograft/PDX systems can also be used as another approach to identify the signaling cascades effected by DPP4 inhibition. Using this information we can then backtrack to the ligand DPP4 targets for degradation. This work also highlights significant implications regarding the interaction of DPP4 inhibitors used to treat Type II diabetes with the androgen deprivation therapies (leuprolide, abiraterone, enzalutamide, etc.) used to treat metastatic prostate cancer.

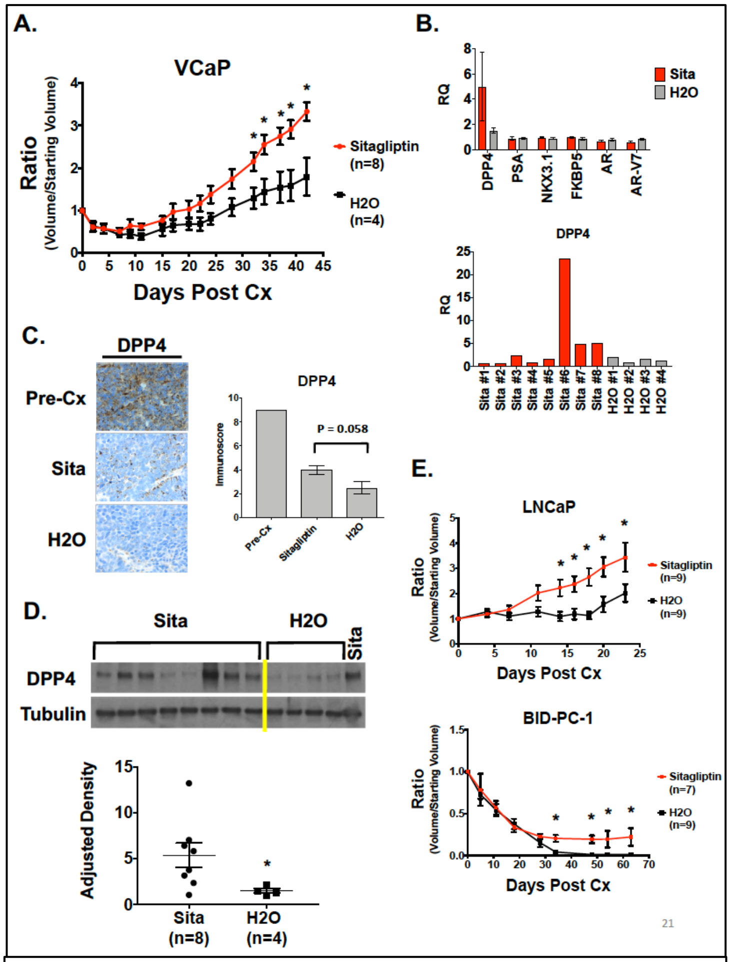


Figure 3 – DPP4 inhibitor increases VCaP tumor resistance to castration. A) VCaP subcutaneous xenografts were grown in intact male mice until tumors reached 500mm^3, then mice were castrated (Cx) and immediately began treatment with sitagliptin (120 mg/kg) or vehicle administered in drinking water. Y axis is the ratio of tumor volume at a given time point divided by the tumor starting volume. Bars = standard error of the mean (SEM). * = P<0.05, Mann-Whitney U. **B)** RT-PCR of *DPP4* and the AR regulated genes *PSA*, *NKX3*.1, and *FKBP5*, as well as the transcripts for AR and AR-V7 in VCaP xenografts harvested at Day 42 of the experiment represented in panel A. Each column represents the expression levels of xenograft tumors from eight mice (Sita) or four mice (H2O), with RT-PCR performed on each in technical triplicate. Bars represent standard error of the mean. H2O = water, Sita = Sitagliptin, RQ = Relative Quantification **C)** Representative high power images of DPP4 immunohistochemistry from Sita and H2O-treated tumors and DPP4 (left) and immunoscoring of DPP4 protein expression (right). P =0.058, Mann-Whitney U. **D)** Western blot of cell lysate from Sita and H2O-treated tumors probed with anti-DPP4 antibody (above) and densitometric quantification of bands (below). Bars = standard error of the mean (SEM). * = P<0.03, Mann Whitney U. **E)** LNCaP and BID-PC-1 subcutaneous xenografts were grown in intact male mice until tumors reached 500mm^3, then mice were castrated (Cx) and immediately began treatment with sitagliptin (120 mg/kg) or vehicle administered in drinking water. Y axis is the ratio of tumor volume at a given time point divided by the tumor starting volume. Bars = standard error of the mean (SEM). * = P<0.05, Mann-Whitney U.

<u>DPP4 downregulation correlates with negative risk factors in primary PCa.</u> DPP4 expression is strikingly decreased in the CRPC setting, but there is also a small fraction of primary prostate cancer that express little to no DPP4 **(Figure 2B)**. To further characterize DPP4 expression in the primary setting I performed IHC on a mixed set of 20 low-grade and 20 high-grade PCa specimens from radical prostatectomies of hormone-naïve patients. While DPP4 protein was still expressed in all primary tumors, high-grade PCa (Gleason Score 8-10) showed a clear reduction in DPP4 protein levels compared to low-grade (Gleason 6) **(Figure 4)**. Further, when I dichotomized the samples by the H-score immunoscoring system into high and low DPP4 expressers **(Table 1)**. I found correlations between low DPP4 levels and a number of negative risk factors including Gleason score > 8, increased tumor volume, higher stage, margin positivity, presence of extra-prostatic extension (EPE), Pre-RP highest Gleason score >8, and increased percent of biopsy core positive for cancer. It is not yet clear what role DPP4 downregulation would play in the primary setting where androgen is plentiful and there is no need for additional pro-survival growth factors and cytokines. However, these DPP4-low primary tumors might represent a more aggressive PCa subtype that have altered AR signaling and that are less likely to respond to ADT therapy if the patient develops metastatic disease. I am expanding these studies into intermediate grade PCa (Gleason 7) disease to assess if DPP4 levels can differentiate intermediate favorable from intermediate unfavorable PCa.

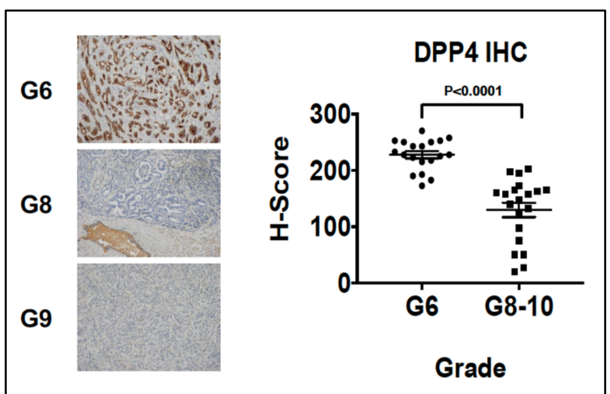


Figure 4 – DPP4 protein expression inversely correlates with tumor grade in hormone-naïve primary PCa. A) Immunohistochemistry for DPP4 protein in a selection of hormone-naïve primary prostate cancers. **B)** H-Score quantification of DPP4 IHC in 20 cases of low-grade and 20 cases of high-grade hormone-naïve primary prostate cancer. P<0.0001, Mann-Whitney U.

VCaP cell lines with doxycycline-inducible DPP4 shRNA and cDNA have been established and placed into the xenograft setting. A major goal of the first year of this award was the establishment of VCaP cell lines with doxycycline-inducible knockdown (shRNA) or overexpression (cDNA) of DPP4. Given my hypothesis that DPP4 is targeting specific prosurvival cytokines/growth factors for degradation, I reasoned that the best place to see alterations in the levels of these cytokines/growth factors and their signaling cascades was in the VCaP xenograft setting where I had already demonstrated that DPP4 activity levels have a significant effect. I have now established these VCaP cell lines and shown when they are induced, they specifically decrease or cause overexpression of DPP4 protein levels **(Figure 5)**.

Table 1

	H-Score >190 (N=20)	H-Score < or =190 (19)	P	Test
Mean H-Score	228.1	124.1	p<0.0001	Mann-Whitney U
Grade LGvsHG				
LG	17 (85)	3 (15)	p<0.0001	Chi X2
HG	2 (11)	17 (89)		
Mean Gleason Score	6.5	8.737	p<0.0001	Mann-Whitney U
Age	58.4	62.79	p=0.0473	Mann-Whitney U
Wt (gram)	48.35	53.37	p=0.1177	Mann-Whitney U
Tumor volume (%)	13.75	26.67	p=0.0079	Mann-Whitney U
pT Stage				
pT2	18 (90)	3 (16)	p<0.0001	Chi X2
pT3	2 (10)	16 (84)		
Margin				
Positive	2 (10)	11 (58)	p<0.05	Chi X2
Negative	18 (90)	8 (42)		
EPE				
Positive	1 (5)	15 (79)	p<0.0001	Chi X2
Negative	19 (95)	4 (21)		
Pre-RP PSA (ng/ml)	5.796	7.521	p=0.5567	Mann-Whitney U
Pre-RP Highest Gleason Score	6.529	8.22	p<0.0001	Mann-Whitney U
# of (+) cores	3.75	5	p=0.3099	Mann-Whitney U
Highest CA % in (+)cores	47.94	72.5	p=0.0124	Mann-Whitney U
Race				
White	13 (65)	13 (70)	for White vs Black p=0.6819, Chi X2	
Black	3 (15)	2 (10)		
Hispanic	1 (5)	1 (5)		
Asian	1 (5)	0		
Native American	1 (5)	0		
Unknown	1 (5)	3 (15)		

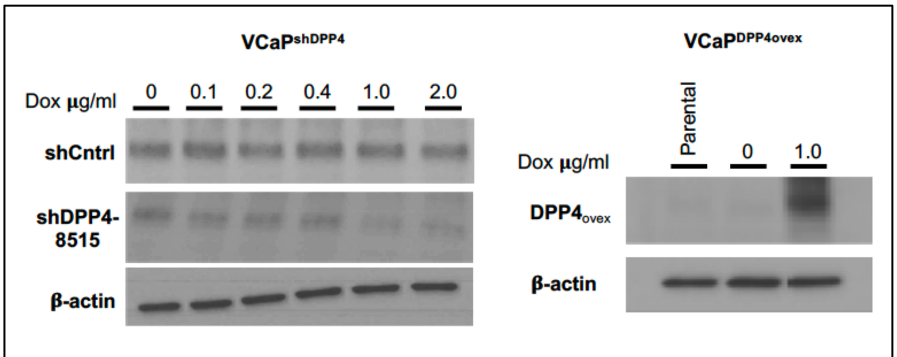


Figure 5 - Dox-Inducible VCaP cell lines stably expressing DPP4 shRNA and cDNA. Immunoblot for DDP4 and β-actin protein levels in whole cell lysates taken from $VCaP^{shCntrl}$, $VCaP^{shDPP4}$, and $VCaP^{DPP4ovex}$ cell lines. $VCaP^{shDPP4}$ cells show significant knockdown of DPP4 protein compared to $VCaP^{shCntrl}$ cells at 1.0-2.0 µg/ml doxycycline. $VCaP^{DPP4ovex}$ cells show significantly increased DPP4 expression compared to uninduced and parental VCaP cells. Dox – doxycycline.

These cells lines will be used to establish dox-inducible xenografts for use in experiments to determine the kinase signaling cascades effected by alterations in DPP4 expression and use this information to backtrack to possible cytokine/growth factors that DPP4 targets for degradation. Briefly, VCaP-shDPP4 and VCaP-DPP4 xenografts will be established in intact immunocompromised mice. VCaP-shDPP4 will be doxycycline-induced for 5 days to knockdown DPP4 protein expression and thereby upregulate the prosurvival cytokine/growth factors targeted for degradation by DPP4 in this setting as well as the kinase signaling cascade the cytokine/growth factor activate **(Figure 6A)**. VCaP-DPP4 xenografts will be castrated, then doxycycline induced to overexpress DPP4 and thereby cause increased degradation of the specific prosurvival cytokines/growth factors as well as the activity of the kinase signaling cascades they regulate. The specific kinase signaling cascades (and possibly the cytokines growth factors) will be identified by mass-spec and phosphoproteome mass-spec or alternatively reverse phase protein array (RPPA). I have established VCaP-shDPP4 xenografts and have successfully tested the ability of the construct to knockdown DPP4 protein levels, getting significant DPP4 protein knockdown with 7 days of doxycycline induction **(Figure 6B)**. A cohort of 20 mice (10 VCaP control-shRNA, 10 VCaP-shDPP4) were started in Feb. 2020 and matured in April 2020 during our hospital's coronavirus shutdown. Unfortunately, due to the moratorium on animal studies forced by the shutdown these mice had to be euthanized in early May due to tumor size restrictions, so the experiment was wasted. The same restrictions have thus far delayed the demonstration of DPP4 overexpression in the xenograft setting for the VCaP-DPP4 cell line.

NPY is a possible pro-survival growth factor in PCa increased by DPP4 downregulation. While stable cell lines are being developed, I also took a likely candidates approach to identifying the growth factor DPP4 targets for degradation and the possible signaling cascades upregulated by DPP4 downregulation. NPY has been shown to positively effect prostate cancer proliferation and is a well characterized degradation target of DPP4. IHC for NPY in serial biopsies of VCaP xenografts at Pre-Cx, CRPC, and Abi/Enza resistance show an increase in NPY protein levels that inversely correlates with DPP4 expression **(Figure 7)**. In reviewing the RNAseq data on these tumors, NPY transcript levels also increase with progression making it possible that the observed increase in NPY protein might be by transcription alone. However, increased NPY protein levels might be a product of increased transcription as well as decreased degradation (via DPP4 downregulation). The xenograft studies using inducible knockdown and overexpression of DPP4 will be helpful in dissecting this.

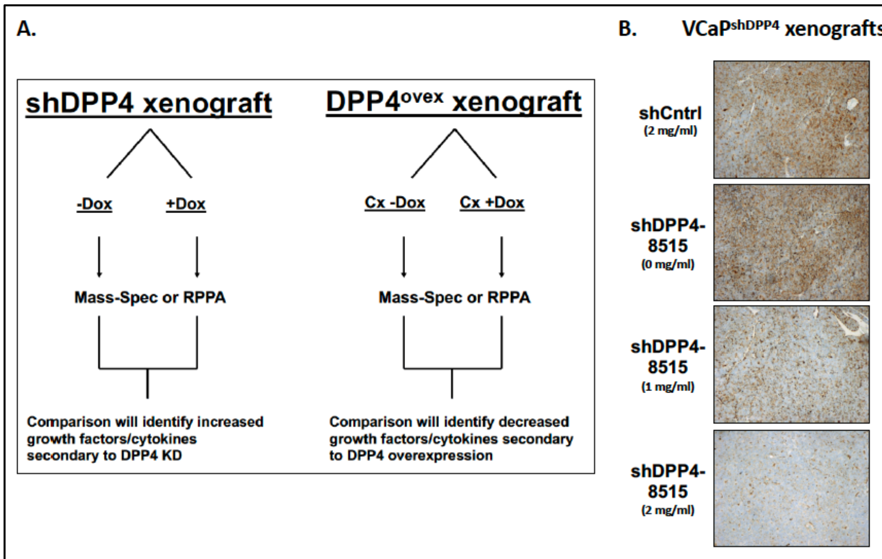


Figure 6 - Dox-Inducible VCaP xenografts. A) Schematic representation of experiments planned for the VCaP^{shDPP4} and VCaP^{DPP4ovex} xenografts. B) IHC for DPP4 protein in VCaP^{shCntr} and VCaP^{shDPP4} xenograft tumors following 1-2mg/ml doxycycline induction for 7 days. shDPP4-8515 is the same cell line labeled VCaP^{shDPP4} in Figure 1.

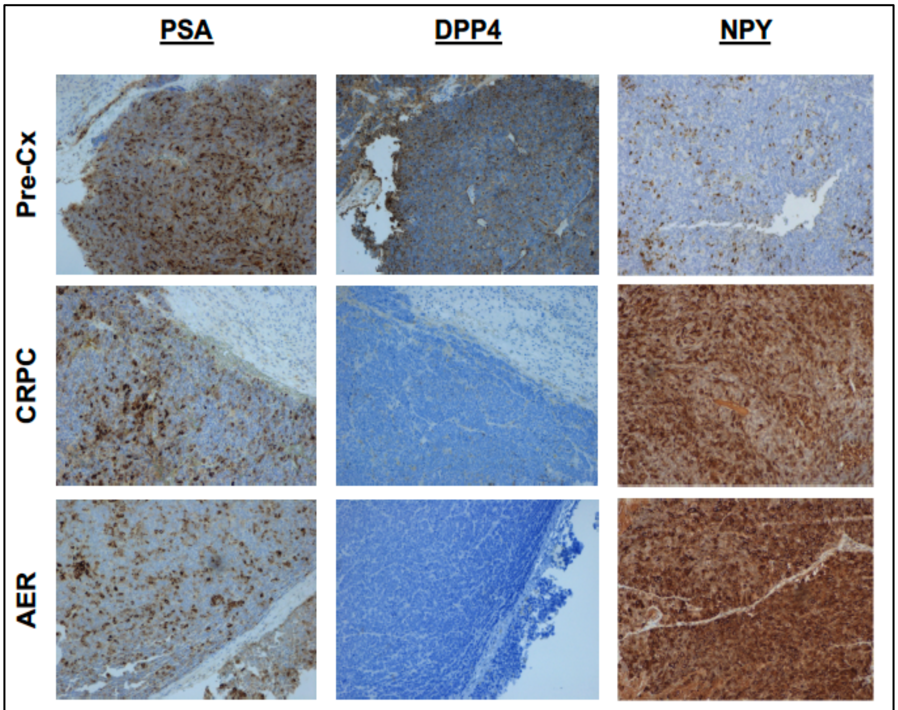


Figure 7 – DPP4 protein expression inversely correlates with NPY protein expression. Immunohistochemistry for PSA, DPP4, and NPY protein in representative serial biopsies of a VCaP xenograft at Pre-Cx, CRPC, and Abi/Enza resistance.

4. **Other Achievements:** Another group within our lab has significant interest in determining the characteristics of early prostate cancer that would make these tumors vulnerable to newly developed immune checkpoint blockade therapies, including anti-PDL1/PD1 therapies. They are working to characterize the "hot immunophenotype" of prostate cancer that will respond to anti-PDL1/PD1 therapies and have recently published a manuscript with their findings (Einstein et al. *Clin Cancer Res*, 2021, PMID: 34168052). It is well known that DPP4, also known as CD26, is expressed on a number of immune cells and can have immunomodulatory effects on T-cells and antigen presenting cells. During my own studies to determine the clinical significance of DPP4 expression and concurrent ADT+DPP4 inhibitor treatment on PCa progression, I found several primary prostate cancers where there were significant foci of tumor infiltrating lymphocytes (TILs) in areas where there was little tumor DPP4 expression by immunohistochemistry (IHC), while adjacent areas of DPP4 positive tumor had no TILs **(Figure 8)**. Further, interrogation of a primary prostate cancer immunophenotype TMA from the Einstein et al. study containing 46 immunologically "hot" tumors and 24 immunologically "cold" tumors showed that DPP4 is significantly decreased in the hot tumors **(Figure 9)**. DPP4 has been shown to degrade a number of stimulatory/chemotactic T-cell cytokines, including CXCL9, 10 11, and 12. Therefore, the downregulation of DPP4 in certain primary prostate cancers might make them more susceptible to T-cell infiltration and immunoresponse (ie "hot" tumors). In addition, DPP4 binds adenosine deaminase, an enzyme that converts the potent T-cell inhibitory nucleoside adenosine to inosine and this could have further immunomodulatory effects. To assess DPP4's role in immune modulation

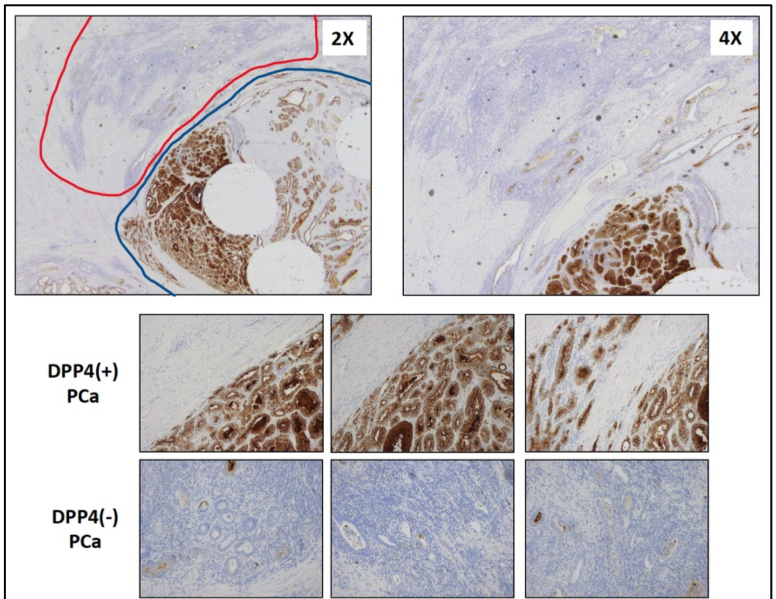


Figure 8 - DPP4 downregulation in tumors associates with TILs. IHC for DPP4 protein expression in adjacent foci of primary prostate cancer. (Upper) Low power images. Red area – foci of primary prostate cancer with no DPP4 staining and significant lymphocyte infiltration. Blue area – adjacent foci of primary prostate cancer with high DPP4 expression and low/absent lymphocyte infiltration. (Lower) Higher power images of DPP4 positive (+) and negative (-) foci.

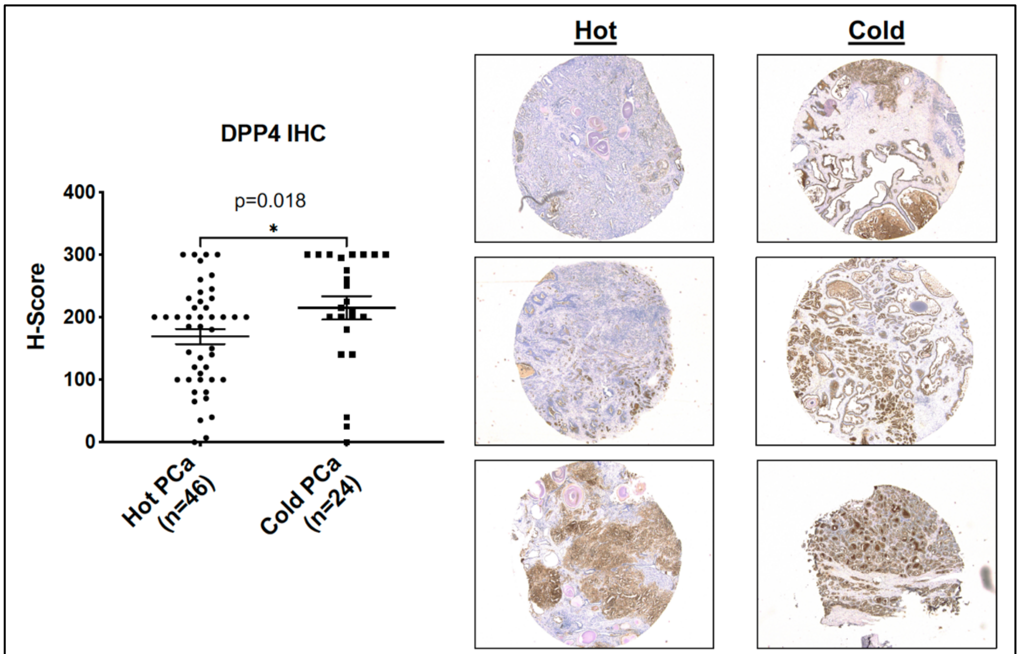


Figure 9 - DPP4 expression in "hot" and cold" primary prostate cancer. IHC for DPP4 protein expression from a TMA of immunophenotypically "hot" and "cold" primary prostate cancer as described in Einstein et al., PMID: 34168052. There were 46 samples of "hot" prostate cancer and 24 samples of "cold" prostate cancer. The DPP4 staining intensity of hot and cold tumor samples was determined using H-score (left). Representative images of DPP4 IHC in selected TMA spots (right). PCA – prostate cancer.

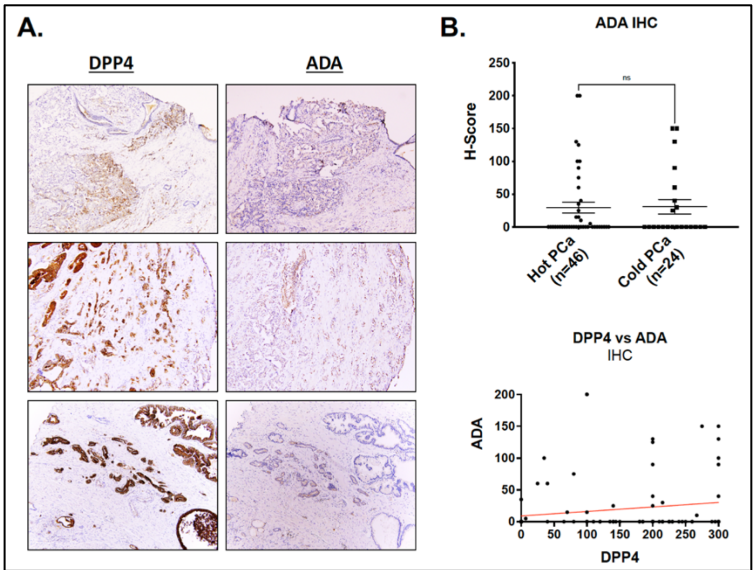


Figure 10 – ADA expression. IHC for DPP4 and adenosine deaminase (ADA) protein expression from a TMA of immunophenotypically "hot" and "cold" primary prostate cancer as described in Einstein et al., PMID: 34168052. There were 46 samples of "hot" prostate cancer and 24 samples of "cold" prostate cancer. **A)** Representative images of DPP4 and ADA IHC in selected TMA spots. **B)** *Upper* The ADA staining intensity of hot and cold tumor samples was determined using H-score. *Lower* Comparison of ADA and DPP4 staining intensity on a per TMA spot basis. Red line depicts linear regression.

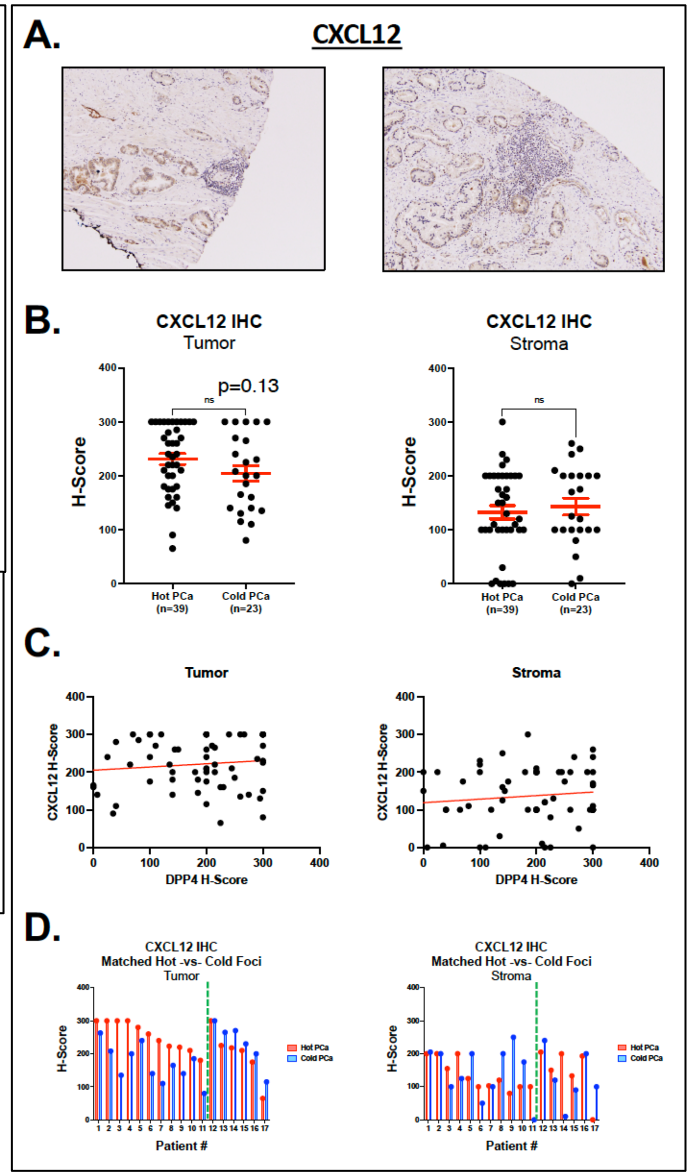


Figure 11 – CXCL12 expression. IHC for CXCL12 from a TMA of immunophenotypically "hot" and "cold" primary prostate cancer as described in Einstein et al., PMID: 34168052. There were 46 samples of "hot" prostate cancer and 24 samples of "cold" prostate cancer. **A)** Representative images of CXCL12 expressed in tumor near foci of tumor infiltrating lymphocytes. **B)** CXCL12 staining intensity of tumor and adjacent stroma from hot and cold tumor samples determined using H-score. **C)** Comparison of CXCL12 and DPP4 staining intensity on a per TMA spot basis. Red line depicts linear regression. **D)** Comparison of CXCL12 staining intensity in tumor and stroma between "hot" and "cold" tumor foci from the patient. Green dotted line demarks those patients with increased tumor CXCL12 levels in "hot" tumor compared to "cold".

in PCa, I have begun studies to correlate DPP4 expression in primary PCa with expression of key immune-modifying proteins. Using the TMA of both "hot" and "cold" primary prostate cancer described in Figure 9, I conducted IHC for adenosine deaminase (ADA) and CXCL12. ADA protein expression was not significantly different between "hot" and "cold" tumors and showed only a weak correlation with DPP4 protein expression **(Figure 10A-B)**. In the case of CXCL12 **(Figure 11A)**, there was a trend towards lower CXCL12 expression in "cold" tumors compared to "hot" tumors (but not stroma) which would be consistent with CXCL12's chemotactic effects **(Figure 11B)**. However, there was not a negative correlation between DPP4 expression and CXCL12 expression, as would be expected if DPP4 regulates CXCL12 expression by degrading CXCL12 **(Figure 11C)**. Interestingly, when CXCL12 expression is broken down on a per patient basis comparing multiple "hot" and cold" foci of tumor from the same patient, more than half the patients sampled showed less CXCL12 in the cold foci **(Figure 11D)**. While DPP4 does not appear to be a regulator of CXCL12 protein levels, this data does indicate that increased CXCL12 expression by the prostate cancer itself might be associated with a "hot" immunophenotype. While not part of this award, the immune-oncology group in our lab is now including DPP4 and CXCL12 in their panel of IHC markers they are using to characterize the "hot immunophenotype" of clinical prostate cancer specimens. In conjunction with these studies, I have also performed IHC for DPP4 on several prostate cancer GEMM models including PTEN$^{-/-}$, high-Myc, and P53$^{-/-}$ **(Figure 12)**. If we decide to move into testing immune modulatory functions of DPP4 in PCa, these in vivo models will likely be used due to GEM models having intact immune systems. Of interest is the finding that DPP4 is expressed at very high levels in mouse prostate stromal cells in all models examined, while its expression decreases in the developing prostate cancer, (similar to what is observed in humans). Also of interest is the finding that unlike the PTEN$^{-/-}$ and high-Myc models, in p53$^{-/-}$ mouse prostate cancer, DPP4 appears to be ubiquitously expressed within the tumor. These studies are not a focus of the research conducted in this award and none of these studies were or will be conducted using funds from this award.

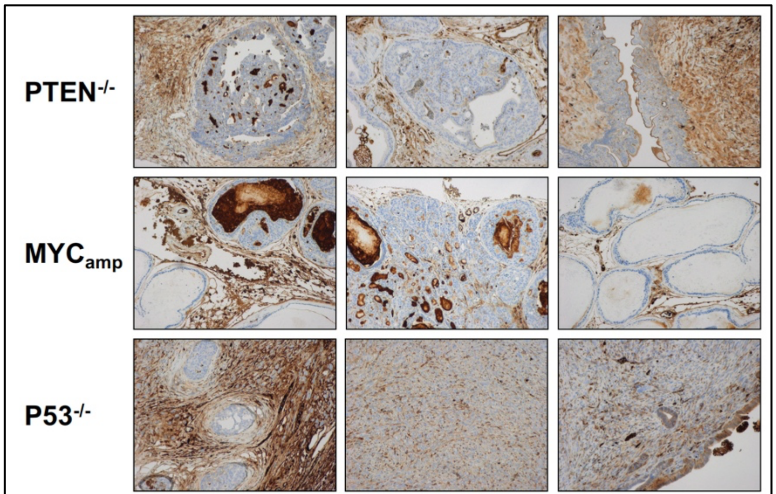


Figure 12 - DPP4 expression in GEMMs. IHC for DPP4 protein expression in genetically modified mouse models (GEMMs). Images of 3 separate tumors each for the PTEN$^{-/-}$, MYC_{amp} (high-Myc), and p53$^{-/-}$ models.

5. **Stated Goals Not Met**: Goal #1 to establish large numbers of xenografts with Dox-inducible expression of a shRNA or cDNA to DPP4 and Goal #2 to determine the signaling cascades effected by DPP4 downregulation/inhibition and the corresponding growth factors/cytokines targeted by DPP4 that are responsible for ADT resistance have not yet been met. The dox-inducible VCaP^{shDPP4} and VCaP^{DPP4ovex} xenografts are the major means by which the studies in both of these goals will be achieved. Unfortunately, animal experiments have been one of the areas of research most negatively affected by the coronavirus pandemic induced lock-downs, animal husbandry labor shortages, xenograft supply shortages (matrigel) and subsequent investigator animal housing restrictions at my institution. Using these xenografts in the experiments outlined above in combination with mass-spec phosphoproteome methods to identify signaling cascades effected by DPP4 downregulation/inhibition will the primary focus of my research efforts moving forward. It is my express hope that the negative effects of the pandemic will lessen over the next year to allow completion of this work. Goal #3 to determine the clinical significance of DPP4 expression and concurrent ADT/DPP4 inhibitor treatment on PCa progression is continuing to proceed. Given the known immune modulatory functions of DPP4, I am extending these studies into the immune-oncology setting.

What opportunities for training and professional development has the project provided?

Through this project I have had extensive one-on-one work with my mentor, Dr. Steven Balk. This includes a weekly progress report style meeting. I also present my work every 6 weeks to the lab group as a whole and once a year to the BIDMC Cancer Center as a whole as part of a "Work in Progress" seminar.

How were the results disseminated to communities of interest?

Results from these studies have been published as a manuscript in the journal Cancer Research (PMID: 30242112) and were presented at the Gordon Research Conference for Hormone-Dependent Cancers in August 2019. They were also accepted for a poster presentation format at the American Association for Cancer Research (AACR) Special Conference on Advances in Prostate Cancer Research that was to be held in March 12-15, 2020 (conference cancelled due to the coronavirus pandemic).

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

The results of the studies conducted through this award have demonstrated that the androgen receptor (AR) regulated gene DPP4, shows differential regulation when AR signaling is restored in castration-resistant prostate cancer (CRPC). This adds to the growing knowledge in the field of prostate cancer that AR signaling has different epigenetic drivers in the primary vs. castration resistant settings. These studies also underscore the importance of DPP4 expression and enzyme activity to castration-resistance in prostate cancer, a novel finding not yet previously reported. Finally, these studies demonstrated a detrimental interaction between castration and DPP4 inhibitor treatment (commonly used to treat Type II diabetes). This is an important finding suggesting that diabetic men undergoing ADT for their metastatic prostate cancer should not be treated with DPP4 inhibitors.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

The coronavirus pandemic continued to have a major impact on my research productivity through July 2020 to July 2021. Beth Israel Deaconess Medical Center (BIDMC) lifted lab occupancy restrictions on August 6^{th}, 2020. However, in the following months with the resumption of work there were severe lab supply shortages (cell culture plastics, media, reagents, buffers, etc.) that continued to curtail productivity. This shortage also extended to ordering immunocompromised mice and due to decreased staffing in our animal facility restrictions were placed on the number of mice our lab group could house at a given time. Starting in late October 2020, Massachusetts experienced a significant surge in coronavirus cases that lasted through March 2021. Despite this surge in cases BIDMC did not reinstitute any COVID safety measures with the exception of continued mask use. By December state-wide case numbers had reached very high levels and BIDMC mitigation levels were non-existent. In addition, lab supplies continued to be in extreme shortage. For these reasons, I began to work from home starting in late December 2020 and continuing until late March 2021 when I was able to get vaccinated.

I have been back at work full time since April 2021, but my work continues to be hampered by shortages and back-ordering of supplies. In recent months our animal facility has developed significant issues due a shortage of trained animal husbandry staff. This has resulted in a C. Bovis bacterial outbreak in our immunocompromised rooms at the animal facility attributed to the animal facility's low staffing numbers and difficulty hiring competent replacements. The C. Bovis outbreak caused the animal facility to limit access to *one person per lab group* in order to cut down the spread of the mouse pathogen. Our lab group currently has 6 people who do xenograft work and until the beginning of Nov. 2021 all animal work had to be done by a single individual form the lab. This has massively curtailed the number of xenograft experiments we were able to run as a group. We are currently searching for some kind of work around if this happens again. This might necessitate individual investigators such as myself starting my own independent IACUC protocol or possibly leaving this institution.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Russo JW, Gao C, Bhasin SS, Voznesensky OS, Calagua C, Arai S, Nelson PS, Montgomery B, Mostaghel EA, Corey E, Taplin ME, Ye H, Bhasin M, Balk SP. Downregulation of Dipeptidyl Peptidase 4 accelerates progression of castration-resistant prostate cancer. (2018) Cancer Research. 78(22):6354-6362. PMID: 30242112.
Status of Publication: Published.
Acknowledgement of Federal Support: Yes

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Einstein DJ, Arai S, Calagua C, Xie F, Voznesensky O, Capaldo BJ, Luffman C, Hecht JL, Balk SP, Sowalsky AG, and **Russo JW**. Metastatic castration-resistant prostate cancer remains dependent on oncogenic drivers found in primary tumors. *JCO Precision Oncology* (2021) 5:1514-1522. DOI: 10.1200/PO.21.00059. PMID: 34568716.
Status of Publication: Published.
Acknowledgement of Federal Support: Yes

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

niques that resulted from the research activities. Describe the technologies or techniques were shared.
Nothing to report.

- **Inventions, patent applications, and/or licenses**

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Joshua W. Russo
Project Role: Principal Investigator
Research Identifier: ORCID ID – 0000-0001-5481-0090
Nearest person month worked: 10
Contribution to Project: Dr. Russo performed all the animal studies and in vitro work.
Funding Support: CDMRP PCRP Early Investigator Award, PCF Young Investigator Award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: