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TITLE: Targeting Mechanisms Driving ErbB2 Activation in Subsets of CRPC

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

Steven Balk

5d. PROJECT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our overall hypothesis is that the ErbB2 pathway is driving a subset of advanced PCa, and that these patients would respond to a therapy that effectively suppresses ErbB2 signaling. Our corresponding overall objective is to generate the preclinical data needed to support a biomarker driven clinical trial of an ErbB2 inhibitor in advanced CRPC. To achieve this objective, Aim 1 will first determine whether the d16ErbB2 splice variant activates a distinct downstream pathway. Aim 2 will in parallel identify mechanisms of intrinsic and acquired resistance to ErbB2 inhibitors, and Aim 3 will translate the results into clinical samples to identify biomarkers that may be predictive of responses to ErbB2 targeted therapies.

15. SUBJECT TERMS
prostate cancer, ErbB2, castration-resistance, biomarkers, tyrosine kinase

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1. INTRODUCTION:

It is becoming clear that activation of multiple pathways may contribute to CRPC and subsequent abiraterone or enzalutamide resistance, through AR-dependent and/or AR-independent mechanisms. Previous studies and our preliminary data indicate that ErbB2 pathway activation is one such mechanism, which may both enhance AR activity and drive PCa progression through other downstream pathways including PI3K and RAS/RAF/MAPK. Although previous clinical trials of ErbB2-targeted therapy have not shown significant activity, we hypothesize that this is because the ErbB2 pathway is a major driver of advanced PCa in only a minority of patients, and that these patients may respond to more effective ErbB2-targeted therapies. Our preliminary data further suggest that expression of the delta16 ErbB2 splice variant may contribute to ErbB2 pathway activation and relative resistance (intrinsic or acquired) to reversible ErbB2 antagonists such as lapatinib. Our overall hypothesis is that the ErbB2 pathway is driving a subset of advanced PCa, and that these patients would respond to a therapy that effectively suppresses ErbB2 signaling. Our corresponding overall objective is to generate the preclinical data needed to support a biomarker driven clinical trial of an ErbB2 inhibitor in advanced CRPC. To achieve this objective, Aim 1 will first determine whether the delta16 ErbB2 splice variant activates a distinct downstream pathway. Aim 2 will in parallel identify mechanisms of intrinsic and acquired resistance to ErbB2 inhibitors, and Aim 3 will translate the results into clinical samples to identify biomarkers that may be predictive of responses to ErbB2 targeted therapies.

2. KEYWORDS:

prostate cancer, ErbB2, castration-resistance, biomarkers, tyrosine kinase

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1. Determine the oncogenic activity and signal transduction pathways downstream of the intact wild-type ErbB2 versus the delta 16 ErbB2 splice variant in PCa cells.

Goal 2. Determine efficacy and mechanisms of resistance to lapatinib versus afatinib/dacomitinib in xenograft and PDX models with ErbB2 activation.

Goal 3. Develop biomarkers based on biopsies and potentially CTCs to identify patients with advanced PCa whose tumors have ErbB2 pathway activation.

What was accomplished under these goals?

1) Major Activities

A major focus has continued to be on obtaining data needed to support a biomarker driven clinical trial of an ErbB2 inhibitor in CRPC. This has included further optimizing our immunohistochemistry (IHC) for phospho-ErbB2 (pErbB2) and phospho-ErbB3 (pErbB3) in tissue samples and on circulating tumor cells (CTCs), and analyses of clinical CRPC samples to

establish the proportion that have ErbB2 pathway activation. Based on these studies over the past year, we have reached an agreement with PUMA to conduct an investigator initiated biomarker driven trial of neratinib in CRPC (see below). We have worked on generating and characterizing a series of cell lines overexpressing the d16 ERBB2 splice variant. We continued to assess clinical samples to identify mechanisms driving ErbB2 activity, and identified NRG1 as an autocrine or paracrine factor driving this activity in a subset of cases (see below). We have carried out further preclinical studies of ErbB2 inhibitors in patient derived xenograft (PDX) models, and extended these to neratinib, in support of a clinical trial.

2) Specific Objectives

Our overall objectives are to test the hypothesis that a subset of CRPC are driven by ErbB2, and to determine whether these tumors can be effectively treated with an ErbB2 inhibitor (as a single agent or possibly in combination).

3) Significant Results or Key Outcomes

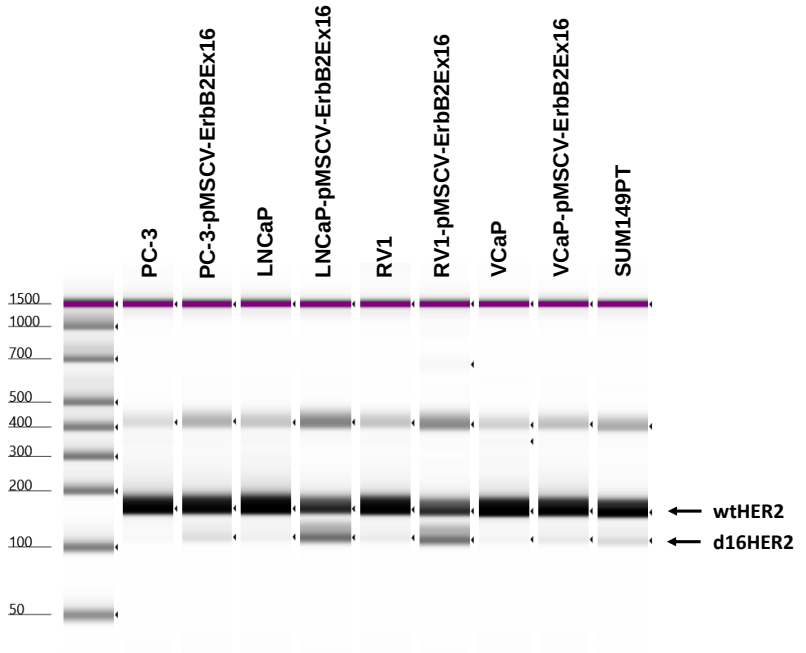


Figure 1. PCa cell lines expressing d16ERBB2. PC3, LNCaP, RV1, and VCaP cells were infected with retrovirus encoding d16ERBB2 (pMSCV-ErbB2dEx16). Relative expression of full length or d16 ERBB2 was determine by qRT-PCR with primers in exon 15 and 17.

<u>Mechanisms driving ERBB2 activity in advanced CRPC.</u> We described previously that ERBB2 was activated in a subset of CRPC, including subsets of mCRPC and in a fraction of tumors that persist in the prostate after neoadjuvant intensive AR targeted therapies. One mechanism for this activation we have been exploring is expression of the exon 16 deleted splice variant of ERBB2 (d16ERBB2) that has increased basal activity due to enhanced homodimerization. We showed previously that this d16ERBB2 was increased in a VCaP xenograft model that became enzalutamide resistant and had markedly increased ERBB2 activation. To determine the actions of this d16ERBB2 in PCa cells we have now generated a series of retrovirally transduced stable PCa cell lines expressing this variant (**Figure 1**). Significantly, by endpoint PCR these lines express modest levels of the d16 variant (relative to full length ERBB2) that are in the range seen with in a breast cancer cell line (SUM149PT) characterized as being activated art least in part via this variant.

Preliminary characterization of these cell lines show variable effects on total HER2 (ERBB2) levels (**Figure 2**). Interestingly, we have not yet been able to observe increased ERBB2 phosphorylation at the Y1221/1222 site. We do not yet know if this is technical, and are currently using parallel approaches (additional phospho-antibodies, phosphotyrosine immunoprecipitation followed by immunoblotting) to assess for ERBB2 and ERBB3 phosphorylation status. However, in 3 of the 4 cell lines we have seen evidence of strong increases in ERBB2/PI3K/AKT pathway activation based on increased phosphor-AKT (**Figure 2**). This result suggests that signaling by the d16ERBB2 variant is going through the conventional pathway for ERBB2 signaling, although more data are clearly needed to address alternative pathways such as through SRC.

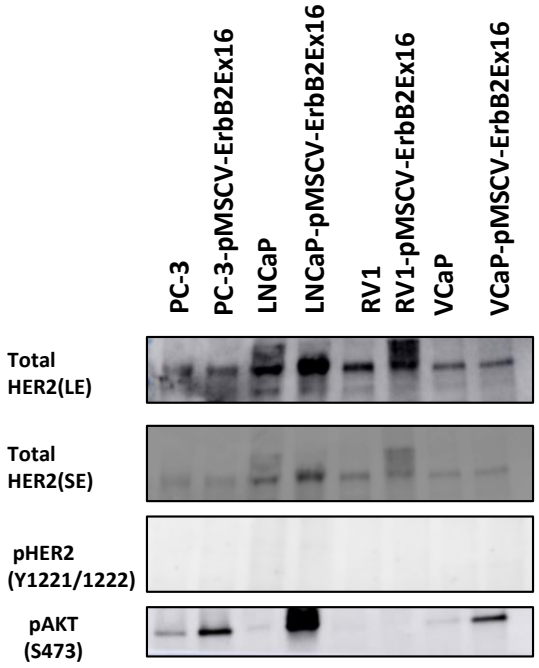


Figure 2. Signaling by d16ERBB2 in PCa cells. Parental or d16ERBB2 over-expressing cells were immunblotted as indicated. Long (LE) and short (SE) exposures for HER2 (ERBB2) are shown

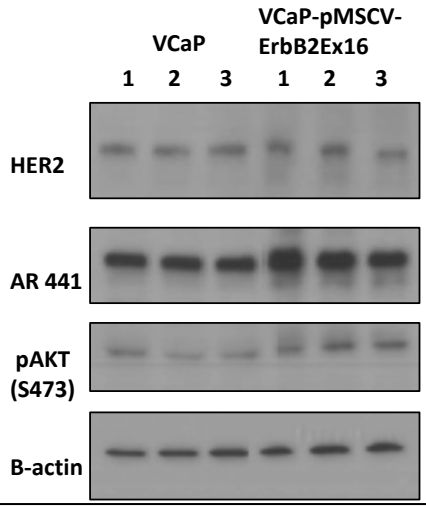


Figure 3. Increased AR expression in d16ERBB2 transduced PCa cells. Parental or d16ERBB2 over-expressing VCaP cells were immunblotted as indicated. AR 441 detects full length AR and splice variants.

Interestingly, in the VCaP cells expressing d16EERBB2 we see a consistent increase in levels of AR (**Figure 3**). This may be related to the increased AKT activity, as we showed previously that AKT activation could decrease AR degradation, probably be decreasing he activity of several ubiquitin ligases.

A second mechanism we had explored previously that may be driving ERBB2 activity in a subset of tumors is increased expression of the ERBB3 ligand NRG1. By immunohistochemistry we had found that NRG1 expression was increased in a subset of tumors that were positive for phospho-ERBB3, suggesting this may be one mechanism. Interestingly, we observed that the NRG1 staining appeared to be in a distinct region of the tumor from the phospho-ERBB3 staining, suggesting a paracrine mechanism (Figure 4). We recently collaborated on a study that examined NRG1 expression in our neoadjuvant cases where residual tumor was positive for phospho-ERBB3 (Zhang et al, Cancer Cell, in press), and in these cases there appears to be a contribution of NRG1

expressed by stromal cells (**Figure 5**). Importantly, we would anticipate that ERBB2 inhibitors will be effective in the case of autocrine or paracrine NRG1.

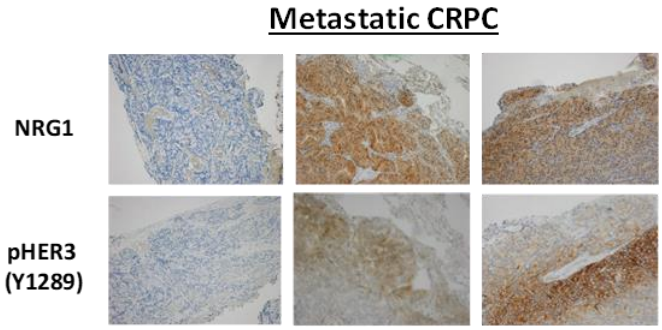
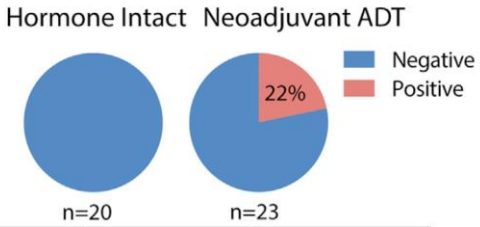


Figure 4. NRG1 expression in mCRPC cases with ERBB2 activation. IHC for NRG1 and pHER3 (pERBB3) in a negative case (left panel) and 2 positive cases. Of note, NRG1 and pHER3 staining appear to be in tumor cells in distinct regions of the tumor.



NRG1	Hormone Intact	Neoadjuvant ADT
Weak	0	2
Strong	0	3
Negative	20	18
Total	20	23
χ^2 test	p = 0.0265 (Two-sided)	

Figure 5. NRG1 expression is increased in residual tumor after neoadjuvant ADT. Signaling by d16ERBB2 in PCa cells. NRG1 staining partially over lapped the 22% of tumors that were pERBB2 positive and a portion was in stroma (from Zhang et al., Cancer Cell, 2020, in press.

PCa with ERBB2 activation responds to ERBB2 inhibitors. We showed previously that enzalutamide-resistant xenografts with ERBB2 activation responded to the ERBB2 inhibitors lapatinib, afatinib, and neratinib, and that the latter covalent inhibitors were more potent in vivo. We have now expanded our analysis to additional PDX models to determine whether ERBB2 pathway activation is a potential biomarker for response to ERBB2 antagonists. Examining the LuCaP series of CRPC we found that ERBB2 was activated in LuCaP70CR and LuCaP96CR, but not in LuCaP35CR (**Figure 6**). To assess biomarker potential we then treated cohorts of mice bearing each PDX with neratinib. Significantly, responses were observed in the VCaP, LuCaP70CR, and LuCaP96CR PDXs, but not in LuCaP35CR (**Figure 7**). These results provide further evidence that a subset of PCa are driven by ERBB2, and support a biomarker driven clinical trial.

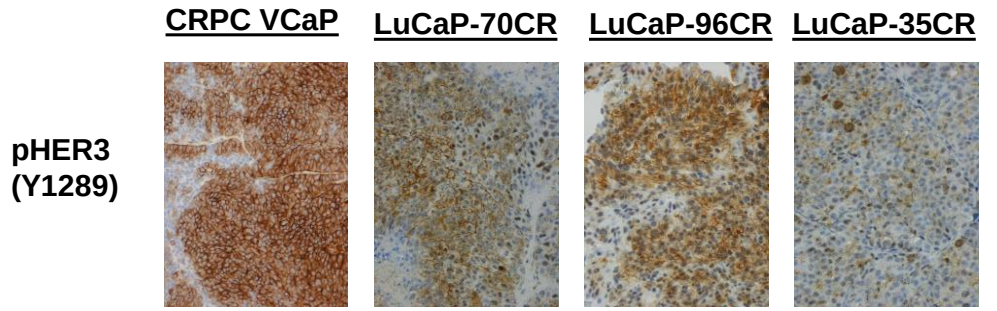


Figure 6. ERBB2 pathway activity in LuCaP PDXs. IHC for pHER3 (pERBB3) was assessed in LuCaP PDXs. LuCaP70CR and 96CR show membrane and cytoplasmic staining while 35CR shows only background nonspecific nuclear staining.

Finally, based on the activity of neratinib in CRPC, we have begun to address whether it may be effective in combination with castration in tumors that are castration sensitive. In preliminary

studies in castration-sensitive VCaP xenografts we have observed that neratinib enhances the response to castration (**Figure 8**).

4) Other Achievements.
We have had a series of discussions with PUMA about a biomarker driven clinical trial. To support this concept we have carried out further validation of pERBB2 and pERBB3 IHC in clinical samples, and have made efforts to detect these by IHC in circulating tumor cells (CTCs) (not shown). Based on these data and results outlined above, PUMA has agreed to support a biomarker driven (phospho-ERBB2) clinical trial of neratinib in mCRPC. The schema is shown below (**Figure 9**).

5) Goals Not Met.
There is still substantial work to be done towards each of the Aims. For Aim 1 we have not yet fully characterized the role of the delta16 ErbB2 splice variant. In Aim 2 we need to better understand mechanisms of resistance in tumors that initially respond to ErbB2 inhibition. In Aim 3 we need to determine whether we can detect ErbB2 activation on CTCs from patients.

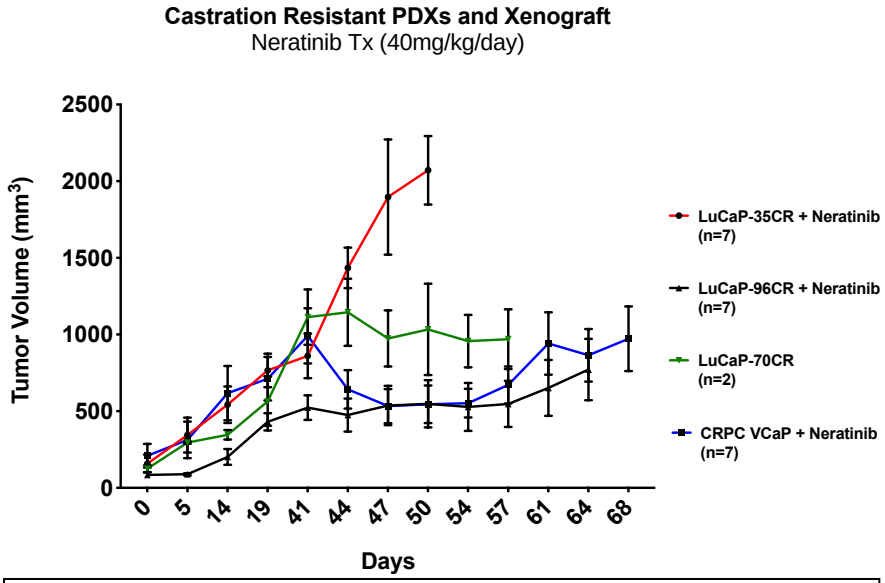


Figure 7. ERBB2 pathway activated PDXs respond to neratinib. Cohorts of mice bearing the indicated PDXs were treated with neratininb and followed for disease progression. Treatment arrested growth in VCaP, LuCaP70CR and LuCaP96CR PDXs (pHER3+), but not LuCaP35CR (pHER3-).

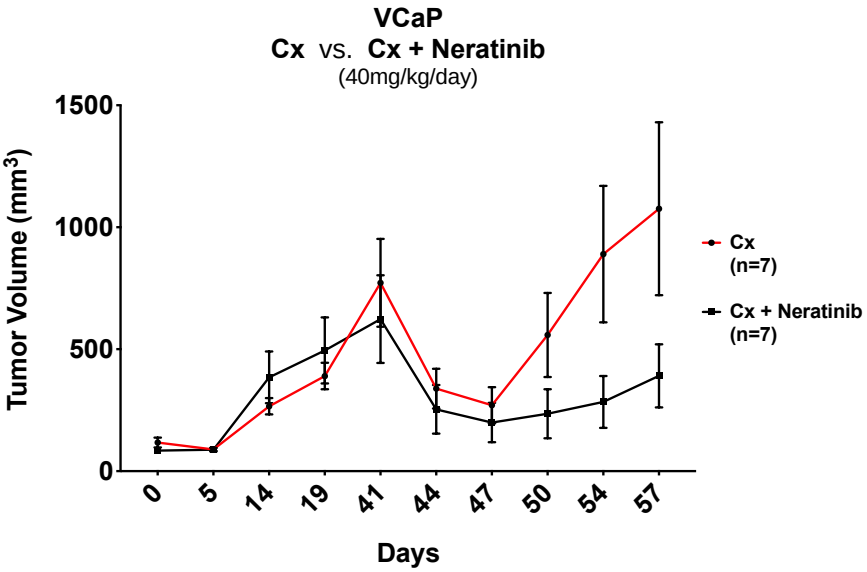


Figure 8. Neratinib enhances responses to castration. Cohorts of intact male mice bearing VCaP xenografts were castrated and treated with neratininb or vehicle, and followed for tumor growth.

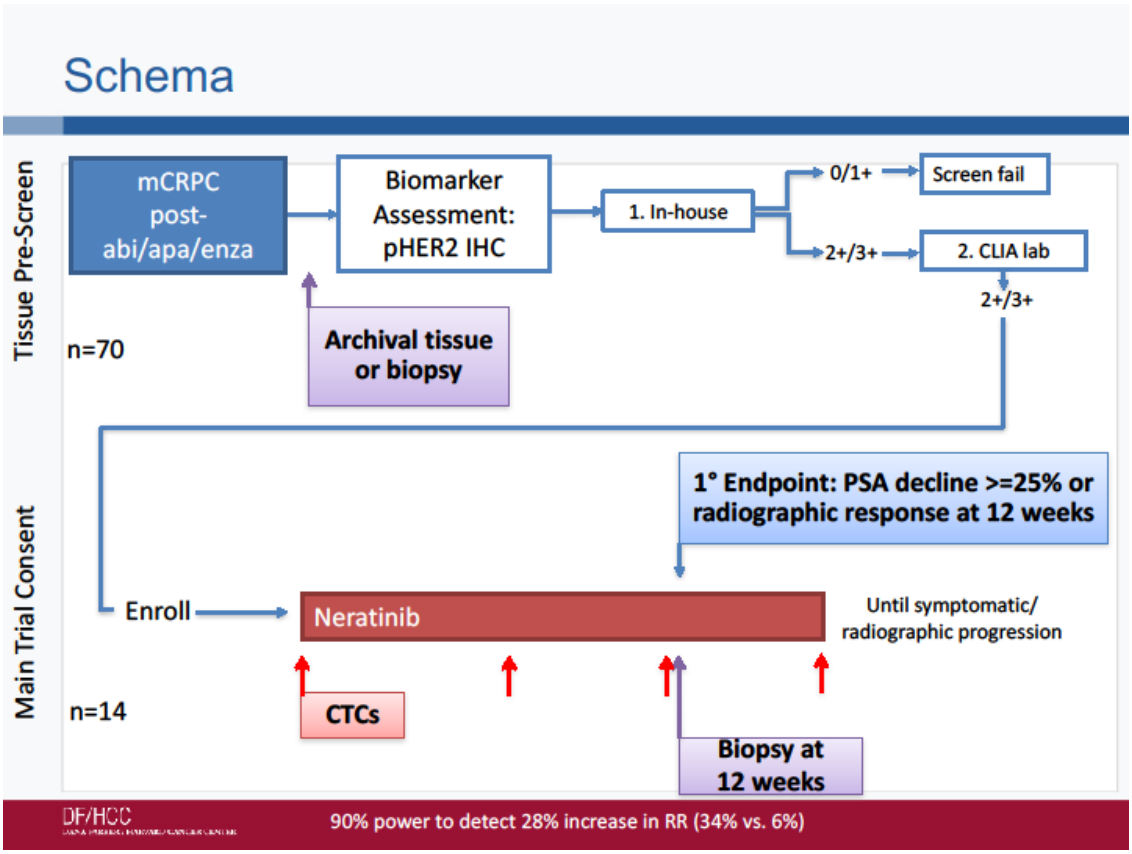


Figure 9. Schema for trial of neratinib in patients with CRPC expressing pHER2 (pERBB2).

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

Nothing to report

- **How were the results disseminated to communities of interest?**

Results from these studies have been presented in seminars and in poster sessions at several meetings.

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

Related to Aim 1, we will continue with our analysis of cell line models expressing the delta16 ErbB2 isoform for signaling studies. We have also completed LCM purification of clinical samples to direct assess expression of this isoform in a series of clinical samples and determine its association with ERBB2 activity. Related to Aim 2 we will now focus mechanisms of acquired resistance to neratinib. We will also further assess the efficacy of using ERBB2 inhibitors earlier in conjunction with castration. For Aim 3 we will further explore the use of CTCs to assess ERBB2 signaling.

4. **IMPACT:**

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

We have provided strong evidence that a subset of CRPC are ERBB2 driven.

- **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**

We were slowed down by the COVID-19 pandemic

- **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**

Nothing to report

- **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**

Zeda Zhang, Wouter R. Karthaus, Young Sun Lee, Vianne R. Gao, Chao Wu, Joshua W. Russo, Menghan Liu, Jose Mauricio Mota, Wassim Abida, Eliot Linton, Eugine Lee, Spencer D. Barnes, Hsuan-An Chen, Ninghui Mao, John Wongvipat, Danielle Choi, Xiaoping Chen, Huiyong Zhao, Katia Manova-Todorova, Elisa de Stanchina, Mary-Ellen Taplin, Steven P. Balk, Dana E. Rathkopf, Anuradha Gopalan, Brett S. Carver, Ping Mu, Xuejun Jiang, Philip A. Watson, and Charles L. Sawyers. Tumor Microenvironment-Derived NRG1 Promotes Antiandrogen Resistance in Prostate Cancer. *Cancer Cell* 2020, in press.

Seiji Arai1, Andreas Varkaris, Mannan Nouri, Sen Chen, Lisha Xie, and Steven P Balk. MARCH5 mediates NOXA-dependent MCL1 degradation driven by kinase inhibitors and integrated stress response activation. *eLIFE* 2020, in press.

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

Nothing to report

- **Technologies or techniques**

Nothing to report

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

Nothing to report

- **Other Products**

Nothing to report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	*Joshua Russo MD, PhD*
Project Role:	investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Russo has performed all of the animal studies and worked with a technician on the in vitro studies.*
Funding Support:	*DoD postdoc award and PCF Young Investigator Award.*

Name:	Jude Owiredu
Project Role:	*Research assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Owiredu has assisted Dr. Russo in the all of the above studies.*
Funding Support:	*This 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?**

The following new grant has been awarded (no overlap with current award):

Title: Drivers and Downstream Effectors of WNT Signaling in Lethal Prostate Cancer
Agency: Department of Defense PCRP Idea Development Award PC190354
Balk – PI $200,000/yr
Goals/specific aims:
Aim 1. Identify and validate downstream effectors of noncanonical versus canonical WNT signaling in PCa.
Aim 2. Assess WNT pathway genes as potential predictive biomarkers for responses to WNT synthesis inhibitors or as therapeutic targets.
Aim 3. Identify WNT/□-catenin independent pathways activated by APC alterations in PCa and corresponding vulnerabilities that may be exploited therapeutically.
Approved for funding, pending negotiation and activation
Contact: Lymor Barnhard; 301-619-7360; Lymor.R.Barnhard.ctr@mail.mil
Effort: 20%
Dates: 09/01/2020 – 08/31/2023

The following grants have ended:

Title: Combined Chimeric Antigen Receptor Therapy (CAR-T) and Active Immunization for Multiple Myeloma
Agency: Multiple Myeloma Research Foundation
Avigan-PI $50,000/yr to Balk lab
Goals: The proposed program will study the combination of chimeric antigen receptor (CAR) therapy and immunization with a novel personalized dendritic cell (DC)/tumor fusion vaccine for multiple myeloma (MM) as a means of overcoming mechanisms of therapeutic resistance.
Dates: 03/1/18-11/30/19

Title: Development of Precision Neoadjuvant-Adjuvant Therapies
Agency: Department of Defense PCRP Impact Award W81XWH-16-1-0431
PI-Balk $354,000/yr to Balk lab

Goals: Aim 1. Identify genomic alterations in tumors from biopsies prior to therapy; Aim 2. Identify genomic alterations in foci of resistant tumor; Aim 3. Determine whether residual tumor foci mediate systemic relapse.
Dates: 09/30/2016-8/31/19 NCE to 8/31/2020
Effort: 10%
Contact: Jennifer Shankle 301-619-2193 jennifer.e.shankle.civ@mail.mil

- **What other organizations were involved as partners?**

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to report

9. **APPENDICES:**

Nothing to report