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14. ABSTRACT We report the progress of our studies focusing on the Discoidin Domain Receptors (DDRs), a set of kinase receptors that signal in response to collagen in prostate cancer (PCa). The project's goal was to define the expression and therapeutic potential of DDRs in prostate cancer. We conducted a comprehensive analyses of DDR1 expression in a 200 case Grade/Stage tissue microarray (TMA) with clinical data, using a highly specific anti-DDR1 antibody. Comparing low [≤7(3+4)] and high [≥7(3+4)] Gleason Score (GS) tumors, we found that PCa malignant progression is associated with reduced expression of DDR1 in the plasma membrane of tumor cells of high GS tumors. However, no significant associations between DDR1 expression and overall survival or biochemical recurrence were found. A selective DDR1 neutralizing antibody blocked collagen-mediated DDR1 activation in cultured PC3 cells. However, it had no impact on intraosseous tumor growth or bone response. However, target inhibition could not be determined. Downregulation of endogenous DDR1 in PC3 cells enhanced growth within bone, suggesting a tumor suppressive effect of DDR1. However, this effect was not conclusive. Studies on DDR1 function and subcellular distribution in culture cells is ongoing. Overall, our studies suggest a complex role of DDR1 in PCa progression that need to be further evaluated.

15. SUBJECT TERMS

Prostate cancer, bone metastases, discoidin domain receptors, kinases, targeted therapies, immunohistochemistry

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1. INTRODUCTION

Subject: Treatment of prostate cancer (PCa) patients with bone metastases remains a challenge due to the limited arsenal of effective therapeutic drugs that reduce disease progression. Therefore, a major goal in PCa research is to identify specific targetable molecules to prevent and/or diminish the ability of PCa cells to survive within the intraosseous environment. The subject of our project is a set of receptor tyrosine kinases (RTKs), known as Discoidin Domain Receptors (DDRs), which signal in response to collagen, the major organic component of the bone extracellular matrix.

Purpose: To investigate the expression, therapeutic potential, and regulation of DDRs in PCa bone metastases.

Scope: Studies are proposed to define the expression of DDRs in PCa tissue specimens, the ability of DDRs to contribute to intraosseous tumor growth and define the regulation of DDRs in PCa cells.

2. KEYWORDS

Discoidin Domain Receptors, prostate cancer, bone metastases, collagen, tyrosine kinase, targeted therapy, extracellular matrix, signaling, antibodies,

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1. To investigate the expression of DDRs in our cohort of human PCa specimens and its association with clinical, pathological, and outcome data.

Task 1: To select and purchase tissue microarrays (TMA) from the Prostate Cancer Biorepository Network (PCBN).

Milestone: Accomplished.

Task 2: Conduct immunohistochemical (IHC) studies to determine the expression of DDRs in samples of human PCa.

Milestone: Accomplished for the analyses of DDR1. We used a highly selective and validated antibody to DDR1. Analyses of DDR2 staining were conducted. However, the staining specificity with the DDR2 antibodies available to us were not reliable, and therefore not pursued further.

Specific Aim 2. To evaluate the anti-cancer effects of DDR1 inhibitors in preclinical human-mouse xenograft models of primary and intraosseous PCa.

Task 3: Evaluate function-blocking antibodies in the orthotopic model of PCa

These studies were not performed due to the fact that we focused initially on the intraosseous model of PCa, which turned out to be challenging and time consuming. These investments and challenges precluded us to switch to the orthotopic model. The departure of Dr. Bonfil also contributed to our inability to conduct these studies.

Task 4: Evaluate function-blocking antibodies against DDR1 in the intraosseous model of PCa.

Milestone: Accomplished.

Specific Aim 3. To define the molecular and cellular bases of DDR regulation and signaling in PCa cell lines in cell based-assays.

Task 5: Analyses of DDR regulation, function, and signaling.

Milestone: Accomplished, in part.

- **What was accomplished under these goals?**

1) Major activities:

Specific Aim 1, Task 1: As we reported in the 2017 report (and here also indicated), we obtained a PCa tissue microarray (TMA) from the Prostate Cancer Biorepository Network (PCBN) at the Johns Hopkins University. The TMA consist of 200 cases laid out in 5 slides containing 1600 core tissues. The TMA provides information on tumor stage and grade and is blinded in relation to patient identification, as required. Clinical data is also available (see Task 2). An IRB (exempt) was approved by Wayne State University for the use of this TMA as requested by the PCBN and provided earlier to the CDMPR. The task of obtaining the TMA was completed.

Specific Aim 1, Task 2: We analyzed the expression of DDR1 in the TMA and correlated its expression with Gleason Score (GS) and clinical data (see below).

Specific Aim 2, Task 4: We evaluated the role of DDR1 in a model of intraosseous tumor growth of PCa in mice. We found no significant effect on tumor growth or bone response.

Specific Aim 3, Task 5: We characterized the expression and activation of DDRs in human PCa cell lines.

2) Specific objectives:

a. To conduct immunohistochemical (IHC) studies to determine the expression of DDRs in samples of human PCa. Focusing on DDR1, our objective was to evaluate the association between subcellular localization of DDR1 and Gleason score and other clinical data available (overall survival, biochemical recurrence). Another objective was to analyze DDR2 expression.

b. Investigate the role of DDR1 in intraosseous growth of human PC3M-Luc2 cells. Used a novel DDR1 neutralizing antibody that blocks collagen-mediated receptor activation to determine

whether targeting DDR1 phosphorylation had any impact on intraosseous growth and host bone response (extent of osteolysis). We used the PC3 xenograft model because this is a well-established model system to examine growth within bone of PCa cells that have reached to the site. We focused our effort on these studies because development of bone metastases is the major clinical complication of PCa patients, which compromise quality of life and overall survival.

3) Significant results or key outcomes:

Task 1. To select and purchase tissue microarrays (TMA) from the Prostate Cancer Biorepository Network (PCBN).

Positive Outcomes: The TMA was obtained, and clinical data were available.

Negative Outcomes: None to report.

Task 2: Conduct immunohistochemical (IHC) studies to determine the expression of DDRs in samples of human PCa.

Positive Outcomes: We succesfully evaluated the expression of DDR1 in a comprehensive TMA of PCa. Following are the major findings:

Figure 1 shows an example of the staining of DDR1 in the tissue cores of the TMA. From these analyses we concluded:

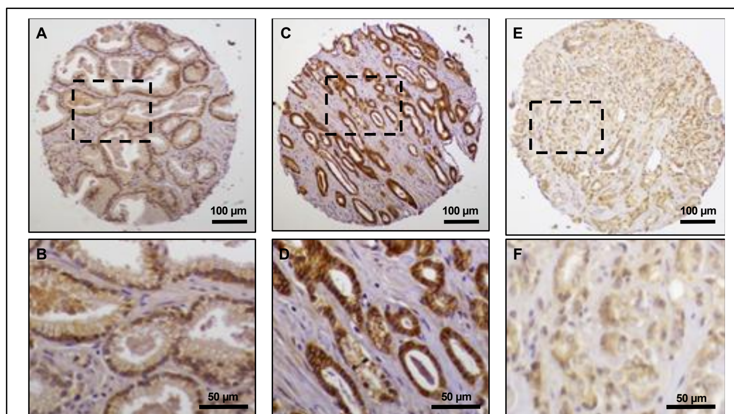

Figure 1. **Immunohistochemical staining of DDR1 in Cancer Tissue Microarray Cores Obtained from Radical Prostatectomy Specimens.** Representative image of a core containing normal prostate glands that display weak basolateral DDR1 protein expression (A), with outlined area shown at higher magnification (B). Gleason score 6 (3+3) lesion with strong membranous DDR1 immunostaining (C and D). High malignant lesion [GS 9 (4+5)] with weak cytoplasmic but non-detectable membranous DDR1 staining (E, F).

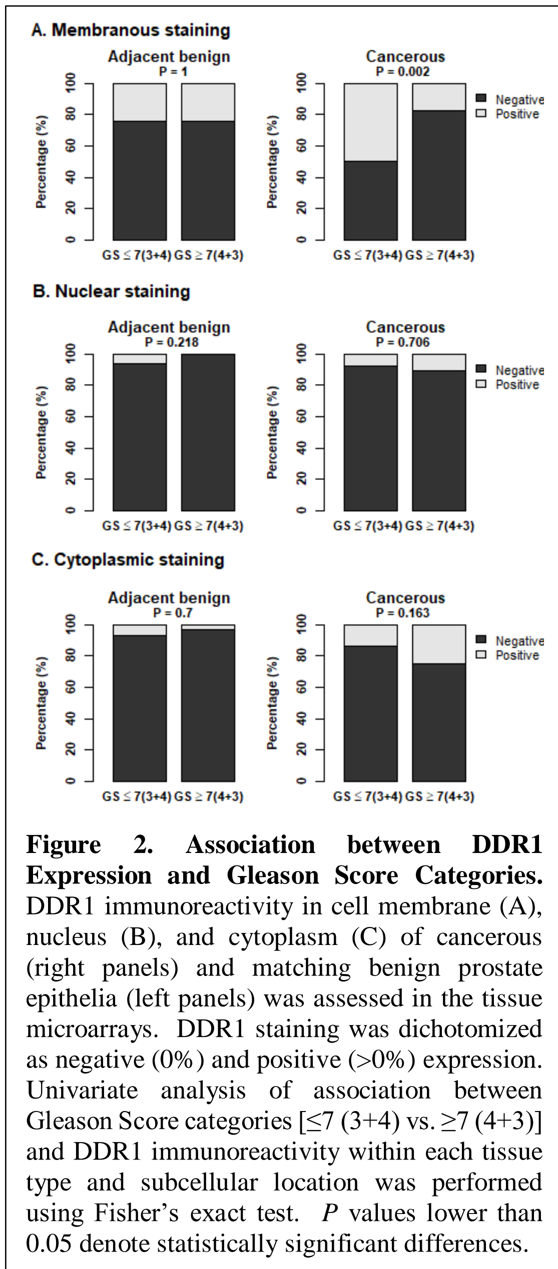

Figure 2. Association between DDR1 Expression and Gleason Score Categories. DDR1 immunoreactivity in cell membrane (A), nucleus (B), and cytoplasm (C) of cancerous (right panels) and matching benign prostate epithelia (left panels) was assessed in the tissue microarrays. DDR1 staining was dichotomized as negative (0%) and positive (>0%) expression. Univariate analysis of association between Gleason Score categories [≤7 (3+4) vs. ≥7 (4+3)] and DDR1 immunoreactivity within each tissue type and subcellular location was performed using Fisher's exact test. *P* values lower than 0.05 denote statistically significant differences.

1. Low GS PCa displays a significantly higher percentage of cancerous glands displaying higher membrane positivity for DDR1 (compared to adjacent benign glands within the same core) when compared to the cancerous glands of high GS (compared to adjacent benign glands within the same core) PCa samples (**Fig. 2**).

2. The proportion of DDR1 overexpression in the membrane of epithelial cells in cancerous compared to that of adjacent benign glands is higher in low GS tumors than in high GS tumors. Thus, in less aggressive PCa, DDR1 expression tends to be more prominent in the cell membrane of the tumor cells of the cancerous glands than in the membrane of the cells of the adjacent benign glands (**Fig. 3**).

3. There is a lack of association between DDR1 expression and tumor aggressiveness in other subcellular localizations (nuclear, cytoplasmic) (**Fig. 3**).

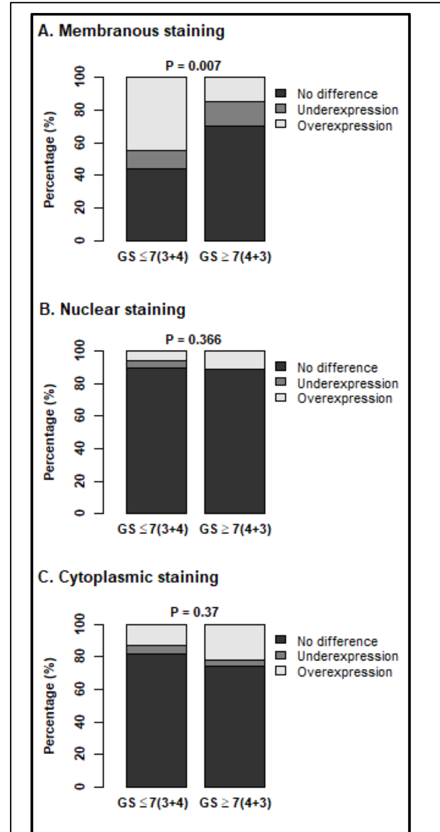

Figure 3. Levels of DDR1 Expression in Cancerous Grands Relative to Adjacent Normal Grands. DDR1 immunostaining in membrane (A), nucleus (B) and cytoplasm (C) of tumor areas corresponding to lower [≤7 (3+4)] and higher [≥7 (4+3)] Gleason scores (GS) was characterized as: "no difference" (equal immunostaining % in cancerous and adjacent benign lesions), "underexpression" (staining % in cancerous lesions lower than in adjacent benign lesions), and "overexpression" (staining in cancerous lesions higher than in adjacent benign lesions). Differences were considered significantly difference when P values were lower than 0.05.

We further analyzed the clinical data from this TMA, and examined whether DDR1 expression was associated with any of the clinical parameters provided. The clinical data included: overall survival (OS), biochemical recurrence free survival (BCRFS), and other covariates (**Table I**). Cause-specific death was available but with a low-event number of deaths due to disease (**Table II**). Associations between staining percentage of DDR1 expression (subcellular localization: nuclear, cytoplasmic and membranous) and OS (**Table III**) and BCRFS (**Table IV**), were analyzed with the univariate Cox model.

As shown in **Table I**, the majority of the samples in this TMA are derived from patients with early disease with a few death (n =3) due to PCs (**Table II**). Analyses of the patient variables (**Table I**) with the localization of DDR1 no association between DDR1 expression and OS (**Table III**) or BCRFS (**Table IV**).

Table IV. Biochemical Recurrence-free Survival and DDR1 localization

Variable	n	HR (95% CI)	P value
Membranous DDR1, Adjacent Benign			
Negative	132	Ref.	
Positive	42	1.57 (0.77-3.19)	0.210
Membranous DDR1, Cancerous			
Negative	96	Ref.	
Positive	68	1.21 (0.61-2.39)	0.580
Membranous DDR1, Paired			
No difference	81	Ref.	
Underexpression	19	1.48 (0.54-4.04)	0.445
Overexpression	61	1.02 (0.48-2.16)	0.953
Cytoplasmic DDR1, Adjacent Benign			
Negative	163	Ref.	
Positive	11	1.61 (0.57-4.54)	0.372
Cytoplasmic DDR1, Cancerous			
Negative	137	Ref.	
Positive	27	1.02 (0.42-2.47)	0.962
Cytoplasmic DDR1, Paired			
No difference	130	Ref.	
Underexpression	7	2.87 (0.99-8.30)	0.052
Overexpression	24	1.34 (0.54-3.29)	0.526
Nuclear DDR1, Adjacent Benign			
Negative	164	Ref.	
Positive	10	0.92 (0.22-3.83)	0.907
Nuclear DDR1, Cancerous			
Negative	150	Ref.	
Positive	14	1.39 (0.49-3.96)	0.534
Nuclear DDR1, Paired			
No difference	143	Ref.	
Underexpression	7	1.30 (0.31-5.46)	0.723

Table I. Patient Characteristics

Variable	Frequency (n=200)
Age at prostatectomy , median (range)	57 (36,73)
Race	
African-American	17 (8)
Asian	2 (1)
Caucasian	173 (86)
Other	8 (4)
Adjuvant treatment	
None	169 (84)
Adjuvant radiation only	2 (1)
Missing	29 (14)
Biopsy Gleason score	
6	155 (78)
3+4	24 (12)
4+3	18 (9)
8	3 (2)
Extraprostatic extension	
No	136 (68)
Yes	63 (32)
Missing	1 (0)
Family history of prostate cancer	
No	112 (56)
Yes	73 (36)
Missing	15 (8)
Received chemotherapy	
No	168 (84)
Yes	3 (2)
Missing	29 (14)
Received chemotherapy	
No	168 (84)
Yes	3 (2)
Missing	29 (14)
Received radiation treatment	
No	160 (80)
Yes	11 (6)
Missing	29 (14)
Lymph node metastases	
No	195 (98)
Yes	3 (2)
Missing	2 (1)
Distant metastasis	
No	165 (82)
Yes	6 (3)
Missing	29. (14)

Variable	Frequency (n=200)
Organ-confined pathology stage	
No	68 (34)
Yes	132 (66)
Prostatectomy Gleason score	
6	110 (55)
3+4	56 (28)
4+3	11 (6)
8	14 (7)
9-10	9 (4)
Surgical margin status	
Negative	169 (84)
Positive	31 (16)
Seminal vesicle involvement	
No	186 (93)
Yes	14 (7)
Clinical stage	
T1c	154 (77)
T2a	35 (18)
T2b	9 (4)
T2c	1 (0)
Tx	1 (0)
Body mass index, median (range)	26.54 (20.04,39.15)
Missing	7
PSA at diagnosis, median (range)	5.995 (1.29,38)

Table III. Overall Survival and DDR1 Localization

Variable	n	HR (95% CI)	P value
Membranous DDR1, Adjacent Benign			
Negative	133	Ref.	
Positive	42	1.56 (0.61-4.04)	0.355
Membranous DDR1, Cancerous			
Negative	96	Ref.	
Positive	69	1.19 (0.47-3.03)	0.709
Membranous DDR1, Paired			
No difference	81	Ref.	
Underexpression	19	2.79 (0.84-9.27)	0.095
Overexpression	62	1.00 (0.35-2.88)	0.997
Cytoplasmic DDR1, Adjacent Benign			
Negative	164	Ref.	
Positive	11	0.50 (0.07-3.72)	0.497
Cytoplasmic DDR1, Cancerous			
Negative	138	Ref.	
Positive	27	1.39 (0.46-4.24)	0.559
Cytoplasmic DDR1, Paired			
No difference	131	Ref.	
Underexpression	7	0.85 (0.11-6.50)	0.875
Overexpression	24	1.58 (0.52-4.86)	0.422
Nuclear DDR1, Adjacent Benign			
Negative	165	Ref.	
Positive	10	0.00 (0.00-Inf)	0.998
Nuclear DDR1, Cancerous			
Negative	151	Ref.	
Positive	14	0.58 (0.08-4.37)	0.598
Nuclear DDR1, Paired			
No difference	144	Ref.	
Underexpression	7	0.00 (0.00-Inf)	0.998
Overexpression	11	0.62 (0.08-4.64)	0.640

Based on these observations, we concluded that the loss or reduced expression of DDR1 in the plasma membrane of the tumor cells in high but not in low GS tumors is consistent with a model in which loss of membrane-associated DDR1, during PCa progression, releases tumor cells from the inhibitory signals produced by the surrounding collagenous matrix. Thus, in primary PCa, membranous DDR1 may behave as a suppressor of malignancy. We previously demonstrated that DDR1 undergoes ectodomain shedding, a proteolytic process that terminates DDR1 activity at the cell surface. Taken together, the results of our TMA analyses suggest the possibility that during the transition from low grade to high grade PCa (as defined by GS), a loss of DDR1 at the membrane caused by proteases may release tumor cells from the suppressive effects of DDR1 in the primary organ. Although this is not part of the current award, we are now exploring this hypothesis in cultured PCa cells. Regardless, our studies raise an important note of caution regarding the utility of targeting DDR1 in patients with PCa. Having said that, our studies do not rule out the possibility that intracellular DDR1 may still play a role in malignancy. Indeed, our studies demonstrate a significant pool of receptor in the cytoplasm and the nucleus. Although the presence of DDR1 in these locale were not associated with Gleason score or other malignant parameters, a potential role of DDR1 in these sites cannot be rule out at this junction. For instance, presence (not necessarily at higher levels) of DDR1 within the nucleus may contribute to PCa malignancy. Thanks to the studies conducted with this award, these possibilities will be explored in future studies. These IHC studies are been completed and are now being written for publication. At this time, we are assembling the final figures, writing the data and the discussion.

Negative Outcomes: In the context of the studies of Task 2, we wished also to examine the expression of DDR2, the second member of the DDR family, by IHC in the TMA. However, disappointingly, we were not satisfied with the quality and specificity of DDR2 staining using several commercially available antibodies. Therefore, this objective was not accomplished.

Task 3: Evaluate function-blocking antibodies in the orthotopic model of PCa.

Positive Outcomes: None to report.

Negative Outcomes: These experiments were not conducted. The intratibial animal studies took most of the time due to the lengthy and complex analyses of mice and the subsequent processing and analyses of multiple bones. We invested considerable effort and time assessing the expression and activation of DDR1 in bone sections.

Task 4: Evaluate function-blocking antibodies against DDR1 in the intraosseous model of PCa.

Positive Outcomes: We evaluated the effect of a DDR1 blocking antibody on intraosseous tumor growth of human PC3M-Luc2 cells in mice. We also evaluated the effect of DDR1 downregulation in human PC3 cells in the same xenograft model. Following are the major findings accomplished within this Task:

To evaluate the potential of DDR1 as a target in PCa cells growing within bone (intraosseous), as proposed in the original application, we proposed to use a new humanized antibody directed against human DDR1 that was designed to block collagen-mediated receptor activation. Through an MTA with Roche, we obtained a neutralizing antibody against DDR1 referred to as RO6849889 antibody. In the application, we reported the ability of the antibody to block DDR1 activation in response to collagen I in cancer cells. With award of the application and the animal protocol, Roche produced large quantities of RO6849889 for the mouse studies. This is a humanized rabbit antibody that only binds to human DDR1 and does not cross-react with mouse DDR1. We confirmed the ability of RO6849889 antibody to block endogenous DDR1 activation in PC3M-Luc2 cells. These cells are highly malignant PCa, and can form rapidly growing intraosseous tumors when inoculated within the tibia of mice. Importantly, these cells express endogenous DDR1.

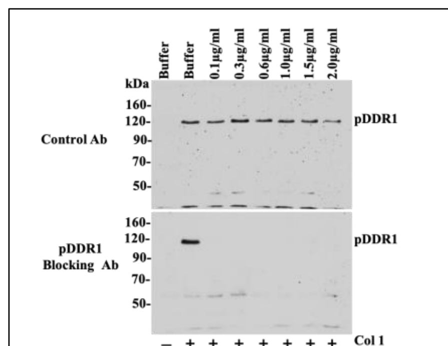

Figure 4. DDR1 Blocking Antibody RO6849889 Inhibits Collagen-Induced DDR1 Activation in PC3M-Luc Cells. Cells were treated (+) or not (-) with collagen I in the absence (Buffer) or presence of increasing amounts of DDR1 blocking antibody. After 24 hrs, the samples were processed for immunoblot analyses using an antibody recognizing phosphorylated tyrosine at position 513 of DDR1b and DDR1c.

The cells were incubated in serum-free media with 2 µg/ml of either RO6849889 antibody or isotype control antibody for 30 min at 37°C before adding 20 µg/ml collagen I. After a 24-h incubation, the cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. The lysates were subjected to reducing 7.5% SDS-PAGE followed by immunoblotting phospho-DDR1 Y513 Ab from Cell Signaling Technology (CST) and the membrane was re-probed with DDR1 Ab, D1G6, from CST for total DDR1. As shown in **Figure 4**, RO6849889 Ab but not the isotype control antibody blocked DDR1-induced collagen I activation. These results gave us the justification to move into the mouse studies.

With the antibody and the cells in hand, we used the intratibial model, one of the most common techniques used to study tumor interaction with the host bone microenvironment, as stated in Task 4 of the SOW.

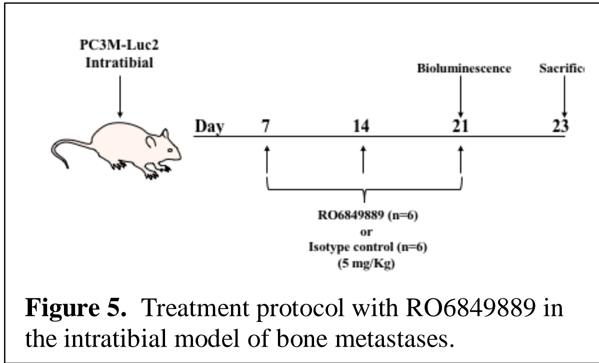

Figure 5. Treatment protocol with RO6849889 in the intratibial model of bone metastases.

Briefly, 7-weeks old male SCID mice were inoculated in the tibiae with $5x10^5$ PC3M-Luc2 cells in a volume of 10 µl and the mice were divided in two groups for administration of either RO6849889 or control antibody (5 mg/Kg). This antibody dose was selected based on the results of the pharmacokinetic data obtained by Roche. Based on these results we decided to administer the compound with the schedule indicated in **Figure 5**. At day 21, the mice were examined for intraosseous tumor burden by bioluminescence after inoculation of luciferin and X-ray. This was performed using the In-Vivo Xtreme imaging system. Both types of images obtained were merged in order to detect and assess tumor burden through the bioluminescent image registered with the X-ray for anatomical context (**Figure 6**). Although the results of the bioluminescence images are not conclusive (without histomorphometry), we noticed an apparent reduced intensity of luciferase in mice treated with RO6849889 (**Figure 3**). We also noticed that some mice had fractures of the proximal tibiae. Therefore, all the animals were sacrificed on day 23. After sacrifice, their tibiae were harvested and subjected to *ex vivo* X-ray imaging using the Trident Digital Specimen Radiography system. The X-ray images were used to determine bone response (osteolytic, osteosclerotic or mixed) in untreated or treated mice.

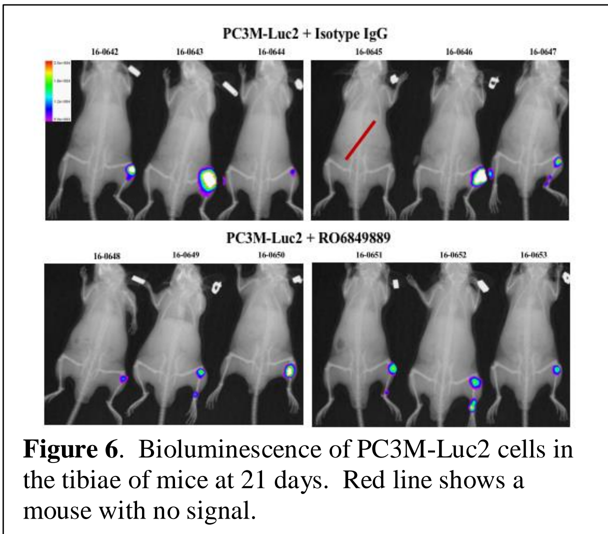

Figure 6. Bioluminescence of PC3M-Luc2 cells in the tibiae of mice at 21 days. Red line shows a mouse with no signal.

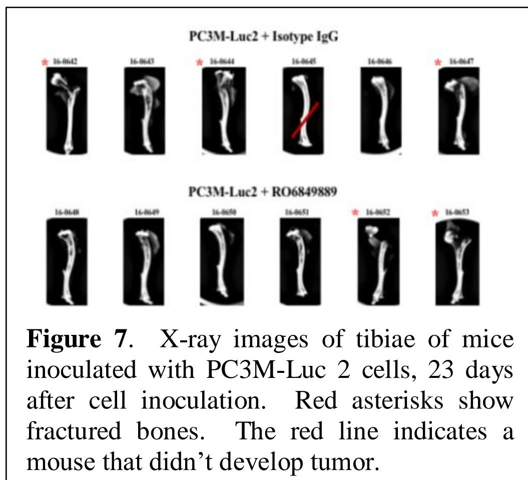

Figure 7. X-ray images of tibiae of mice inoculated with PC3M-Luc 2 cells, 23 days after cell inoculation. Red asterisks show fractured bones. The red line indicates a mouse that didn't develop tumor.

As shown in **Figure 7**, the X-ray images showed osteolytic response in all the tibiae that, in several cases were fractured, as shown earlier with whole body imaging. The tibiae injected with the tumor cells and the contralateral tibiae (controls) were fixed, decalcified, and paraffin-embedded for ulterior longitudinal sectioning for H&E staining and iIHC for pan-cytokeratin, using our established protocols. Because of the presence of multiple tibial fractures that disrupted the continuity of the tumor tissue, we could not evaluate tumor burden by histomorphometry. Therefore, the results of this first study were inconclusive.

Because of the problems with this first study, we set to change two key parameters of the experiment: 1. The number of cell to be inoculated from $5x10^5$ to $2.5x10^5$ PC3M-Luc2 cells per

mouse, and 2. We compared two distinct antibody administration times: a. 2 days after tumor cell inoculation and b. one day prior to tumor cell inoculation. The protocol is depicted in **Figure 8**. Briefly, in the first experiment (**Fig. 8A**), 2.5×10^5 PC3M-Luc2 cells were inoculated intratibially and antibody administration was given on days 2, 9, and 16 days after tumor cell inoculation. In the second experiment (**Fig. 8B**) mice were inoculated with tumors cells that were pre-incubated (60 min at 4ºC) with the blocking antibody or the control IgG before inoculation. Inhibitor was then administered on days 2, 9, and 16 days after tumor cell inoculation. Antibody RO6849889 or control IgG dose were 5mg/Kg, weekly (IV tail vein). The antibodies were administered in a solution of 20 nM Histidine, 140 mM NaCl, pH 6. Whole body BLI and X-rays were performed at week 1, 2 and 3 after tumor injection. Mice were euthanized at day 24, their tibiae were harvested and subjected to *ex vivo* X-ray imaging using the Trident Digital Specimen Radiography system. The X-ray images were used to determine bone response (osteolytic, osteosclerotic or mixed) in untreated or treated mice.

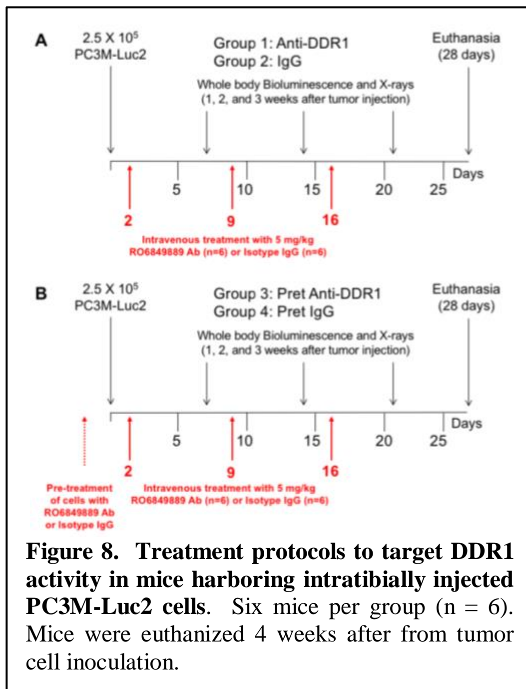

Figure 8. Treatment protocols to target DDR1 activity in mice harboring intratibially injected PC3M-Luc2 cells. Six mice per group (n = 6). Mice were euthanized 4 weeks after from tumor cell inoculation.

Bioluminescence (BLI) Analyses: Tumor sizes were calculated using the sum of total photon flux emission (photons/second/mm2) in the regions of interest (ROI) covering the entire tumors. The tumor growth rates across four time points (Week 1 to 4) were compared using the linear mixed-effects model after tumor sizes were log-transformed. The model allowed for subject-specific baseline tumor size and tumor growth rate considering the correlation between time-dependent observations within the same subject. To assess the tumor size at the last time point (Week 4), unpaired t-test was used after log-transformation. All data were summarized as mean ± standard error of mean (SEM) under a log-normal distribution.

Histomorphometry Analyses: Ex-vivo tibia were fixed in 4% paraformaldehyde and imbedded in paraffin blocks. Paraffin sections (5 µm) derived from bone tumors were immunostained with Pan-cytokeratin and counterstained with hematoxylin. Digital photomicrographs of the entire histological section were captured at 5× magnification and stored as jpeg files. The entire image was then reconstructed using Adobe Photoshop. Tumor tissue (cytokeratin positive areas) and trabecular bone were isolated into separate layers and separately thresholded to black. The whole tissue cross sectional area (considered 100%) was then highlighted and the area occupied by either tumor or bone was automatically calculated.

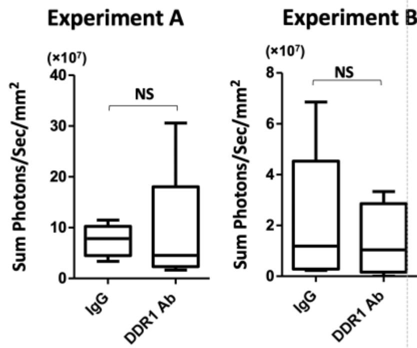

Figure 9. **Quantitation of PC3M-Luc2 Intraosseous Tumor Burden by BLI**. In Experiment A, the DDR1 neutralizing antibody was administered at days 2, 9, and 16 days after tumor cell inoculation. In Experiment B, cells were treated with the antibody prior to tumor cell inoculation, as described in Fig. 1. Data were analyzed using the sum of total photon flux emission (photons/second/mm2) in the regions of interest (ROI) covering the entire tumors. The comparison was performed by the Wilcoxon rank sum test. The mean ±SEM, control IgG (n = 5) and DDR1 Ab (n=6).

BLI Results: As shown in **Figure 9**, administration of the DDR1 neutralizing antibody, under these conditions, had not statistically significant impact on tumor burden within the bone. However, the data suggested a tendency towards reduced tumor burden when PC3M-Luc2 cells were pre-treated with the inhibitor before inoculation (Experiment B).

Histomorphometry Results: Based on the results of Experiment B, as described above, we decided to do histomorphometry analyses on those samples. As shown in **Figure 10**, these analyses showed no significant differences in tumor burden between untreated (IgG) and treated (Neutralizing antibody) mice.

Although disappointing at first sights, these results cannot be interpreted at this junction to conclude that DDR1 is not involved in intraosseous growth of PCa cells. It should be noted that the inhibitor used (a neutralizing antibody that blocks collagen-induced activation) is an effective inhibitor of receptor activation in PC3M-Luc2 cells. This raised the question of DDR1 expression and activation in PC3M-Luc2 cells within the bone and the ability of the anti-DDR1 antibody to block receptor activation within the bone microenvironment. Therefore, studies were conducted to examine DDR1 expression and activation in bone sections obtained from untreated and treated mice.

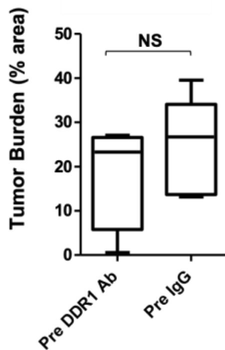

Figure 10. Quantitation of PC3M-Luc2 Intraosseous Tumor Burden by Histomorphometry. Bones from Experiment B were processed for histomorphometry analyses. Data were analyzed using the sum of percentage of tumor and bone area of tibiae injected with cells. The comparison was performed by the Wilcoxon rank sum test. The mean ±SEM, Pre anti-DDR1 Ab (n = 4) and Pre control IgG (n=5).

Expression and Activation of DDR1 in Intraosseous PC3M-Luc tumors.

First, we conducted an IHC analyses of DDR1 expression in bones harboring PC3M-Luc tumors. We used a DDR1 antibody that only recognizes human DDR1 (kindly provided by Dr. Prunotto, Roche). As shown in **Figure 11**, DDR1 was highly expressed in the tumor cells. Thus, we verified that PC3M-Luc cells express DDR1 within the bone microenvironment.

Next, we wished to determine whether DDR1 was activated by examining receptor phosphorylation. This was a challenging issue because detection of phosphorylated proteins in tissues, and especially in bone sections, is not trivial due to the fast turnover of protein phosphorylation, the harsh processing of bones, and the availability of reliable antibodies to phospho-tyrosine residues. To this end, we utilized two approaches: **1**.

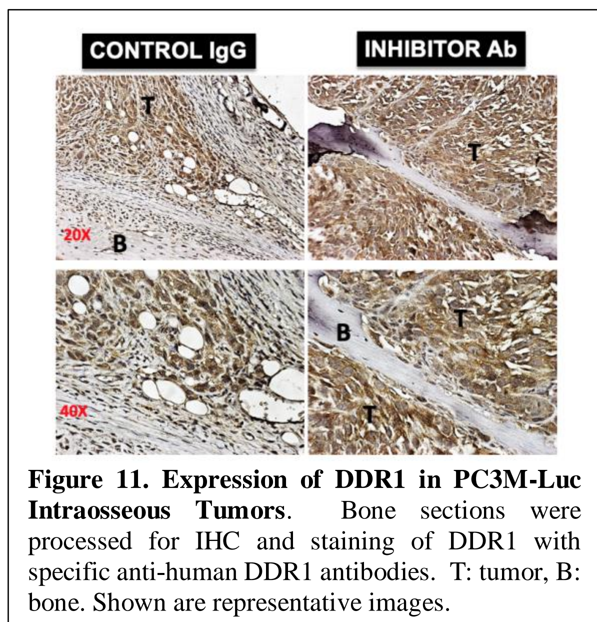

Figure 11. Expression of DDR1 in PC3M-Luc Intraosseous Tumors. Bone sections were processed for IHC and staining of DDR1 with specific anti-human DDR1 antibodies. T: tumor, B: bone. Shown are representative images.

IHC using commercially available antibodies to phosphorylated DDR1, and **2**. Immunoblots of PC3M-Luc cells flushed from bones of treated and untreated mice.

IHC of phospho-DDR1. For the IHC approach, we obtained a rabbit monoclonal antibody recognizing the phosphorylated Tyr at position 513 of DDR1b and DDR1c (Y513 Ab), and a polyclonal antibody that recognizes phosphorylated Tyr at position 792 of DDR1 (Y792 Ab). Both antibodies were purchased from Cell Signaling Technology, Danvers, MA. Although these antibodies are not indicated for IHC by the manufacturer, several published studies reported use of these antibodies for detection of phosphorylated DDR1 in tumor tissues.

To test the reliability of the phospho-DDR1 antibodies by IHC, we utilized several tissues available to us with known expression of DDR1. In addition, we used sections of bones containing PC3M-Luc tumors. For these studies, we have worked several conditions for the staining including antibody concentrations, incubation times, etc. At the time of this writing, we cannot make a rigorous assessment of the IHC results, therefore these studies are ongoing. At this time, however, we can only provide a preliminary evaluation. Based on these preliminary analyses (data not shown), we found that Ab Y513 is not reliable for IHC due to an apparent lack of specificity (staining in all tissue compartments). In contrast, Ab Y792 appears to specifically stain the tumor cells and not the surrounding tissues.

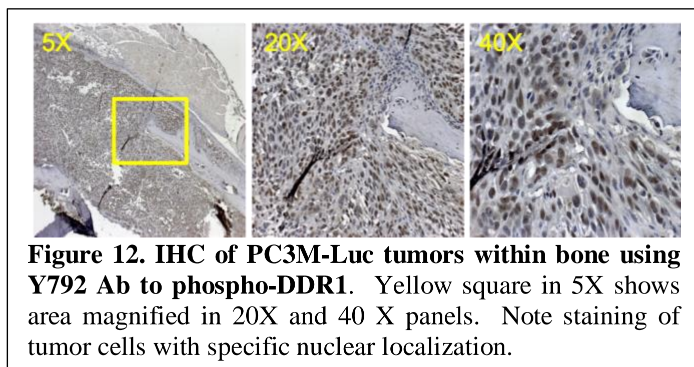

Figure 12. IHC of PC3M-Luc tumors within bone using Y792 Ab to phospho-DDR1. Yellow square in 5X shows area magnified in 20X and 40 X panels. Note staining of tumor cells with specific nuclear localization.

Figure 12 shows a representative section of PC3M-Luc tumors stained with Ab Y792. The staining appears specific for the tumor cells (detected with pan-cyto keratin antibodies, data not shown) and is not detected in the stroma. Interestingly, this antibody strongly stains the nucleus, an unusual localization for phosphorylated DDR1. However, as we reported previously, our studies with cultured human PC3 cells suggest that DDR1 may localize in the nucleus. We are currently examining with details the subcellular distribution of DDR1 (described in Task 5).

Detection of phospho-DDR1 in flushed bones: We decided to evaluate whether we could isolate tumors cells from the tibiae of mice inoculated with PC3M-Luc cells and detect receptor activation. Briefly, tibiae were isolated from sacrificed mice under sterile conditions. The tibiae were rinsed with sterile PBS supplemented with a cocktail of protease inhibitors and phosphatase inhibitors

and then placed in the corresponding well containing 1 ml PBS supplemented with protease inhibitors and phosphatase inhibitors. Both ends of the tibiae were cut to expose the bone marrow. The cut tibiae were then flushed by inserting a 1ml syringe with a gauge 27 needle containing 0.5 ml of PBS supplemented with protease inhibitors and phosphatase inhibitors. This process was repeated 2-3 times until bone marrow was cleared from the bones. Protein concentration was determined and 100 µg of total protein was precipitated in the presence of cold methanol and resolved by SDS-PAGE followed by immunoblot analyses. Disappointingly, we could not detect a positive signal for either total or phosphorylated DDR1.

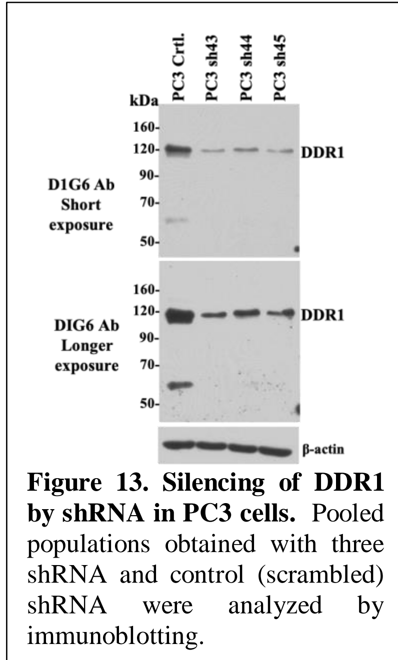

Figure 13. Silencing of DDR1 by shRNA in PC3 cells. Pooled populations obtained with three shRNA and control (scrambled) shRNA were analyzed by immunoblotting.

DDR1 Downregulation in Human PC3 cells and Effect on Intraosseous Tumor Growth: We generated human PC3 cells with downregulated DDR1 expression via shRNA to evaluate the role of DDR1 in intraosseous tumor growth. **Figure 13** depicts an immunoblots of the PC3 variants (control and shRNA) obtained. The PC3 control and the sh43 and sh45 PC3 cell populations were then examined for their ability to form intraosseous tumors in mice.

Animal Protocol: 2.5×10^5 PC3 cells (Control n=6, sh43 n=7, and sh45 n=7) were inoculated intratibially in 5-weeks old CB-17 Scid mice. Mice were subjected to whole body X-ray every week. On day 19, the mice were sacrificed, and the harvested tibiae were ex-vivo X-rayed. Bones were fixated, decalcified, paraffin-embedded and processed for H&E, stained for cytokeratin (**Fig. 14**) and analyzed by histomorphometry

Histomorphometry Results: As shown in **Figure 15**, the tumor area occupied by PC3 sh43 cells was larger than the area occupied by the control Scr PC3 cells. This effect was not significant with the sh45 variant. There was no statistically significant difference in the bone area between Scr and sh 43 tumors, but the bone area was much smaller in sh 45 tumors.

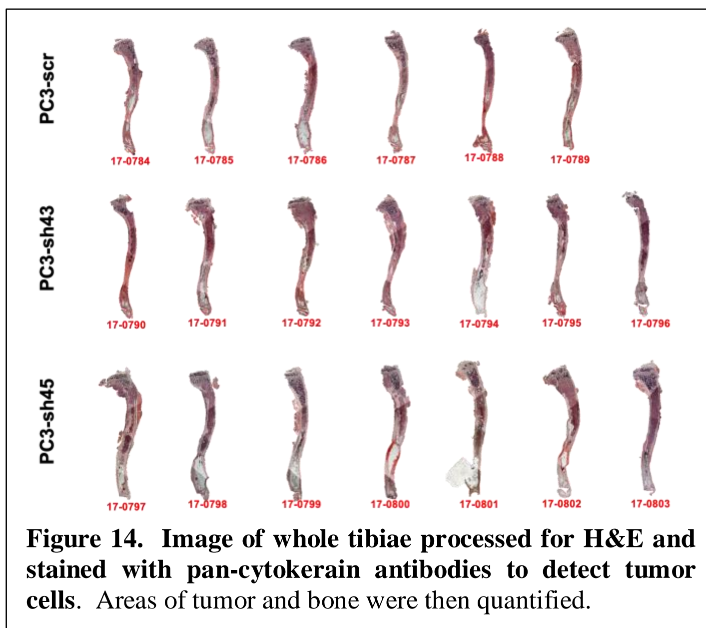

Figure 14. Image of whole tibiae processed for H&E and stained with pan-cytokerain antibodies to detect tumor cells. Areas of tumor and bone were then quantified.

These preliminary results suggest that downregulation of DDR1 enhances intraosseous growth of human PC3 cells. However, this was only observed with one shRNA population (sh 43). At present the reason for these different results with two shRNAs is unclear. Further analyses of DDR1 expression in bone sections are required. Thus, although there were discrepancies, these

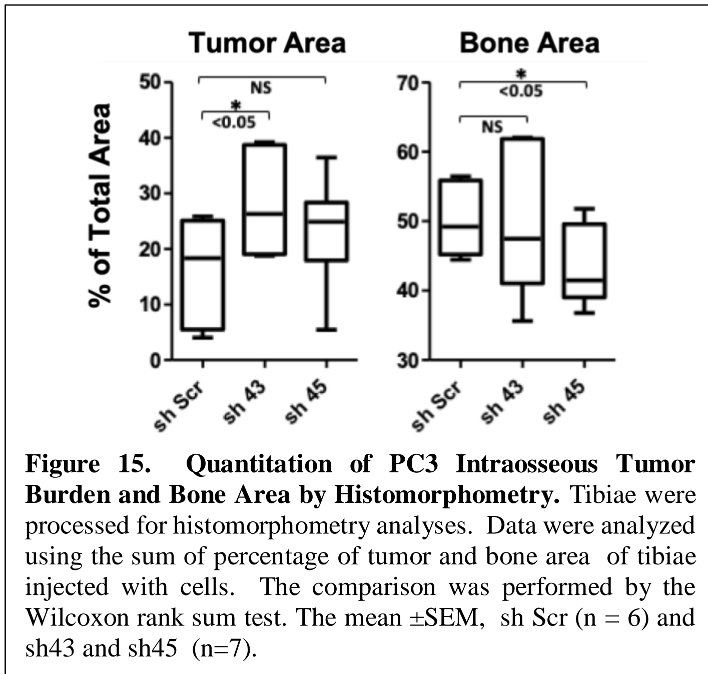

Figure 15. Quantitation of PC3 Intraosseous Tumor Burden and Bone Area by Histomorphometry. Tibiae were processed for histomorphometry analyses. Data were analyzed using the sum of percentage of tumor and bone area of tibiae injected with cells. The comparison was performed by the Wilcoxon rank sum test. The mean ±SEM, sh Scr (n = 6) and sh43 and sh45 (n=7).

results suggest a new paradigm in which DDR1 may elicit a tumor suppressor effect within the bone microenvironment. However, more experimentation is required to test this hypothesis.

Negative Outcomes: There were several negative outcomes in this Task, including the inability to determine whether the neutralizing DDR1 antibody actually blocked receptor activation in the tumor cells growing within the bone. We are now testing a new antibody against phosphorylated DDR1 (at Tyr513) from another company and which has been used in a recent publication. However, these IHC studies are ongoing. Another challenge we encountered was the discrepancy on the effects of the two DDR1 shRNAs.

4) Other achievements.

Nothing to Report.

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

Nothing to Report

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

Nothing to Report

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

Nothing to Report, this is the final report.

4. IMPACT

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

We have evaluated the expression of DDR1 in a comprehensive cohort of PCa samples and conducted animal studies to evaluate the role of DDR1 in intraosseous tumor growth using a newly developed neutralizing antibody. The results we obtained are complex but we believe they

are based on a rigorous experimental design and data analyses. We demonstrated that the level of DDR1 at the plasma membrane is associated with disease progression (as defined by low and high GS). We believe these analyses provide new information on DDR1 in PCa that will contribute to the understanding on the expression of DDR1. A publication of these results is under preparation. We hope that this publication will have an impact in the field of PCa markers, histopathology, DDRs, and receptor tyrosine kinases. Our animal studies showed that targeting DDR1 has no apparent benefit on intraosseous tumor growth. These results are disappointing, yet they are important because they suggest that DDR1 may not be a key target in this type of conditions: tumors that are growing within bone. However, due to the difficulties we encountered assessing target status, namely receptor activation, it is unclear whether the lack of therapeutic effect was due to the inability of the blocking antibody to reach its target or, more importantly, to the lack of a role of DDR1 activation in intraosseous tumor growth. There are many other issues that can be learned from our results that we are currently evaluating and compiling. Thus, we believe that once these results are reported, our studies will have an impact for the design and analyses of preclinical studies in the area of oncology.

- **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**

Throughout the funding period we tried to follow the SOW, and therefore there were no significant changes in approach. However, due to the unforeseen complexities of several studies (described above), some of the Tasks were not conducted. Our effort focused on addressing the challenges, both technical and conceptual, that we encountered, which compromised our inability to accomplish some of the Tasks.

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

This is a final report.

- **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**

There were no changes.

6. PRODUCTS:

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

An abstract was presented at the 2018 American Association for Cancer Research (AACR) Annual Meeting in Chicago, IL.

Title: Discoidin domain receptor 1 (DDR1): A potential suppressor of prostate cancer progression

R. Daniel Bonfil[1], Anjum Sohail[2], Semir Vranic[3], Daniel S. M. Oliveira[2], Dongping Shi[2], Wei Chen[2], Hyejeong Jang[2], Allen D. Saliganan[2], Benjamin D. Wasinski[2], Hyeong-Reh Kim[2], **Rafael Fridman[2]**. [1]College of Medical Sciences, Nova Southeastern University, Fort Lauderdale, FL; [2]Wayne State University School of Medicine, Detroit, MI; [3]College of Medicine, Qatar University, Doha, Qatar.

- **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?**

Period 2015-2016

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Daniel Bonfil	Co-PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.12	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Allen Saliganan	Research Scientist	5.40	Animal studies, immunostaining	This grant

Period 2016-2017

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Daniel Bonfil	Co-PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.12	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Allen Saliganan	Research Scientist	5.40	Animal studies, immunostaining	This grant
Anjum Sohail	Research Scientist	5.40	Studies in cell culture	This grant
Benjamin Wasinski	Research Assistant	9	Studies in cell culture	This grant

Period 2017-2018

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Daniel Bonfil	Co-PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.12	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Allen Saliganan	Research Scientist	5.40	Animal studies, immunostaining	This grant
Anjum Sohail	Research Scientist	5.40	Studies in cell culture	This grant
Benjamin Wasinski	Research Assistant	9	Studies in cell culture	This grant

Period 2018-2019 (August 31)

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.06	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Anjum Sohail	Research Scientist	4.2	Studies in cell culture	This grant

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

No changes in Other Support to report for this period for the PI or any other senior/key personnel.

- **What other organizations were involved as partners?**
    - **Organization Name:** Hoffmann-La Roche
    - **Location of Organization:** Basel, Switzerland
    - **Partner's contribution to the project**
        - **Other:** Supplied the neutralizing antibody to DDR1, referred to as RO6849889.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Nothing to Report