

AWARD NUMBER: W81XWH-14-1-0321

TITLE: Wnt Signaling in Prostate Cancer Bone Metastases

PRINCIPAL INVESTIGATOR: Wachiraphan Supsavhad

CONTRACTING ORGANIZATION: The Ohio State University
Columbus, OH 43210

REPORT DATE: September 2015

TYPE OF REPORT: Annual progress report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE September 2015		2. REPORT TYPE Annual Report		3. DATES COVERED 1 Sep 2014 - 31 Aug 2015	
4. TITLE AND SUBTITLE Wnt Signaling in Prostate Cancer Bone Metastases				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0321	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wachiraphan Supsavhad E-Mail: Supsavhad.1@osu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) College of Veterinary Medicine, The Ohio State University 1925 Coffey Road Columbus OH 43210				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Ace-1-Dkk-1, a canine prostate cancer overexpressing Dkk-1 is used in this study to investigate how enhanced Wnt/JNK signaling could alter metastasis and the bone microenvironment. Evidence was found that Dkk-1 up-regulated the non-canonical Wnt/JNK pathway resulting in downstream alterations in gene expression important in osteoblast stimulation, cell proliferation and epithelial to mesenchymal transformation of tumor cells. Inhibiting non-canonical Wnt/JNK signaling using SP600125, a JNK inhibitor, did not cause the canonical Wnt signaling to resume in Ace-1-Dkk1 cells. However, SP600125 significantly increased the mRNA expression of genes that induce osteoblast differentiation as well as decreased osteolytic genes (decreased RANKL:OPG ratio) in both Ace-1-Dkk1 and Ace-1-Vector cells.					
15. SUBJECT TERMS Prostate cancer, Bone Metastasis, Wnt signaling					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4-9
4. Impact.....	9
5. Changes/Problems.....	9
6. Products.....	10
7. Participants & Other Collaborating Organizations.....	10
8. Special Reporting Requirements.....	10
9. Appendices.....	

1.Introduction

Prostate cancer is the second most common cancer in men worldwide. Approximately 17% of patients with prostate cancer die from metastatic disease, and bone is the most common metastatic site. Even though studies have shown the promising connections between bone metastasis and bone microenvironment in this cancer, much more information needs to be clarified. Canonical Wnt signaling pathway is an important pathway in bone microenvironment regulation as well as in new bone formation and osteoblast differentiation. In prostate cancer, canonical Wnt signaling has also been speculated to have a role in osteoblast differentiation and the regulation of osteoblastic bone metastases. How canonical Wnt signaling regulates new bone formation and how other non-canonical Wnt signaling pathways function in bone metastatic cancers are poorly understood. The roles of Dkk-1 (Dickkopf-1) in canonical Wnt signaling inhibition and non-canonical Wnt/JNK pathway up-regulation have been reported. Alteration of Dkk-1 expression in prostate cancer could change the metastatic phenotype and tumor growth in vivo. Ace-1-Dkk-1, a canine prostate cancer overexpressing Dkk-1, previously developed in our lab was used in this study to investigate the role of the Wnt signaling pathways in prostate cancer bone metastases. This study aims to elucidating how enhanced Wnt/JNK signaling could alter metastasis and the bone microenvironment.

2.Keywords

Prostate cancer, bone metastases, Wnt signaling

3.Accomplishments

What were the major goals of the projects?

Aim 1. Determine the role of the non-canonical Wnt pathway on prostate cancer cell proliferation.

Aim 1.1: Investigate the expression of Dkk-1, non-canonical Wnt/JNK and canonical Wnt signaling pathways, and proliferation in the Ace-1 and the Dkk-1-transfected Ace-1 (Ace-1-Dkk-1) cell lines in vitro and in vivo.

Aim 1.2: Investigate the gene expression effect of inhibiting the non-canonical Wnt/JNK signaling pathway in Ace-1-Vector and Ace-1-Dkk-1 cell lines.

(1) Inhibit non-canonical Wnt/JNK signaling pathway in Ace-1-Vector and Ace-1-Dkk-1 cell lines with the JNK inhibitor (SP600125).

(2) Inhibit non-canonical Wnt/JNK signaling pathway in Ace-1-VectorAP-1 and Ace-1-Dkk-1AP-1 cell lines with the anti-Dkk1-antibody.

Aim 2. Determine the role of the canonical Wnt pathway on the bone microenvironment in prostate cancer.

Aim 2.1: Measure canonical Wnt signaling in osteoblasts from calvarial bone of mouse pups that have been co-cultured with or without Dkk-1 conditioned media.

Aim 2.2: Measure osteoblast and osteoclast function in calvaria co-cultured with and without Dkk-1.

Aim 3. Determine the effects of the bone microenvironment on prostate cancer cell proliferation and gene expression

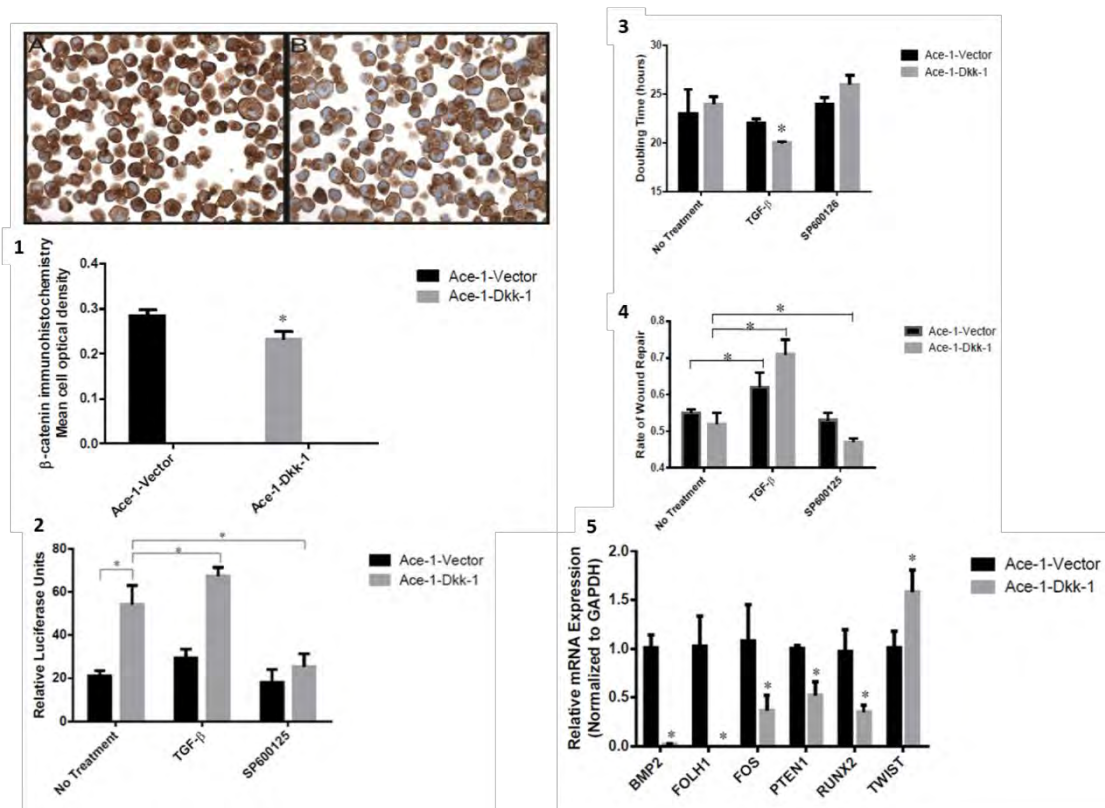
Timeline

Plans	2015									2016						
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Aim 1.1																
Aim 1.2																
Aim 2.1																
Aim 2.2																
Aim 3																
Analyze data																
Writing paper																

What was accomplished under these goals?

Aim 1.1: Investigate the expression of Dkk-1, non-canonical Wnt/JNK and canonical Wnt signaling pathways, and proliferation in the Ace-1 and the Dkk-1-transfected Ace-1 (Ace-1-Dkk-1) cell lines in vitro and in vivo.

Dr. Jessica Simmons found that Ace-1-Dkk-1 has significantly lower beta-catenin immunostaining intensity than Ace-1-vector cells and AP-1 activity in Ace-1-Dkk1 cells was significantly greater than in Ace-1-vector cells (Figure 1). AP-1 activity as well as cell migration rate in Ace-1-Dkk-1 cells were significantly decreased after treated with SP600125 (Figure 2 and 4). Moreover, mRNA expression levels of bone-related genes, including BMP2 and Rux2, as well as prostate specific membrane antigen gene (FOLH) were significantly decreased in Ace-1-Dkk-1 compared to Ace-1-vector cells (Figure 5).

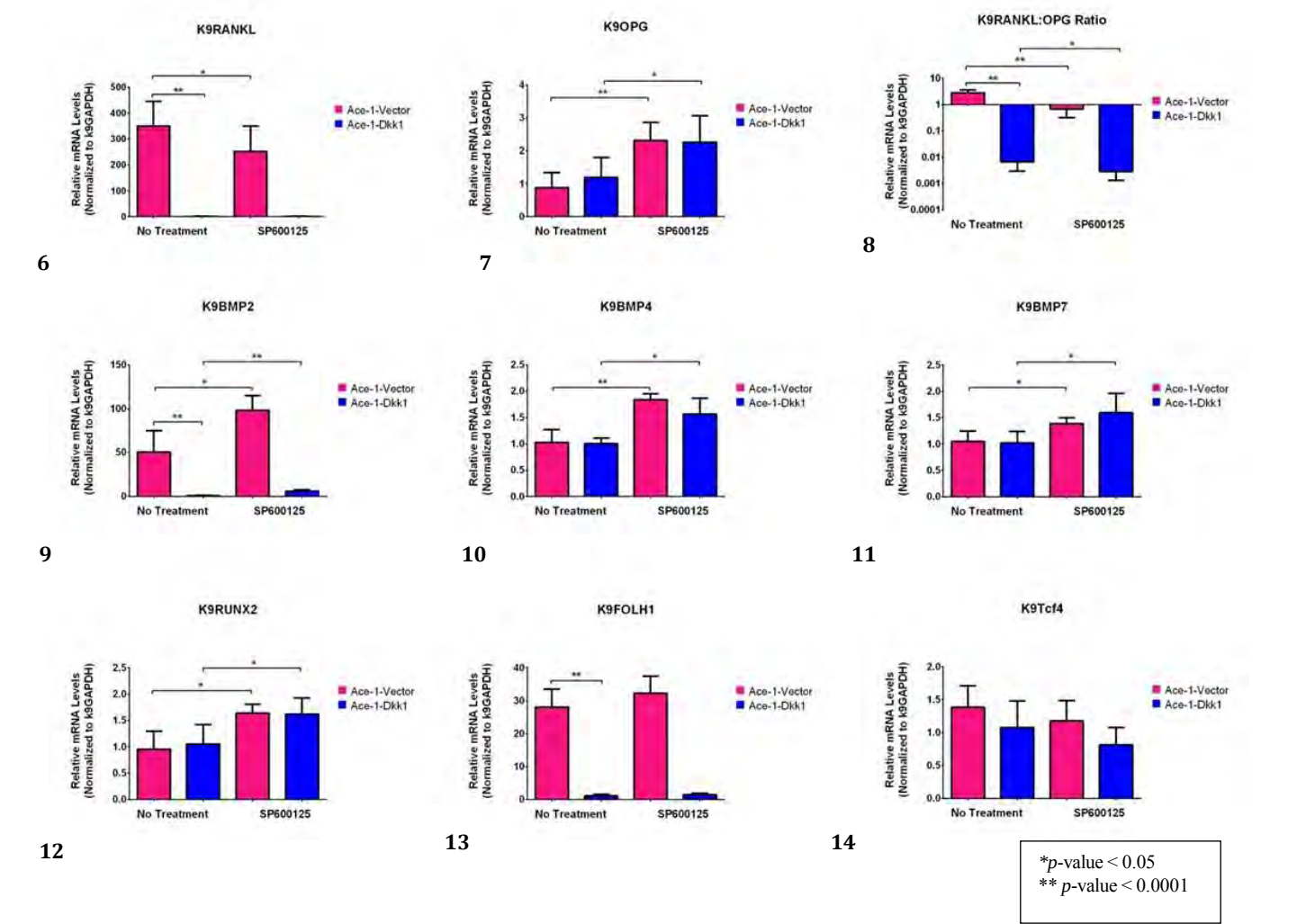


Based on these results, we hypothesized that:

- 1) Overexpress of Dkk1 would cause a switch from canonical Wnt to non-canonical Wnt signaling in Ace-1 cells.
- 2) Inhibiting non-canonical Wnt signaling using a JNK inhibitor or anti-Dkk1-antibody would resume canonical signaling in Ace-1-Dkk1 cells.
- 3) Targeting the Wnt/JNK signaling pathway will provide a novel therapeutic pathway to reduce prostate cancer proliferation and metastasis in dogs and men.
- 4) Treatment of Ace-1-Dkk1 with a JNK inhibitor will decrease metastasis of cancer cells to bone.

Aim 1.2: Investigate the effect of inhibiting the non-canonical Wnt/JNK signaling pathway in Ace-1-Vector and Ace-1-Dkk-1 cell lines on gene expression.

SP600125 significantly increased the relative mRNA expression levels of genes that related to osteoblast differentiation (BMP2, BMP4, BMP7, and RUNX2) in both Ace-1Dkk-1 and Ace-1-Vector cells (figure 9-12). This drug decreased Tcf4 mRNA in both Ace-1-Dkk-1 and Ace-1-pcDNA cells but had no effect on K9FOLH1 mRNA expression levels (Figure 13, 14). Interestingly, the RANKL:OPG mRNA expression ratio, an indicator of osteolytic bone resorption, was significantly decreased after treatment with SP600125 in both cell lines (Figure 6-8).



Conclusion:

SP600125 which inhibited the non-canonical Wnt/JNK signaling did not cause the canonical Wnt signaling to resume in Ace-1-Dkk1 cells. However, we hypothesize that SP600125 treatment can be used to increase bone formation, decreased bone lysis, and inhibit bone metastasis in both Ace-1-Vector and Ace-1-Dkk1 cells in vivo.

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

This project has provided me the opportunities to development new laboratory skills related to investigations on bone, including in vitro bone resorption experiments. In addition, I have been able to spend more times to discuss my work results and progress with my mentor, Dr. Thomas Rosol.

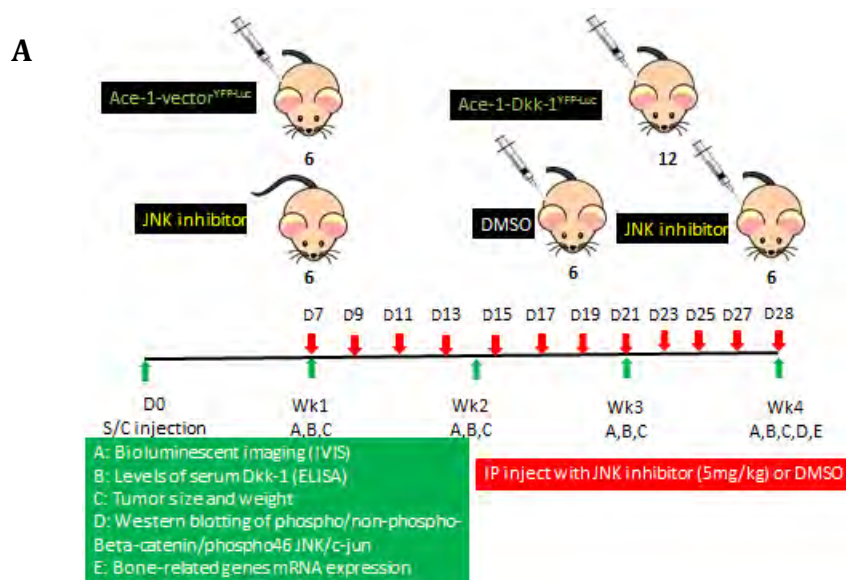
How were results disseminated to communities of interest?

The research results have been shared at the 2014 Annual Meeting of the Society for Toxicologic Pathologists in Washington DC.

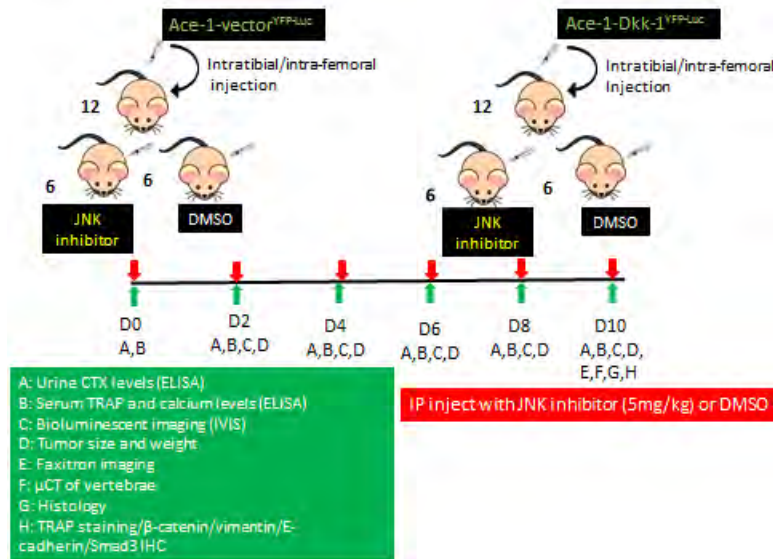
What do you plan to do during the next reporting period to accomplish the goals?

Plans:

(1) In vivo study: Ace-1-Dkk1^{YFP-Luc} and Ace-1-vector^{YFP-Luc} cells will be injected subcutaneously (A) and intratibially (B) injected into male nude mice and then treated with SP600125 (5mg/kg, intraperitoneal (IP)) for studying the effect of the JNK inhibitor (SP600125) on tumor growth rate, bone metastasis, mRNA expression levels as well as biological and bone microenvironment effects in vivo.



B



(2) Inhibit non-canonical Wnt/JNK signaling pathway in Ace-1-VectorAP-1 and Ace-1-Dkk-1AP-1 cell lines with the natural ligand blocking Anti-Dkk1-antibody.

No.	Works	
1	Perform titration experiment for natural ligand binding anti-Dkk1-antibody	3 week
2	Grow Ace-1-Dkk1 and Ace-1-Vector cell lines in 6 well-plates and then treat 70% confluence of Ace-1-Dkk1 and Ace-1-Vector cell lines with aniti-Dkk1-antibody	3 weeks
3	Repeat work (1) in 2 different passages of cell lines	2 weeks
4	Isolate RNA sample from treatment and non-treatment cell lines (from 1 and 2)	1 week
5	RNA purification	
6	RNA quantification	
7	Covert RNA to complimentary DNA (cDNA) using Reverse transcriptase PCR (RT-PCR)	1 week
8	Quantify mRNA expression levels of interested bone-related genes by using real time PCR	2 weeks
9	Analyze data	1 week

(3) Measure canonical Wnt signaling in osteoblasts from calvarial bones of mouse pups that have been co-cultured with or without Dkk-1 conditioned media.

(4) Measure osteoblast and osteoclast function in calvaria co-culture with and without Dkk-1.

No.	Works	
1	Grow Ace-1-Dkk1 and Ace-1-Vector cell lines in 75cm3 Flasks	3 weeks
2	Perform condition cell culture medium with non-FBS medium for 48 hours then freeze the conditioned medium in -80°C	3 weeks

3	Collect calvarial bone samples from 7 days old mice pups	
4	Co-culture calvarial bones (from 3) in the mixture between conditioned medium and bone culture medium in 48 wells plate for 4-7 days	1 week
5	Take Faxitron radiographic imaging for all samples	1 week
6	Quantify osteoclast activity in calvaria samples using TRAP staining	1 week
7	Visualize and measure osteoblast by alkaline phosphatase (ALP) staining	1 week
8	Measure calcium levels in calvaria co-culture medium using ELISA kit	1 week
9	Measure OPG:RANKL ratio in calvaria co-culture medium using ELISA and Western blot	1 week
10	Analyze data	1 week

4.Impact

What was the impact on the development of the principle discipline 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 sciences and technology?

Nothing to Report

5.Changes/Problems

Change in approach and reasons for change

Nothing to Report

Actual or anticipated problem or delay and action plans to solve them

Nothing to Report

Change that had significant impact on expenditures

Nothing to Report

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

No change

6.Products

Publications, conference paper, and presentations

Journal publication: Nothing to Report

Book or other non-periodical, one-time publications: Nothing to Report

Other publications, conference papers, and presentations:

Wnt Signaling in Prostate Cancer Bone Metastasis, Jessica Simmons, Wessel Dirksen, Thomas Rosol, The Ohio State University, Columbus, OH, USA (Poster presentation at the 2014 Annual Meeting of the Society for Toxicologic Pathologists in Washington DC.)

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?

PI has been changed from Dr. Jessica Simmons to Dr. Wachiraphan Supsavhad since April 1st 2015.

Name:	Wachiraphan Supsavhad, DVM, MS
Project Role:	PhD Graduate Student
Research ID:	0000-0001-6462-5139
Nearest person month	6 months
Contribution to project:	Provides majority of laboratory work for the project. Mentored by Dr.
Funding support:	Nothing in addition to the DOD fellowship.

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

Nothing to report

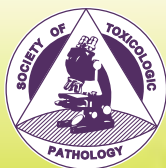
Quad chart

Nothing to report

9. Appendice

Poster abstract; 33rd annual symposium Society of Toxicologic Pathology

Wnt Signaling in Prostate Cancer Bone Metastasis by Jessica Simmons, Wessel Dirksen, Thomas Rosol. The Ohio State University, Columbus, OH, USA



Poster Abstracts

33rd Annual Symposium



TRANSLATIONAL PATHOLOGY:
Relevance of Toxicologic Pathology
to Human Health

Washington, DC

Marriott Wardman Park Hotel

June 22–26, 2014

11

Wnt Signaling in Prostate Cancer Bone Metastasis

Category: Oncology/Carcinogenesis

Jessica Simmons¹, Wessel Dirksen¹, Thomas Rosol¹

¹The Ohio State University, Columbus, OH, USA

Introduction: The molecular mechanisms by which prostate cancer cells metastasize and grow in bone are not fully understood, however we hypothesized that the Wnt signaling pathways play an important role in the pathogenesis. To investigate the contribution of the Wnt signaling pathways in prostate cancer bone metastases, we over-expressed the Wnt/JNK pathway agonist, Dkk-1, in the mixed osteoblastic and osteolytic Ace-1 prostate cancer cells. Previous work had shown that Dkk-1 expression increased the number and lytic nature of bone metastases in vivo. This study focused on elucidating how enhanced Wnt/JNK signaling could be altering metastasis and the bone microenvironment.

Methods and Experimental Design: Ace-1 cells stably expressing human DKK-1 or empty vector were cultured in vitro. Wnt/JNK signaling was investigated by AP-1 reporter activity, Affymetrix mRNA microarray, and qRT-PCR. Treatment with a non-canonical Wnt agonist and antagonist were performed and the resultant changes in reporter activity, gene expression, proliferation and migration were investigated.

Results: DKK-1 significantly increased non-canonical Wnt/JNK signaling. Subsequent gene expression alterations include a dramatic decrease in mRNA expression of genes important in osteoblast maturation. Treatment with a Wnt/JNK agonist enhanced tumor cell proliferation and migration; this effect was reversed with the antagonist treatment.

Conclusion The present study showed that DKK-1 is a potent activator of non-canonical Wnt/JNK signaling and provides possible mechanisms whereby DKK-1 expression inhibits bone growth and enhance tumorigenesis in prostate cancer metastases.

Impact Statement: This research highlights a potential pathway to target to reduce the morbidity and mortality of prostate cancer bone metastases.