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TITLE: Notch Signaling in Prostate Cancer Cells Promotes Osteoblastic Metastasis

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<b>14. ABSTRACT</b> Better understanding of the host/tumor interactions that trigger and drive metastatic processes could provide avenues for improved therapeutic intervention. Overexpression of the activated form of Notch3 (NICD3) in PC3 cells decreased osteolytic lesions and decreased the number of osteoclasts in the tumor-bone microenvironment. Conversely, inhibition of Notch3 in PC3, 22rv1 and C42B cells with shRNA, promoted prostate cancer-induced osteolytic lesions when injected in the tibiae. Conditioned medium from PC3-NICD3 cells generated ALP-positive osteoblasts, and increased osteoblast proliferation in vitro, and this was associated with increase expression of Cyclins A, D and E. Conditioned medium from PC3-NICD3 cells also decreased osteoclasts and inhibited osteoclastogenesis, but had no effect on osteoclast apoptosis. PC3-NICD3 cells injected into tibiae expressed more human-specific MMP3 than tibiae injected with control cells. Conversely, PCa cells expressing Notch 3sh RNA expressed less of human-specific MMP-3. Notch signaling in PCa tumors probably favors osteoblastic metastasis by stimulating the production of MMP3 in the tumor microenvironment to inhibit osteoclast function and number while inducing osteoblast proliferation. Our results suggest that Notch signaling from cancer cells promotes osteoblastic metastasis and thus may be a therapeutic target for such metastatic lesions.						
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## 1. Introduction:

To address the clinical problem of disease progression in prostate cancer- induced bone metastasis, the fundamental question that my fellowship was designed to investigate is whether notch signaling from cancer cells promotes changes in the bone-tumor microenvironment, resulting in osteoblastic metastasis. Data from this designed project will also shed light on how the tumor microenvironment plays an important role in progression of PCa-induced osteoblastic metastasis.

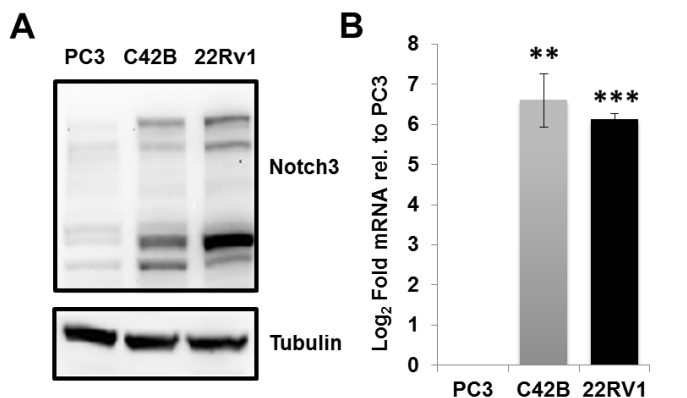
## 2. Keywords:

Prostate Cancer; Notch3; MMP3; Bone-Tumor microenvironment; Osteoblastic and Osteoclastic metastasis.

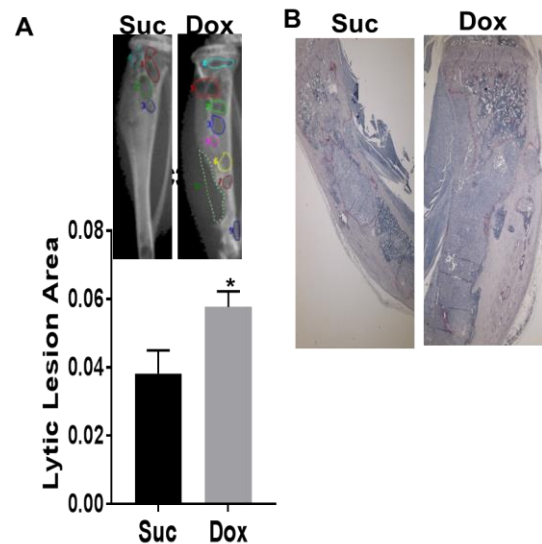
## 3. Accomplishments:

The major goal of this project is to investigate the role of Notch in prostate cancer induced-bone metastasis and investigate the mechanism how Notch signaling from prostate cancer cells crosstalk with the bone microenvironment and interact with osteoblasts and osteoclasts in promoting metastatic lesions.

I have accomplished in this first year of funding most of what had been outlined in my statement of work for the first year. The major objectives for first 12 months of the fellowship were to confirm my preliminary results and then investigate the mechanism by which Notch promotes metastatic lesions.



**Figure 1:** Osteoclastic lesion producing cells PC3 has the least amount of Notch3 expression. (A) PC3, 22Rv1 and C42B cells were lysed and whole cell lysate was subjected to SDS-PAGE analysis (B) and extracted RNA was subjected to q-RT-PCR. Mean  $\pm$  S.E.M,  $n \leq 3$ . \* $0.01 \leq p < 0.05$ ; \*\* $0.001 \leq p < 0.01$ ; \*\*\* $p < 0.001$



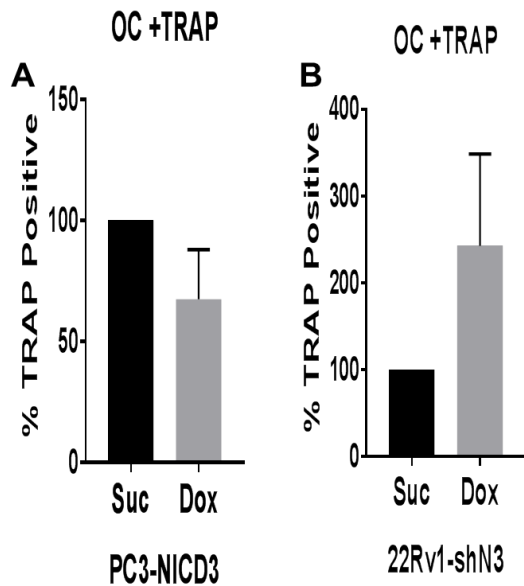
**Figure 2:** Inhibition of Notch3 promotes PC3-induced Osteolytic metastasis. A. Osteolytic lesions were measured on X-ray images using Metamorph software. Representative image at 3 weeks post-injection are shown  $n=8$ ,  $P^* < 0.05$  by student t test. B. TRAP staining of PC3-Notch3sh injected mice (+Dox) has more number of red stained multinucleate Ocs as compared to control mice (Sucrose).

Our findings during this past year have established the role of Notch3 in prostate cancer induced bone metastasis. We showed that Notch 3 expression was lower in osteolytic PC3 cells as compared to osteoblastic 22RV1 and C42B cells (Figure 1).

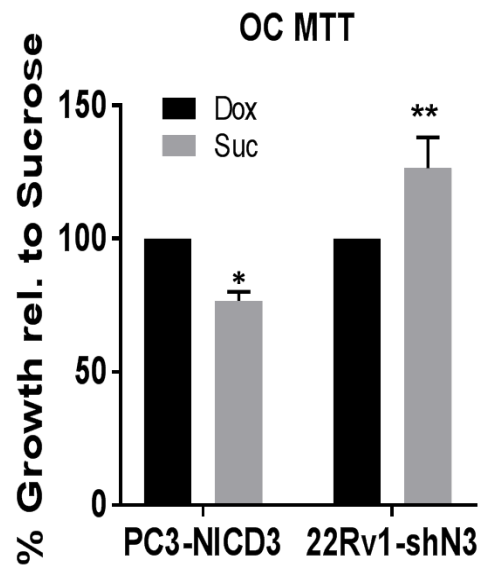
Our preliminary results during the fellowship application demonstrated intra-tibial injection of an overexpressed constitutive active form of Notch3 (NICD3) decreased bone lesions (shown in original application). We also conducted loss of function studies in which we showed that expression of a dox inducible Notch3 shRNA in PC3 (Figure 2) and C42B cells (shown in original application) promoted more osteoclastic lesions. All these data indicate that Notch3 decreased osteoclastic lesion to promote osteoblastic lesion development. The bones from these experiments were collected and scanned into micro-CT machine and I will be conducting analysis to get BT/TV and new total bone area in the next year of funding.

When we intratibially injected an overexpressed constitutive active form of Notch1 (NICD1) in PC3 cells, we did not see any significant changes in lesion development (not shown), indicating that all the Notch family members do not play the same roles in prostate induced bone lesion development. The project thus focused on the effect of Notch3 on prostate cancer-induced bone lesion development.

TRAP staining of tibiae harvested after injection of PC3NICD3, cells showed that there is an increased



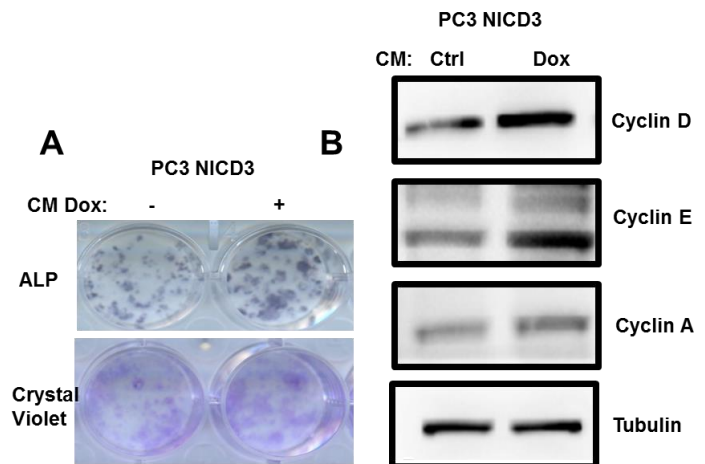
**Figure 3.** Primary osteoclasts were differentiated from flushed bone marrow of wild type mice in the presence of conditioned media from PC3-NICD3 expression cells (A) and conditioned media from 22Rv1 cells expressing Notch3sh (B) for 4 days in the presence of mCSF and RANKL. Mean  $\pm$  S.E.M, n  $\leq$  3. \*0.01  $\leq$  p < 0.05; \*\*0.001  $\leq$  p < 0.01; \*\*\*p < 0.001



**Figure 4:** Notch3 inhibits proliferation of Osteoclast cells. MTT assay was performed on primary osteoclasts cultures treated with conditioned media for 4 days from PC3-NICD3 cells (left) and 22Rv1-Notch3sh cells (right) respectively. Mean  $\pm$  S.E.M, n  $\leq$  3. \*0.01  $\leq$  p < 0.05; \*\*0.001  $\leq$  p < 0.01; \*\*\*p < 0.001

number of osteoclasts PC3NICD3 (shown in original application) and fewer number of TRAP positive osteoclasts were observed in tibiae injected with C42B and PC3Notch3sh injected tibiae (Figure 2B top). I will be performing Bioquant analysis of these stained tibiae to calculate the total number of osteoclasts. Consistent with our TRAP results from cancer injected tibiae we found out that there was a significant decrease in TRAP positive osteoclasts when bone marrow cells were differentiated into osteoclasts in the presence of conditioned media from PC3NICD3 cells (Figure 3A). Conditioned media from 22Rv1 cells expressing Notch3shRNA promoted the number of TRAP positive osteoclasts, consistent with our *in vivo* findings (Figure 3B). PC3NICD3 conditioned media also inhibited osteoclast proliferation; however, no apoptosis was observed. (Figure 4).

To investigate whether signaling from Notch3 in cancer cells promoted the functional activity of osteoblasts, conditioned media from PC3NICD3 were incubated with *in vitro*



**Figure 5.** NICD3 promotes Osteoblast activity and proliferation. Primary osteoblasts were differentiated from flushed bone marrow of wild type mice in the presence of conditioned media from PC3-NICD3 expression cells for 7 days in the presence of Vitamin C and stained for ALP and Crystal Violet (A). (B) Extracted protein was subjected to SDS-PAGE and blotted for Cyclin A, D and E.

differentiated osteoblasts. ALP activity, a marker for osteoblasts differentiation was increased in osteoblasts when incubated with NICD3 conditioned media accompanied by an increase in osteoblast proliferation. NICD3 conditioned media also promoted the expression of Cyclins. (Figure 5).

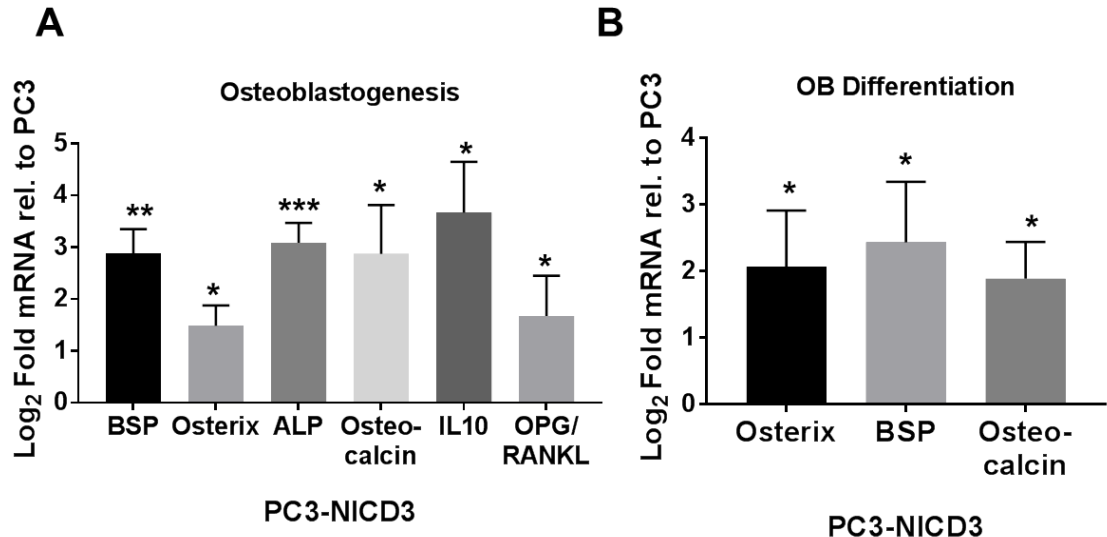
To investigate the effect of Notch3 signaling from prostate cancer cells to osteoblast and osteoclast differentiated cells *in vivo*, RNA was extracted from tibiae injected with PC3NICD3 cells. Using mouse-specific primers we found that PC3NICD3-harboring tibiae had significantly higher expression of osteoblastic markers and higher OPG/RANKL expression (marker for reduced osteoclastogenesis and increased osteoblastogenesis) and IL-10

(inhibitor of osteoclastogenesis) (Figure 6A). Consistent with our *in vivo* findings there was an increase of osteoblastogenesis markers when conditioned media from PC3NICD3 expressing cells were incubated with *in vitro* differentiated osteoblasts extracted from mouse bone marrow (Figure 6B).

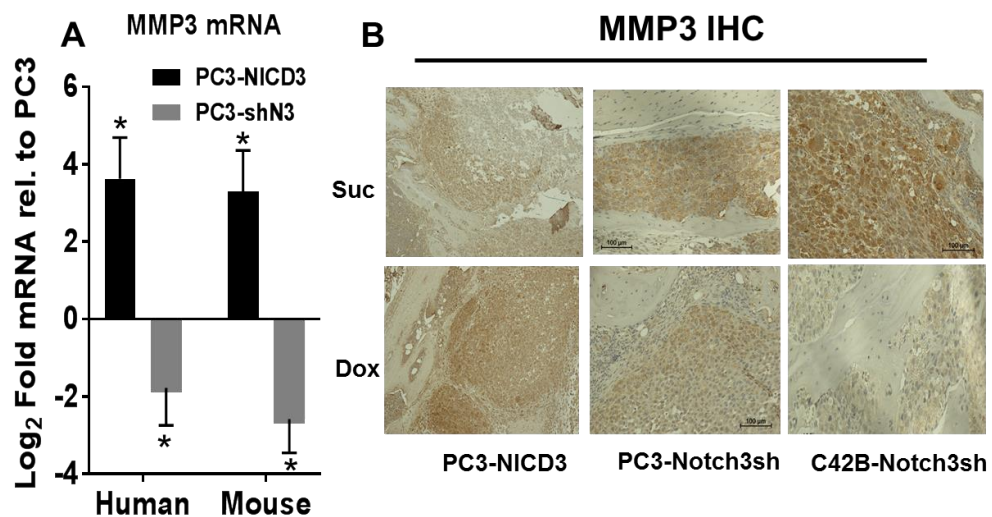
Additionally conditioned media from NICD3 cells decreased osteoclastogenesis markers when incubated with *in vitro* differentiated osteoclasts from mouse bone marrow (shown in original application). All these results indicate that Notch3 signaling from prostate cancer cells inhibits

osteoclastogenesis and promotes osteoblastogenesis to promote osteoblastic bone lesion development.

To examine the mechanism of Notch3-induced bone metastasis we found out that PC3NICD3 injected tibiae express more human specific MMP3 expression (both by RNA and IHC) and mouse-



**Figure 6.** NICD3 promotes osteoblast differentiation. (A) PC3 cells harboring Doxycycline-induced NICD3 were injected in tibiae of 5-6 week and RNA was extracted from harvested and subjected to qRT-PCR with mouse-specific primers. (B) Primary osteoblasts were differentiated from flushed bone marrow of wild type mice in the presence and absence of conditioned media from PC3-NICD3 expression cells for 7 days in the presence of Vitamin C extracted RNA was subjected to qRT-PCR. Mean  $\pm$  S.E.M, n  $\leq$  3. \*0.01  $\leq$  p < 0.05; \*\*0.001  $\leq$  p < 0.01; \*\*\*p < 0.001



**Figure 7.** Notch3 promotes the expression of MMP3. (A) RNA extracted from PC3-NICD3 and PC3Notch3sh injected tibiae with osteolytic lesions were subjected to qRT-PCR with human- and mouse-specific MMP-3 primers. (B) Harvested tibiae were formalin fixed and stained with MMP-3 antibody which recognizes human MMP-3. Mean  $\pm$  S.E.M, n  $\leq$  3. \*0.01  $\leq$  p < 0.05; \*\*0.001  $\leq$  p < 0.01; \*\*\*p < 0.001

specific MMP3 transcript (**Figure 7A**). Loss of function studies with PC3Notch3sh and C42B Notch3sh injected tibiae also showed reduced expression of MMP3 by IHC (**Figure 7B**).

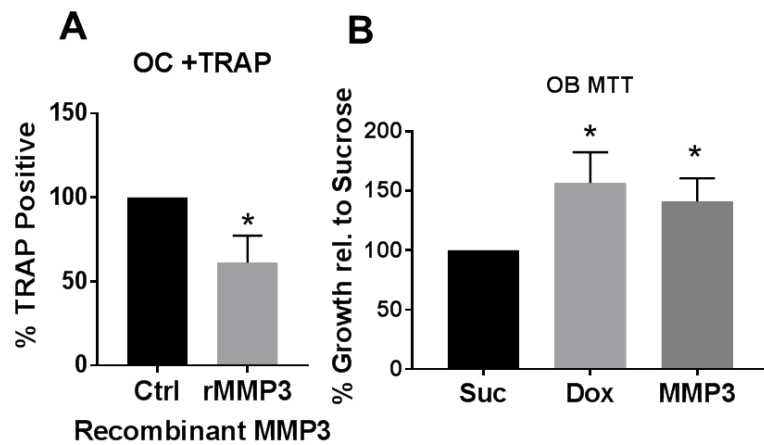
Using recombinant MMP3 protein in bone marrow cultures we also found out that MMP3 inhibits the expression of TRAP positive osteoclasts and also promotes osteoblast proliferation (**Figure 8**). These findings, of the effect of MMP3 on osteoblasts and osteoclasts cells, are consistent with our findings with the conditioned medium from NICD3 expressing cells (Figure 4 and 6B), indicating that Notch3 may exert its effects on the bone microenvironment in a MMP-3 dependent manner. To investigate whether Notch3 promotes bone lesion development in MMP3 dependent manner I am in the process of stably expressing MMP3sh in PC3NICD3 cells. These cells will be injected in mouse tibiae and we will investigate if it can rescue the block in osteoclastic lesion development caused by PC3NICD3 expressing cells. Also other in vitro osteoblast and osteoclast differentiation assay will be conducted with conditioned media from PC3NICD3-MMP3sh cells to investigate if Notch3 inhibits osteoclastogenesis and promotes osteoblastogenesis and osteoblast proliferation in a MMP3-dependent manner.

Professional Development: As part of the professional development and training during the course of the fellowship, both my mentors have given me ample opportunities for professional development. We have one-to one meetings once a week where we discuss data and future directions for this project. Both my mentors have always encouraged me and guided me for my progress in this and various other projects that I have taken.

I also participated in Society of Basic Urologic conference in 2016 as part of my professional development and plan to go to the same meeting this year. In our institute, other professional development courses were held (Career day workshop for post-docs, grant writing workshop) in which I also participated. As stated in my SOW for other training and educational development, I attended weekly meetings at the Skeletal Program meeting in our institute and also attended in our biweekly lab meeting. I have also presented my work in these institute meetings.

Dissemination of Data: My data was presented as a poster at the recent Cancer And Bone Society (CABS) meeting on metastatic and bone cancers. Abstract is in the Appendix. I am in the process of drafting a manuscript and will present a poster at the upcoming SBUR meeting. There is also an upcoming AACR Prostate Cancer Meeting in January, which I will consider attending to present a poster.

Future: In the next funding year, I plan to determine whether Notch3 and MMP3 expression are correlated in human samples via IHC. I have acquired samples from my collaborator Dr. Evan Keller (University of Michigan) and other TMA samples from PCBN network. I am in the process of optimizing the antibodies and will then stain the TMAs. I have mentioned in my SOW that I will investigate how MMP3 promotes osteoclast apoptosis; however we have confirmed that MMP3 do not promote osteoclast apoptosis rather promotes osteoblast proliferation and inhibit osteoclast proliferation. I will determine the mechanism by which MMP3 and Notch3 promotes osteoblast proliferation. All the work presented here will go towards my first manuscript. Additionally as mentioned in my SOW, I will also determine how Notch3-MMP3 promotes its effect on bone



**Figure 8.** MMP3 inhibits osteoclastogenesis and promotes osteoblast proliferation, (A) Primary osteoclasts were differentiated from flushed bone marrow of wild type mice in the presence recombinant MMP3 for 4 days in the presence of mCSF and RANKL, and stained for TRAP. TRAP positive cells were counted. (B) MTT assay was performed on primary osteoblasts cultures treated with conditioned media from PC3-NICD3 cells or rMMP3. Mean  $\pm$  S.E.M,  $n = 3$ . \* $0.01 \leq p < 0.05$ ; \*\* $0.001 \leq p < 0.01$ ; \*\*\* $p < 0.001$

microenvironment in a bFGF- dependent manner. Additionally I will determine how Notch3 promotes the expression of MMP3 in prostate cancer cells.

#### **4. Impact:**

Death from metastatic bone disease is due in part to the lack of effective therapies, the development of which requires knowing the signaling pathways that lead to this osteoblastic phenotype. Results from this project will determine how prostate cancer cells, when residing in the bone, will influence its surroundings, called the bone tumor microenvironment, in the formation of osteoblastic metastasis. Findings from this project will enhance the existing knowledge in the field of the prostate cancer-induced bone metastasis. Results from this study will shed light on how Notch3 signaling from cancer cells promote the expression of MMP3 which in turn effect the cells of the bone microenvironment to promote metastatic lesions. Results obtained from the proposed study will open the doors for future development of new therapies for prostate cancer patients with metastatic disease by targeting both the cancer cells and the associated bone microenvironment. Targeting the bone microenvironment can provide additional benefit, because unlike cancer cells the tumor microenvironment is not as genetically unstable and thus less likely to evade targeted therapeutic intervention. Even though the findings will be novel and clinically relevant, additional studies will be needed before a patient-related outcome can be achieved. However, findings from this grant will open new avenues for determining how cancer cells modulate the bone tumor microenvironment in the promotion of prostate cancer-induced osteoblastic metastasis to identify additional targets in the bone tumor microenvironment that could be used to treat patients with prostate cancer bone metastasis and thus improving the patients' quality of life.

I have nothing to report for impact towards other disciplines, impact on technology transfer and on society beyond science and technology.

#### **5. Changes/Problems:**

I have nothing to report for changes/problems encountered during the time of this fellowship.

#### **6. Products:**

CABS Abstract:

Ganguly, S.1, Li, X., and Miranti, C.K. 2017. Notch3 promotes Prostate Cancer-Induced Osteoblastic Bone Metastasis, Cancer and Bone Society Annual Meeting, Indianapolis, IN, May 4-6.

#### **7. Participants and other collaborating organizations:**

I initiated collaboration with Dr. Evan Keller at University of Michigan to interrogate PCa bone metastasis TMA for expression of Notch 3 and MMP3.

**Organization name:** University of Michigan.

**Location:** Ann Arbor, Michigan.

**Partner's Contribution to project:** In- kind support of providing TMAs to stain for Notch3 and MMP3.

#### **8. Special Reporting Requirements**

I have nothing to report.

## 9. Appendices

CABS Annual Conference: May 4-6, 2017 in Indianapolis, IN

### Notch3 promotes Prostate Cancer-Induced Osteoblastic Bone Metastasis

Ganguly, S.<sup>1</sup>, Li, X.<sup>1</sup>, and Miranti, C.K.<sup>1,2</sup>

<sup>1</sup>Van Andel Research Institute, Grand Rapids, MI

<sup>2</sup>University of Arizona Cancer Center, Tucson, AZ

**Background:** Notch signaling is dysregulated in bone metastatic prostate cancer (PCa), but how it contributes to bone metastasis is unknown. PCa bone metastasis is typically osteoblastic. The molecular basis for osteoblastic lesion formation remains poorly understood. In this study, we demonstrate that Notch3 activity in PCa tumor cells is responsible for driving an osteoblastic phenotype.

**Methods:** Several PCa cell lines, in which Notch3 signaling was suppressed by Tet-inducible shRNA or enhanced by expression of Tet-inducible NCID3, were injected into the tibiae of SCID mice. X-ray was used to monitor bone lesion development and osteolytic lesion area measured using Metamorph software. Harvested tibiae were subjected to histological analyses, qRT-PCR, or immunoblotting. We used cultured bone marrow from naïve mice to differentiate osteoblasts or osteoclast *in vitro* in the presence or absence of conditioned medium from Notch3 expressing cancer cells. The proliferation of osteoblasts or osteoclasts were measured by Crystal violet staining or MTT assays and differentiation monitored by ALP or TRAP staining, respectively.

**Results:** PCa cell lines that promote mixed osteoblastic bone lesions (C42B and 22RV1) express more Notch3 after intra-tibia injection relative to cell lines that promote osteolytic bone lesions (PC3). Overexpression of active Notch3 (NICD3) in PC3 cells decreased osteolytic lesions and decreased the number of osteoclasts in the tumor-bone microenvironment. Conversely, inhibition of Notch3 in PC3, 22rv1, or C42B cells with shRNA, promoted osteolytic lesions. Conditioned medium from PC3-NICD3 cells increased osteoblast proliferation *in vitro*, while conditioned medium from PC3-NICD3 cell inhibited osteoclastogenesis, but had no effect on osteoclast proliferation or apoptosis. Human MMP3 levels were elevated in tibia injected with PC3-NICD3 cells, whereas Notch3 shRNA tibia tumors expressed less MMP-3. Recombinant MMP3 blocked osteoclastogenesis and stimulated osteoblast proliferation *in vitro*.

**Conclusions:** Notch signaling in PCa tumors favors osteoblastic metastasis by stimulating the production of MMP3 and release into the tumor microenvironment to inhibit osteoclastogenesis while also inducing osteoblast proliferation.