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TITLE: Discoidin Domain Receptors: Novel Targets in Breast Cancer Bone Metastasis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Here we report major findings for our project aimed at studying the expression of Discoidin Domain Receptors (DDRs) in breast cancer (BrCa) tissues and their functional contribution to the formation of BrCa bone metastases. We also aim at testing the feasibility of targeting DDRs for the treatment of BrCa bone metastases. During the first funding period, we performed immunohistochemical analysis of DDR1 in 120 samples of invasive BrCa cases with different molecular subtypes, and found a significant inverse association between cytoplasmic DDR1 localization and progesterone receptor expression in ER+ tumors. We plan to continue these tissue analyses to elucidate the significance of these findings, and to further examine the association between DDR expression in primary tumors and development of BrCa bone metastasis. In our experimental studies, we have screened and defined the expression and activation of DDRs in different human BrCa cell lines, confirmed the selectivity and inhibitory action of a new DDR1 tyrosine kinase inhibitor, and validated the ability of MCF7-Luc BrCa cells to grow within the tibiae of immunodeficient mice when supplemented with estrogen. With this information, we plan to test the role of DDR1 in intraosseous growth of these cells in mice treated with the kinase inhibitor. We will also test the role of DDR2 in intraosseous BrCa growth. The results of these studies will be used to investigate the role of tumor-associated DDRs on the regulation of pro- and anti-osteolytic genes *in vitro.*

15. SUBJECT TERMS
Breast cancer, bone metastasis, discoidin domain receptors, kinases, targeted therapies, immunohistochemistry

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1. INTRODUCTION

Different treatments are currently used to treat bone metastasis, the main cause of morbidity and mortality in patients with advanced breast cancer (BrCa). However, although currently available therapies can be effective to relieve pain, prevent complications, and improve quality of life in these patients, are not curative. The identification of novel molecules involved in the establishment and expansion of BrCa metastatic cells within the bone is, therefore, crucial for the development of new prognostic biomarkers and therapeutic agents to prevent and/or inhibit skeletal metastases. Discoidin domain receptors (DDRs) are expressed in invasive BrCa, and represent the only receptor tyrosine kinases (RTKs) that uniquely signal in response to collagen, a major organic component of the bone microenvironment. Based on these facts, the purpose of the research proposed in this application is to test our hypothesis that DDRs mediate the survival of metastatic BrCa cells within the skeletal niche and consequently represent promising targets for intervention in BrCa patients with bone metastasis. The scope of research involves the analysis of DDR expression in primary tumor and bone metastatic tissues from BrCa patients, the evaluation of therapeutic efficacy of DDR inhibition in a preclinical model of intraosseous BrCa growth, and the study of tumor-derived DDRs' role in the regulation of BrCa pro-osteolytic programs using *in vitro* systems.

2. KEYWORDS

Discoidin domain receptors, breast cancer, bone metastasis, receptor tyrosine kinases, collagen, biomarkers, targeted therapy.

3. ACCOMPLISHMENTS

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

Specific Aim 1. To conduct a histopathological analysis of DDR expression in samples of primary BrCa tissues with different subtypes and their matching bone metastasis.

Task 1: Select BrCa tissues for analyses and construct tissue microarrays (TMAs).

Task 2: Analyses of DDR expression.

Specific Aim 2. To evaluate the therapeutic efficacy of DDR inhibition in a preclinical xenograft model of intraosseous BrCa growth.

Task 1: Analyze DDR expression/activation and generate modified BrCa cell lines.

Task 2: Animal Studies.

Specific Aim 3. To investigate the role of tumor-derived DDRs in regulation of BrCa pro-osteolytic programs in cell culture systems.

Task 1: Evaluate role of DDRs in regulation of pro-osteolytic factors.

Task 2: Conduct *in vitro* osteoclastogenesis studies.

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

1) Major activities:

Specific Aim 1.

Task 1: Select BrCa tissues for analyses and construct tissue microarrays (TMAs).

During the first year of funding, we selected BrCa tissues from the tissue bank at University of Michigan and generated a tissue microarray (TMA) containing triplicate samples of 120 invasive breast carcinomas including all BrCa subtypes with comprehensive clinical and pathological information, including the presence of distant metastasis. Sixteen cases had presence of bone metastases.

Task 2: Analyses of DDR expression.

To investigate DDR expression in the newly generated TMA, we first focused on DDR1, a member of the DDR family of collagen receptors. To this end, we utilized a newly developed antibody against human DDR1 that we obtained from Roche trough a Material Transfer Agreement with Wayne State University. This antibody binds the extracellular juxtamembrane region of all DDR1 isoforms and thus identifies receptor present on the cell surface. The antibody works under denaturing conditions and thus works well for immunohistochemical (IHC) analyses in paraffin embedded tissues. Consistently, the antibody does not work for immunoblotting analysis. We tested the antibody in various IHC protocols and tissue samples known to express DDR1 or lack the receptor, as well as in fixed cells that express or lack DDR1. These analyses confirmed its specificity for DDR1 and lack of reactivity with DDR2. We also worked out the optimal staining conditions for IHC.

With this antibody in hand and an optimized protocol, we conducted an IHC study with the BrCa TMA, as proposed in task 2 of Aim 1. The staining was then evaluated by Dr. Kleer using the criteria of intensity of staining as 0 (negative), 1 (weak), 2 (moderate), 3 (strong); as well as the staining pattern (cytoplasmic, cell membrane, or both). **Figure 1** shows representative examples of DDR1 expression in invasive BrCa carcinomas. DDR1 was localized in the tumor cells mostly in the cytoplasm (~48%) but also in the membrane (~22%).

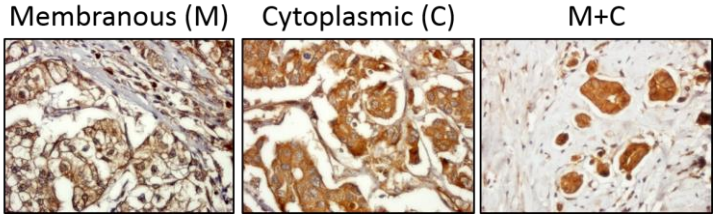

Figure 1. Representative images of invasive breast carcinomas showing expression of DDR1 in the membrane (M), cytoplasm (C), and both membrane and cytoplasm (M+C).

Most of the samples (~49%) showed moderate staining for DDR1 (**Table 1**). No significant staining was detected in the tumor stroma. When the staining data was analyzed in relation to the histopathological features of the tissues, we found a lack of association between DDR1 expression levels and age, race, menopausal status, estrogen receptor (ER), progesterone receptor (PR), HER2-neu expression, tumor grade, tumor stage, or lymph node metastasis. Interestingly, however, we found a **significant association between DDR1 protein localization and PR status, namely cytoplasmic DDR1 was significantly associated with negative PR expression (p=0.015) (Table 2).** The biological significance of this finding is still unclear and thus remains to be determined.

Table 1. Expression of DDR1

DDR1 Immunostaining	N	%
INTENSITY (n=120)		
No Staining	7	5.8
Weak	38	31.7
Moderate	59	49.2
Strong	16	13.3
CELLULAR LOCALIZATION (n=113)		
Cytoplasmic	54	47.8
Membranous	25	22.1
Both	34	30.1

Table 2. Association between DDR1 cellular localization and categorical variables.

	Cellular Localization						
	Cytoplasmic (C)		Membranous (M)		C + M		
Variable	N	%	N	%	N	%	p-value
ER							0.40
Neg	19	38.0	8	32.0	7	23.3	
Pos	31	62.0	17	68.0	23	76.7	
PR							0.015
Neg	30	60.0	11	44.0	8	26.7	
Pos	20	40.0	14	56.0	22	73.3	
HER2							0.20
0	32	64.0	17	68.0	24	80.0	
1	9	18.0	3	12.0	1	3.3	
2	0	0	1	4.0	2	6.7	
3	9	18.0	4	16.0	3	10.0	
Menopause							0.36
Peri	6	12.2	1	4.0	4	13.8	
Post	36	73.5	17	68.0	17	58.6	
Pre	7	14.3	7	28.0	8	27.6	
Grade							0.88
1	6	11.8	1	4.0	3	10.7	
2	22	43.1	13	52.0	13	46.4	
3	23	45.1	11	44.0	12	42.9	
N Stage							0.96
0	22	52.4	10	47.6	16	55.2	
1	11	26.2	6	28.6	9	31.0	
2	7	16.7	3	14.3	3	10.3	
3	2	4.8	2	9.5	1	3.5	
Stage							0.87
1	15		9		11		
2	16		8		13		
3	11		6		4		
4	2		0		1		

Next, we conducted analyses to examine the relationship between DDR1 expression/cellular localization and patient survival. These preliminary analyses showed that cytoplasmic DDR1 was associated with shorter time to recurrence or death compared to C+M DDR1 (hazard ratio [HR] 2.03, 95% confidence interval [CI] 1.12-3.69, p=0.021). Interestingly, membranous DDR1 was associated with shorter time to recurrence or death compared to C+M DDR1 (HR 2.22, 95% CI 1.12-4.37, p=0.022) (**Fig. 2**).

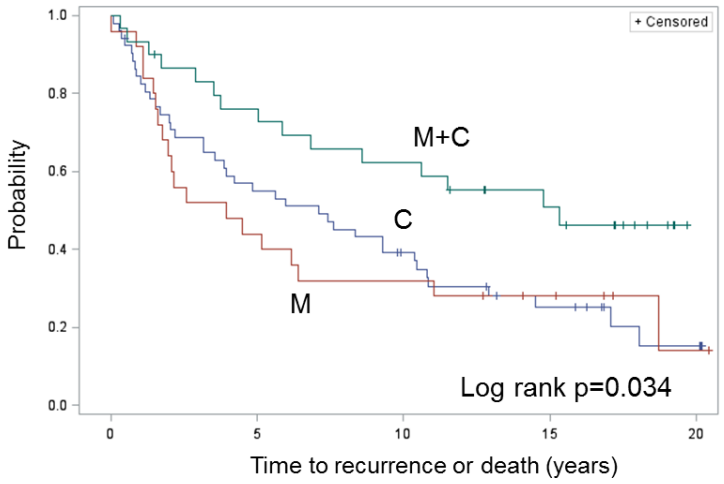

Figure 2. Kaplan Meier survival analyses showed that DDR1 localization is significantly associated with time to tumor recurrence or death.

Of the 120 cases of primary invasive carcinoma analyzed, 16 cases had presence of bone metastasis. Therefore, we wished to determine whether DDR1 expression in the primary tumor was associated with bone metastasis. However, this limited number of cases precluded achievement of statistical power and thus this issue remains unresolved. We are currently in the process of identifying primary carcinomas and matching bone metastasis to study the expression of DDR1 in these samples, which are planned for this year.

Specific Aim 2.

Task 1: Analyze DDR expression/activation and generate modified BrCa cell lines.

Our goal is to investigate the role of DDRs in intraosseous growth of BrCa cells, as proposed in the application. Because BrCa metastases is most frequently diagnosed in women with ER+ breast tumors, we proposed to use MCF7 cell line, which is ER+ and also can grow in bones of immunodeficient mice. Importantly, MCF7 cells express DDR1. Thus, these cells will be utilized to examine the role of DDR1 in intraosseous tumor growth of ER+ breast tumor cells, as we proposed in the application. Women with triple negative BrCa (TNBC) also can develop bone metastases. To test the role of DDRs in intraosseous growth of TNBCs, we proposed to use MD-MB-231 cells. To this end, we purchased MCF7 and MDA-MB-231 cells labeled with luciferase from Cell Biolabs Inc. (San Diego, CA). Upon arrival, the cells were expanded and grown under the culture conditions recommended for these cell lines. Importantly, we submitted the cells for authentication by Short Tandem Repeat (STR) analyses to our core facility, which certified their nature as human MCF7 and MDA-MB-231 cells. We then examined expression of DDRs by immunoblotting using specific antibodies. We compared the luciferase-overexpressing cells with control MCF7 and MDA-MB-231 cells. These analyses demonstrated that the MCF7-Luc cells express readily detectable DDR1, albeit at lower levels than the original MCF7 cells. MDA-MB-231-Luc cells showed no expression of DDR1 or DDR2, in our hands. In contrast, parental MDA-MB-231 showed

low levels of DDR1 but not DDR2 (Fig. 3). Therefore, we decided to use the MCF7-Luc cells, which express and active DDR1 I response to collagen I, for our studies aimed at evaluating the contribution of DDR1 to intraosseous tumor growth, using a small molecule kinase inhibitor (described below) that targets DDR1.

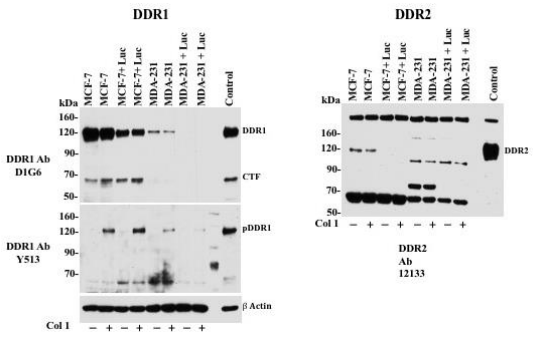

Figure 3. Expression of DDRs in MCF7 and MDA-MB-231 cells treated without or with collagen I. Cell lysates were processed for immunoblot analyses and examined for expression and activation of DDRs. The DDR1 Ab DIG6 recognizes total DDR1 whereas the Ab Y513 recognizes activated DDR1.

A New DDR1 Kinase Inhibitor: In the original application, we proposed to use a kinase inhibitor designated 7rj (J. Medical Chemistry 25; 3281-95, 2013). However, after the award of the application, we received a novel tyrosine kinase inhibitor (TKI) from Roche with excellent pharmacological and pharmacokinetics (pKa) characteristics. This proprietary inhibitor (referred to as Compound A here) exhibits high selectivity for DDR1 with an IC_{50} for DDR1 binding of 0.026 μM. In contrast, the IC_{50} for DDR2 binding is 2.3 μM, an 89-fold difference. Compound A also inhibits kinase activity of DDR1 with an IC_{50} of 0.018 μM. In kinase selectivity assays (468 targets), the DDR1 inhibitor preferentially targeted DDR1 with a K_d of 0.002 μM. In contrast, Compound A exhibited a K_d of 0.023 μM towards DDR2. When compared to the other targets, Compound A displayed >338-fold selectivity for DDR1. Roche also conducted pKa analyses in mice, which they have shared with us. We received this compound and tested it for inhibition of collagen I-induced activation of DDR1 and DDR2 in MCF7-Luc and BT549 cells, respectively. To this end, the cells were serum-starved and the next day incubated (30 min) with Compound A (various concentrations) or vehicle (DMSO 0.2%) and then stimulated with collagen I for 2 hrs. The cells were then lysed and analyzed for DDR1 activation using Ab Y513. For DDR2, the cell lysates were immunoprecipitated with an antibody to DDR2 (AF2538) and the precipitates were analyzed by immunoblotting with antibody Y740 recognizing phosphorylated DDR2. As shown in **Figure 4**, Compound A strongly and preferentially inhibited DDR1 collagen-induced activation but had no effect on DDR2 activation, under the same conditions.

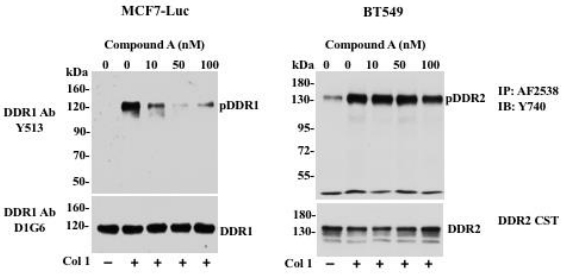

Figure 4. Compound A inhibits collagen-induced DDR1 activation in MCF7-Luc cells. The compound had minimal effects on DDR2 activation at the concentrations tested.

Task 2: Animal Studies.

Having validated the expression and activation of DDR1 in MCF7-Luc cells, it was important to test their ability to produce tumors when inoculated into the tibiae of immunodeficient mice. We have already submitted an animal protocol, for which we obtained approval from Wayne State University Institutional Animal Care and Use Committee (IACUC) and from the USAMRMC Animal Care and Use Review Office (ACURO). Briefly, $1x10^5$ MCF-7-Luc cells/mouse were inoculated into the tibiae of female nude mice which were implanted subcutaneously with 0.18 mg/90-day extended release 17β-estradiol pellets (Innovative Research of America, Sarasota, FL; Cat. # NE-121), or in female nude mice without estrogen supplementation. We found that all mice receiving 17β-estradiol supplementation formed intraosseous tumors that were evident by bioluminescence (BLI) by week 4. In contrast, no signal was detected in the absence of 17β-estradiol supplementation (**Figure 5**). *Ex vivo* x-rays showed an apparent increase in bone mass in tibiae derived from mice with 17β-estradiol supplementation, as compared with those harvested from mice without hormone supplementation (**Figure 6**). This effect was independent from tumor growth, and is likely to be due to a reduced activation of bone remodeling in mice treated with estrogens. In summary, the MCF7-Luc system appears to be appropriate to test the role of DDR1 in intraosseous tumor growth.

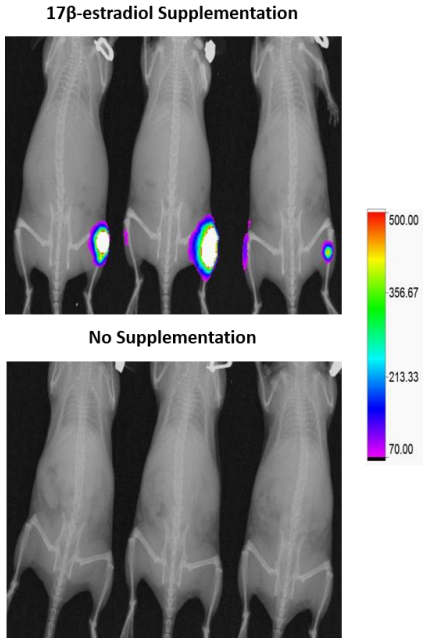

Figure 5. Representative whole-body bioluminescent and x-ray merged images of female nude mice supplemented with 17β-estradiol (top) or not (bottom) 4 weeks after intratibial injection with 10^5 MCF-7-Luc cells. The color scale indicates the intensity of photon emissions.

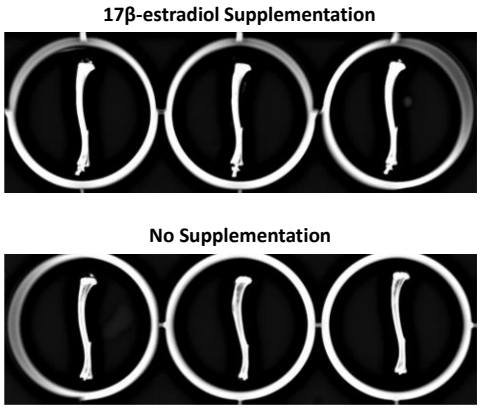

Figure 6. *Ex vivo* x-rays of tibiae injected with MCF-7/Luc cells in female nude mice supplemented with 17β-estradiol (top) or not (bottom) at week 4. Note the higher density of the bone in mice supplemented with

Specific Aim 3.

Task 1: Evaluate role of DDRs in regulation of pro-osteolytic factors.

Nothing to report

Task 2: *In vitro* osteoclastogenesis studies.

Nothing to report

2) Specific objectives:

The objectives during the period covered by this report were:

a. Select a TMA with containing triplicate samples of 120 invasive breast carcinomas including all BrCa subtypes with comprehensive clinical and pathological information.

b. Immunostaining of the selected TMA for DDR1, and analysis of the association of its expression levels with ER, PR, HER2-neu expression, tumor grade, tumor stage, or lymph node metastasis.

c. Define DDR1 and DDR2 expression and activation upon exposure to collagen in different BrCa cell lines, and confirm the inhibitory effect of a small molecule inhibitor.

d. Identify the appropriate preclinical xenograft models to evaluate the therapeutic efficacy of DDR inhibition.

3) Significant results or key outcomes:

Specific Aim 1, Tasks 1 and 2: A TMA was generated with 120 cases of invasive BrCa including all BrCa subtypes. We observed positive DDR1 expression using a highly specific DDR1 antibody, and completed the study of DDR1 expression and association with disease progression with one TMA. We found a significant inverse association between DDR1 cytoplasmic expression and PR expression in the BrCa tissues analyzed.

Specific Aim 2, Task 1 and 2: We defined expression and activation of DDRs in BrCa cell lines to be used in the subsequent studies of the application. We obtained cell lines labeled with luciferase for animal studies and analyzed DDR expression and activation. We found that MCF7-Luc cells express DDR1 but not MDA-MB-231-Luc. We tested a new DDR1 kinase inhibitor in MCF7-Luc and BT549 cells and confirmed its ability to selectively target DDR1 over DDR2. We tested the MCF7-Luc cells for their ability to grow into the tibiae of female mice in the presence of estrogen supplementation, and found that these cells form tumors within the bone in the presence of estrogen.

Specific Aim 3, Task 1 and Task 2: not initiated yet.

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?**

For the next period, we plan to perform the following studies, as per the SOW:

Specific Aim 1, Tasks 1 and 2: We will continue with our analyses aimed at defining the association of DDR expression in primary tumors with presence of bone metastases (Dr. Kleer). We also plan to evaluate DDR2 expression in the same TMA already analyzed by IHC for DDR1, and evaluate its association with malignant progression and development for bone metastatic disease. We are currently in the process of identifying primary carcinomas and matching bone metastasis to study the expression of DDR1 in these samples, which will be performed in the next grant cycle. All collected data will be analyzed by the biostatistician to determine the associations between DDR 1 and DDR2 expressions (in isolation or in combination) and molecular subtypes of BrCa, development of bone metastasis, and disease-free and overall survival.

Specific Aim 2.

Task 1: We will examine the role of DDR1 in MCF7-Luc cells in various *in vitro* assays of cell behavior. We will downregulate DDR1 expression in the MCF7-Luc cells to complement the studies with the kinase inhibitor. Upon selection, of a DDR2-expressing BrCa cell line that can grow within the bone, we will conduct similar studies. In this regard, we recently received an MDA-MB-231-derivative "MDA GFP Bone C2" kindly provided by Dr. Julie Anne Sterling, Vanderbilt University School of Medicine. These MDA-MB-231 variant displays selective growth in bone over the parental cells. Indeed, these cells grow rapidly intraosseously, as evidenced by *in vivo* fluorescence imaging, and cause osteolysis in about 3 weeks after intratibial injection in nude mice (Dr. J. A. Sterling, Vanderbilt University School of Medicine, personal communication). We are currently testing these cells for DDR expression and activation. If these cells express DDR2 we will downregulate the receptor to test them in mice.

Task 2: In this report, we demonstrated the ability of the small molecule inhibitor for DDR1 obtained from Roche to efficiently block the activation of DDR1 in response to collagen. Based on these *in vitro* results and the pilot intratibial studies performed with the MCF-7-Luc cells, we plan to test the role of DDR1 in intraosseous growth of these cells in mice treated with the kinase inhibitor.

Specific Aim 3.

Task 1: We will follow with the studies proposed in SOW examining the role of DDR activation on the expression of pro-osteolytic factors in the BrCa cells .

Task 2: We plan to conduct the studies proposed. Specifically, we will evaluate the role of conditioned media (CM) obtained from BrCa cells with defined/controlled DDR1 or DDR2

expression and activation on the regulation of osteoclastogenesis in murine pre-osteoclastic RAW264.7 and primary monocytic bone marrow cells. We will also compare relative gene expression of markers of osteoclast differentiation in BrCa with defined/controlled DDR1 or DDR2 expression/activation using real-time PCR. In addition, we will investigate the effect of BrCa-derived CM on osteoblast differentiation using mouse pre-osteoblastic MC3T3-E1 cells in mineralization assays, and assess their effect on the expression of osteoblast differentiation markers.

4. IMPACT

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

Nothing to report.

- **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

Our goal is to examine the role of both DDR1 and DDR2 in intraosseous tumor growth in both ER+, and ER- (TNBC) cells. While we are now set to test ER+ DDR1-expressing MCF7-Luc cells, we had problems with the MDA-MB-231-Luc cells we obtained from Cell Biolabs in regards to their lack of DDR expression. Therefore, these cells cannot be used to evaluate DDR's role if they do not express these receptors. We know that the parental MDA-MB-231 cells do express DDR1 but not DDR2. Therefore it appears that during the generation of the transfection of luciferase, these cells lost DDR1 expression. As stated earlier, we just obtained a new set of MDA-MB-231 cells from Dr. Sterling, which we are testing for DDR expression. If these cells express and activate DDRs, then we will use them in our mouse studies. If DDRs are not expressed, we will express luciferase in our MDA-MB-231 cells, which express DDR1. Alternately, we could introduce DDR1 in the cells from Dr. Sterling. Once this has been accomplished, we will test then for growth within bone.

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

See above section.

- **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**

Nothing to report.

- **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	Project Role	Nearest Person Months Worked	Contribution to the Project	Funding Support
Ricardo Daniel Bonfil	Initiating PI	1.2	Design of experiments and data analyses	This grant
Rafael Fridman	Partnering PI	0.72	Design of experiments and data analyses	This grant
Allen Saliganan	Research Assistant	6.6	Animal studies, tissue processing, immunohistochemical (IHC) assays	This grant
Anjum Sohail	Research Scientist	4.2	Expression/activation analysis of BrCa cells, analysis of pro-osteolytic factors	This grant
Celina Kleer	Co-I	0.6	Evaluation and analysis of IHC data obtained for TMA and association with histopathological features	This grant (subcontract)
Maria E. Gonzalez	Research Associate	4.8	TMA construction, evaluation of IHC data	This grant (subcontract)

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

Ricardo Daniel Bonfil, Initiating PI in this grant:

Agency: National Cancer Institute (NCI), National Institutes of Health (NIH)
Grant # RO1 CA123362-05
PI: H-R. Kim
Title: PDGF D and prostate cancer progression.
Period: 05/01/2016-04/30/21
Effort: 0.6 Calendar Months
Role: Co-Investigator

Rafael Fridman, Partnering PI in this grant:

Nothing to report.

Celina Kleer, Co-Investigator in this grant:

Agency: NCI/NIH
Grant#: R01 CA107469
Title: Role of EZH2 in breast cancer progression
Period: 2/1/05-6/30/21
Effort: 2.50 Calendar Months
Role: PI

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

Organization Name:	Hoffmann-La Roche
Location of organization:	Basel, Switzerland
Partner's contribution to the project:	Supplied antibodies for DDR1 and a small molecule inhibitor for DDR1.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.