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PRINCIPAL INVESTIGATOR: Sourik S. Ganguly

CONTRACTING ORGANIZATION: Van Andel Research Institute
Grand Rapids, MI 49503

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**6. AUTHOR(S)**

Sourik S. Ganguly, Xiaohong Li and Cindy K Miranti

E-Mail: sourik.ganguly@vai.org, xiaohong.li@vai,org, and cmiranti@email.arizona.edu

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Van Andel Research Institute,
333 Bostwick Avenue NE.
Grand Rapids, MI-49503

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**14. ABSTRACT**

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 osteoclastgenesis, 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.

**15. SUBJECT TERMS**

Prostate Cancer-induced bone metastasis; Notch3; MMP3; Osteoblasts and Osteoclasts

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## 1. Introduction:

To address the clinical problem of prostate cancer- induced bone metastasis, the fundamental question that my fellowship was designed to investigate is whether Notch3 signaling from cancer cells promotes changes in the bone-tumor microenvironment by inducing changes in the bone cells (osteoblasts and osteoclasts), resulting in osteoblastic metastasis. Data from this project will also shed light on the mechanism of how the cancer cells modulate the bone-tumor microenvironment, which plays an important role in progression of PCa-induced osteoblastic metastasis. We will also investigate whether the identified Notch3 and MMP3 proteins are upregulated in PCa-induced bone metastasis patient samples as compared to visceral metastasis.

## 2. Keywords:

Prostate Cancer-induced bone metastasis; Notch3; MMP3; Osteoblasts and Osteoclasts.

## 3. Accomplishments:

The major goal of this project is to investigate the role of Notch3 and MMP3 axis in prostate cancer induced-bone metastasis and investigate the mechanism for how Notch3 signaling from prostate cancer cells crosstalks with the bone microenvironment and interacts with the bone cells (osteoblasts and osteoclasts) in promoting bone metastatic lesions.

I have accomplished, what had been outlined in my statement of work during the time frame off my fellowship. I have one prepared manuscript, which encompasses most of all the findings from the fellowship. The manuscript was submitted to Clinical Cancer Research in May 2018 and was rejected after review and is now in review in Oncogene.

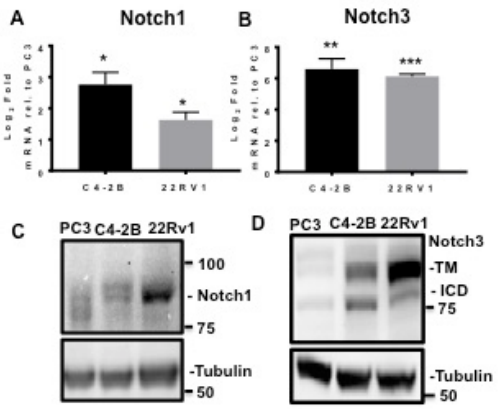


**Figure 1**: Osteoclastic lesion producing cells PC3 has the least amount of Notch1 and 3 expression. **(A -B)**PC3, 22Rv1 and C42B cells were lysed and extracted RNA was subjected to q-RT-PCR for Notch1 and 3 respectively (C-D) and whole cell lysate was subjected to SDS-PAGE analysis for Notch1 and Notch3 respectively. Mean ± S.E.M, n ≤ 3. *0.01 ≤ p <0.05; **0.001 ≤p<0.01; ***p<0.001

Our findings have established a role for the Notch3-MMP3 axis in prostate cancer induced bone metastasis. We showed that Notch1 and 3 expression is lower in osteolytic-inducing PC3 cells as compared to osteoblastic inducing 22RV1 and C42B cells, both at mRNA and protein levels (**Figure1**), indicating that Notch might be an important factor is inducing osteoblastic metastasis.

My 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) (**Figure 2A-C**). We subsequently conducted corollary loss of function studies in which we show that expression of a Dox inducible Notch3 shRNA in PC3 (**Figure 2 D-F**), 22Rv1, and C42B cells (**Figure 3 A-E**) promoted more osteoclastic lesion development. To determine whether the effects on osteolytic lesion development by NICD3 or Notch3sh were due to changes in tumor proliferation, we monitored Ki67 expression by IHC staining. There was no difference in proliferation between control cells and NICD3-expressing tumors or Notch3sh-expressiong tumors (data not shown). Also the observed changes in lesion area and development were not due to non-specific effects of doxycycline, as doxycycline treatment of mice injected with non-NICD expressing parental PC3 cells did not alter osteolytic lesion formation. (**Figure 4A).** All these findings indicate that Notch3 decreased osteoclastic lesion to promote osteoblastic lesion development. Active osteoclasts on the bone tumor interface were quantified and PC3-NICD3 significantly inhibited the osteoclast number along the bone tumor interface, while

shNotch3 expression in PC3, 22rV1, and C42B increased the number of mature multinucleate osteoclasts in the bone tumor microenvironment (**Figure. 2C,F and 3E**).

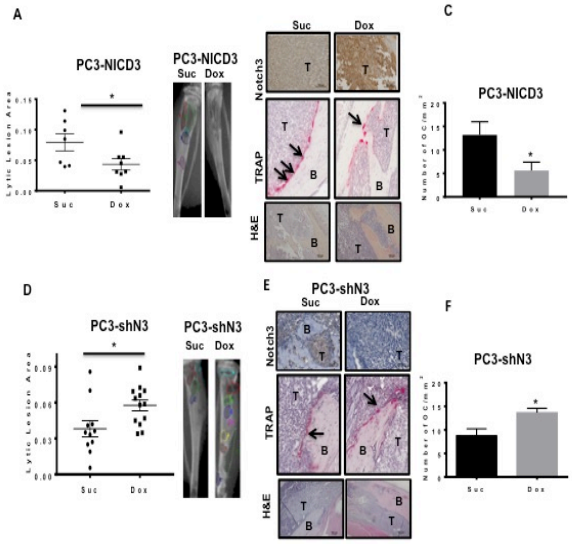


**Figure 2. NICD3 inhibits osteolytic lesion area (A-C)** PC3 cells harboring Tet-inducible NICD3 or (**D-F**) Notch3 shRNA (shN3) injected into tibiae of mice treated with sucrose (Suc) or doxycycline (Dox). X-rayed lytic lesion area (outlined) quantified. (**B,E**) Tibiae from (B) and (E) were stained with H&E (top), anti-Notch3 (middle), TRAP (bottom), and number of osteoclasts quantified (**C,F**). T=tumor; B=bone; arrows indicate TRAP+ osteoclasts. Error bars are S.E.M, n≥11; *0.01≤p<0.05; **0.001≤p<0.01; ***p<0.001.

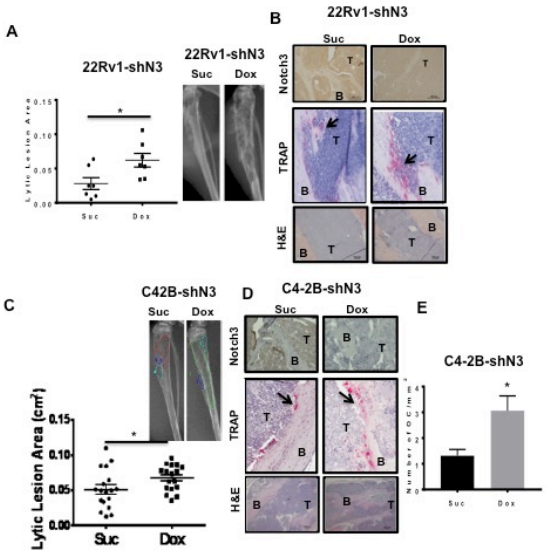


**Figure 3. Inhibiting Notch3 promotes osteolytic lesions.** (**A-B**) 22Rv1 or (**C-E**) C4-2B cells harboring doxycycline-induced Notch3 shRNA (shN3) injected into tibiae of mice treated with sucrose (Suc) or doxycycline (Dox). (**A, C**) X-rayed lytic lesion area (outlined) quantified. **(B, D)** Tibiae were stained with H& E (top), anti-Notch3 (middle), TRAP (bottom). (**E**) Number of osteoclasts quantified. T=tumor; B=bone; arrows indicate TRAP+ osteoclasts. Error bars are S.E.M, n≥7; *0.01≤p<0.05.

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 **(Figure 4B**), indicating that all the Notch family members do not play the same roles in prostate cancer-induced bone lesion development and demonstrating the specificity of Notch3 in the development of osteoblastic lesions.

Consistent with our *in vivo* TRAP staining results (**Figure 2-3**) from cancer injected tibiae we found 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 PC3-NICD3 (**Figure 5A**). Conversely, conditioned media from 22Rv1 and C42B cells expressing Notch3 shRNA increased the number of TRAP positive osteoclasts, consistent with our *in vivo* findings (**Figure 5 B-C**). We did not see any osteoclast apoptosis in these cultures (not shown).

To investigate how Notch3 signaling from cancer cells affects osteoblast function and activity, conditioned media from PC3-NICD3 were incubated with *in vitro* differentiated osteoblasts. ALP activity, a marker for osteoblast differentiation, was increased in osteoblasts when incubated with NICD3 conditioned media, accompanied by an increase in osteoblast proliferation as assessed by MTT assay. NICD3 conditioned media also promoted the expression of Cyclins in mature osteoblasts. **(Figure 6)**.

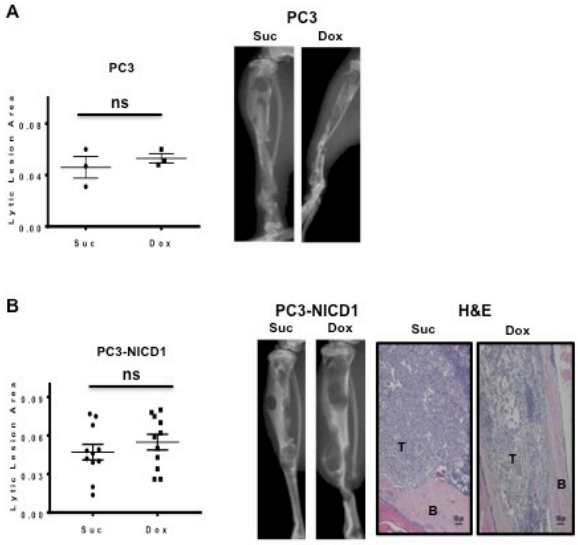


**Figure 4. NICD1 does not affect osteolytic lesion development.** (**A**) PC3 cells harboring Tet-induced NICD1 (PC3-NICD1) were injected into tibiae of 5-6 week old male NSG mice, treated with sucrose (Suc) and doxycycline (Dox) for 4 weeks, X-rayed, and lytic lesion area quantified. Representative X-ray images shown. (**B**) Parental PC3 cells were injected into the tibiae of mice and treated with sucrose (Suc) or doxycycline (Dox) for 3 weeks, X-rayed, and lytic lesions quantified. Representative X-ray images shown. Harvested tibiae were formalin fixed, decalcified and stained with H&E. T = tumor; B = bone. Error bars are S.E.M, (**C**) n = 3, (**D**) n ≥ 11. *0.01 ≤ p <0.05; **0.001 ≤p<0.01; ***p<0.001

To investigate the effect of Notch3 signaling from prostate cancer cells on osteoblast and osteoclast differentiated cells in vivo, RNA was extracted from tibiae injected with PC3-NICD3 cells. Using mouse-specific primers we found that PC3-NICD3-harboring tibiae had significantly higher expression of OPG/RANKL expression (marker for reduced osteoclastogensis and increased osteoblastogenesis) and IL-10 (inhibitor of osteoclastogenesis) (**Figure 7A-B**), and higher expression of osteoblastic markers (Bone-sialoprotein, ALP, and osteocalcin) (**Figure 7C**). Consistent with our *in vivo* findings there was also an increase of osteoblastogenesis markers when conditioned medium from PC3-NICD3 expressing cells were incubated with *in vitro* differentiated osteoblasts extracted from mouse bone marrow (**Figure 7D**). Osteoclacin, which is only expressed in mature osteoblasts, was upregulated by NICD3 conditioned medium indicating that NICD3 favors osteogenesis. Additionally, conditioned medium from NICD3 cells decreased osteoclastogensis markers, specifically Calcitonin Receptor, when incubated with *in vitro* differentiated osteoclasts from mouse bone marrow (**Figure7E**). All these results indicate that Notch3 signaling from prostate cancer cells inhibits osteoclastogenesis and enhances osteoblastogensis to promote osteoblastic bone lesion development

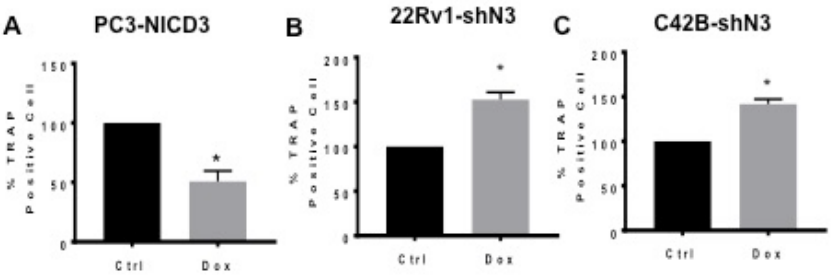


**Figure 5 NICD3 inhibits osteoclastogenesis. (A-C)** Bone marrow-derived osteoclasts differentiated in the presence of conditioned medium (CM) from doxycycline (Dox) or vehicle-treated (Ctrl) (**A**).PC3-NICD3 cells **(B)** 22Rv1-shN3 (Notch3 shRNA), **(C)** C42B-shN3.TRAP+ cells with 2 ≥ nuclei quantified.. Error bars are S.E.M, n=3; *0.01≤p<0.05;

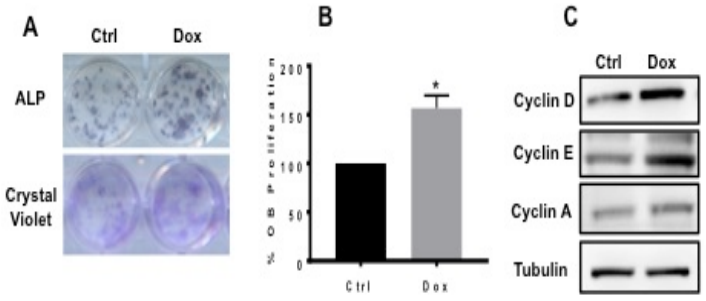


**Figure 6. NICD3 promotes osteoblastogenesis. (A-C).**Bone marrow-derived osteoblasts differentiated in the presence of conditioned medium (CM) from PC3-NICD3 cells treated with doxycycline (Dox) or vehicle (Ctrl). **(A)** Colonies were stained for ALP or crystal violet (CV). **(B)** MTT assay of treated osteoblast cultures. **(C)** Levels of Cyclin A, D, E and tubulin (Tub) from treated osteoblast cultures assessed by immunoblotting. Error bars are S.E.M, n=3; *0.01≤p<0.05.

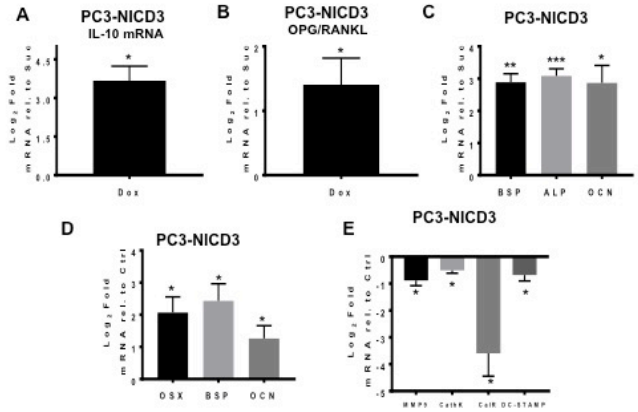


**Figure 7. NICD3 inhibits markers of osteoclastogenesis and promotes osteoblastogenesis markers. (A-C)** Expression of mouse-specific mRNA from tibiae of mice injected with PC3-NICD3 cells and treated with sucrose (Suc) or doxycycline (Dox): **(A)** IL-10, **(B)** OPG/RANKL ratio, and **(C)** bone sialoprotein (BSP), alkaline phosphatase (ALP), or osteocalcin (OCN). Expressed $Log_2$ fold relative to sucrose controls. **(D)** Bone marrow-derived osteoblasts differentiated in the presence of conditioned medium (CM) from PC3-NICD3 cells treated with doxycycline (Dox) or vehicle (Ctrl) were taken and expression of mouse-specific mRNA: osterix (OX), bone sialoprotein (BSP), or osteocalcin (OCN) were evaluated. Expressed $Log_2$ fold relative to vehicle controls. **(E)** Bone marrow-derived osteoclasts differentiated in the presence of conditioned medium (CM) from PC3NICD3 cells treated with doxycycline (Dox) or vehicle (Ctrl) were taken and expression of mouse-specific mRNA: MMP9, Cathepsin K, Calcitonin Receptor and DC-STAMP was evaluated . Error bars are S.E.M, n=3; *0.01≤p<0.05; **0.001≤p<0.01; ***p<0.001.

To examine the mechanism by which Notch3 promotes bone metastasis, we found that PC3-NICD3 injected tibiae express more human specific MMP-3 (by both qRT-RNA and IHC) (**Figure 8A,D**). Loss of function studies with PC3-Notch3sh (**Figure 8 B,E)** and C42B-Notch3sh (**Figure 8 C, F**) injected tibiae also showed reduced expression of MMP-3 by IHC and a-RT-RNA. PC3-NICD3 conditioned mediium also had higher expression of active-MMP3 as compared to its control, as detected by ELISA (not shown)

To study the effect of MMP-3 on bone cell differentiation/maturation, we used recombinant MMP-3 protein in bone marrow cultures. rMMP3 inhibits the expression of TRAP positive osteoclasts and promotes osteoblast proliferation; however, it did not have any effect on osteoblast differentiation (**Figure 9**). These findings (except osteoblast differentiation) are consistent with our findings with the conditioned medium from NICD3 expressing cells, indicating that Notch3 may exert its effects on the bone microenvironment in a MMP-3-dependent manner. To investigate the dependency of NICD3 on MMP-3 for its inhibitory effects on osteoclasts and promoting osteoblast proliferation, PC3-NICD3 cells were engineered to stably express MMP3 shRNA (shMMP3). Conditioned medium from doxycycline-treated PC3-NICD3-shMMP3 cells rescued the block in TRAP-positive multinucleate cell differentiation induced by conditioned medium from doxycycline-treated PC3-NICD3 cells (**Figure 10A**) and prevented the promotion of osteoblast proliferation (**Figure 10B**). We next tested whether inhibition of MMP-3 could also rescue the inhibition of NICD3-induced osteolytic lesion formation. Doxycycline-treated PC3-NICD3-shMMP3 tibial tumors had increased osteolytic lesion area relative to doxycycline-treated PC3-NICD3 (**Figure 10 C-D**). Altogether, these data indicate MMP-3 is required for Notch3-mediated inhibition of osteolytic bone lesion development by prostate tumors.

To measure the levels of Notch3 and MMP-

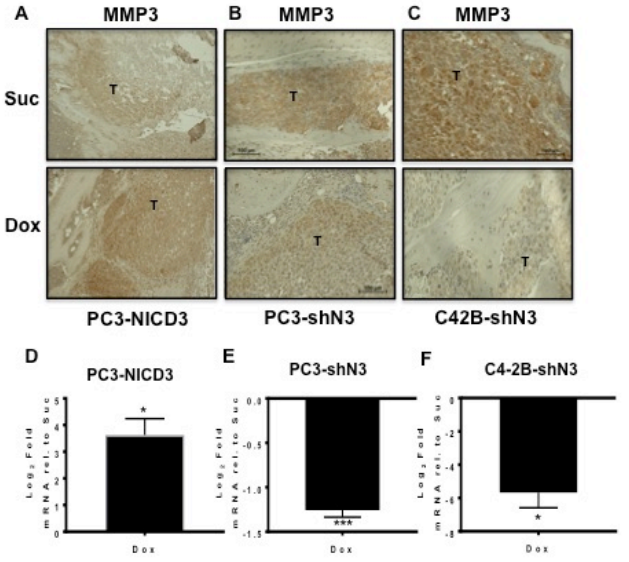


**Figure 8. Notch3 promotes the expression of MMP3.** **(A)** Tibiae from mice injected with PC3-NICD3, PC3-shN3, or C4-2B-shN3 cells treated with sucrose (Suc) or doxycycline (Dox) were stained with human-specific MMP-3 antibody. T=tumor **(B-D)** Levels of human-specific MMP-3 mRNA from tibiae in (A) assessed by qRT-PCR. Expressed $Log_2$ fold relative to sucrose controls. Error bars are S.E.M. n>4: *0.01≤p<0.05.

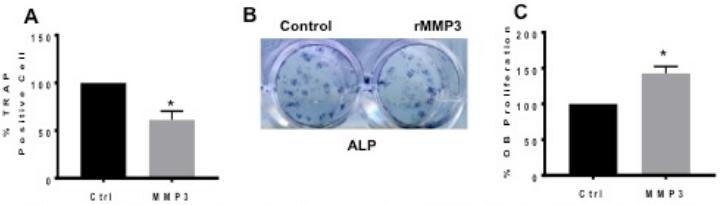


**Figure 9. MMP3 promotes osteoclastogensis and Osteoblast proliferation.** **(A)** Bone marrow-derived osteoclasts differentiated in the presence of 25 ng/ml recombinant human MMP-3 (rMMP3). Percentage of TRAP+ cells with 2 ≥ nuclei quantified. **(B-C)** Bone marrow-derived osteoblasts differentiated in the presence of recombinant MMP3 (rMMP3) and **(B)** immunostained for ALP or **(C)** proliferation measured by MTT assay.

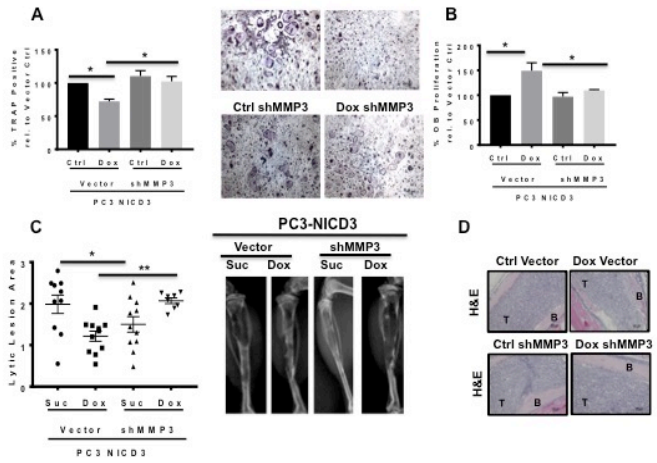


**Figure 10. Notch3 promotes osteoblastic lesion development in an MMP3-dependent manner.** Tet-inducible PC3-NICD3 cells engineered to stably express MMP-3 shRNA (PC3-NICD3-shMMP3) were generated and **(A)** Bone marrow-derived osteoclasts or **(B)** osteoblasts differentiated in the presence of conditioned medium (CM) from doxycycline-treated (Dox) or vehicle-treated (Ctrl) PC3-NICD3-vector or PC3-NICD3-shMMP3 cells. **(A)** Percentage of TRAP+ cells quantified. **(B)** MTT assay of treated osteoblasts. **(C)** Tet-inducible PC3-NICD3-vector and PC3-NICD3-shMMP3 injected into tibiae of mice treated with doxycycline (Dox) or sucrose (Suc). Lytic lesion area on X-ray quantified. **(D)** Tibial tumors from (C) were stained with H&E. Error bars are S.E.M, n≥8; *0.01≤ p<0.05; **0.001≤p<0.01.

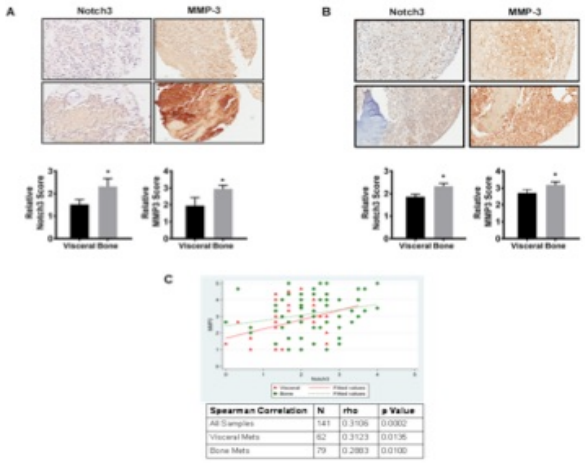


**Figure 11. Notch3 and MMP-3 expression are higher in osteoblastic bone metastases relative to visceral metastasis.** Tissue microarrays **(A)** TMA-170 and **(B)** UWTMA79 probed for Notch3 and MMP-3 expression by IHC. Levels of expression were compared between patient-matched visceral and bone metastases. **(C)** Frequency of Notch3 and MMP-3 co-expression across all samples. Error bars are S.E.M; *0.01≤ p<0.05.

3 in human prostate cancer bone metastases, two independent human tissue microarrays, TMA-170 (acquired from collaborator Dr. Evan Keller) with 10 matched visceral and bone metastases, and UWTMA79 (available from PCBN) with 30 matched visceral and bone metastases, were analyzed by IHC with antibodies to Notch3 or MMP3. Bone metastases expressed more Notch3 and MMP-3 compared to matched patient visceral metastases in both the arrays (**Figure 10 A,B**). Furthermore, there was a significant correlation between expression of Notch3 and MMP-3 in all metastases (**Figure 10 C**). Altogether, our findings indicate that a Notch3-MMP-3 axis plays an integral part in favoring osteoblastic lesion development.

Professional Development: As part of the professional development and training during the course of the fellowship, my mentor Dr. Cindy Miranti has given me ample opportunities for professional development. We have had one-on-one meetings once a week where we discuss data and future directions for this project. She has always encouraged me and guided me during my progress in this project and in various other projects that I have undertaken.

I also participated in Society of Basic Urologic conferences in 2016 and 2017 as part of my professional development. In 2017, at SBUR I presented the work funded by my fellowship and also received a travel award to attend the conference. In our institute, other professional development courses were held, Career Day Workshop for Post-docs and a grant-writing workshop, in which I also participated. As stated in my SOW for other training and educational development, I attended weekly meetings of the Skeletal Program in our institute and also attended our biweekly lab meetings. I presented my work in these institute meetings.

Dissemination of Data: My data was presented as a poster at the 2017 Cancer and Bone Society (CABS) meeting on metastatic and bone cancers and Annual SBUR meeting, 2017. Abstracts for CABS and SBUR-2017 are in the Appendix.

## 4. Impact:

Death from prostate cancer-induced bone metastatic is due in part to the lack of effective therapies, the development of which requires the understanding of the interaction of cancer cells with the bone microenvironment. Knowledge of the signaling pathways that lead to the unique osteoblastic phenotype in prostate cancer bone metastasis is an important factor for determining 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. Results from this project will determine how prostate cancer cells, when residing in the bone, will influence its bone tumor microenvironment, in the formation of osteoblastic metastasis. Results from this study indictate that the Notch3-MMP-3 axis induces changes in the bone microenvironment, to promote osteoblastic lesion development. These results 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 has additional advantages, because unlike cancer cells the host-tumor microenvironment is not as genetically unstable and thus is less likely to evade targeted therapeutic intervention. Findings from this study will open new possibilities for determining how prostate cancer cells modulate the bone cells in the promotion of prostate cancer-induced osteoblastic metastasis.

I have nothing to report for impact towards other disciplines, impact on technology transfer, and impacy 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.S., 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.

SBUR Abstract:
Ganguly, S.S., Li, X., and Miranti, C.K. 2017. Notch3 promotes Prostate Cancer-Induced Osteoblastic Bone Metastasis in a MMP3 dependent manner, Society of Basic Urologic meeting, Tampa, FL Nov 9-12.

Submitted Manuscript:
Ganguly, S.S., Hostetter, G., Tang, L., Frank, S.B., Saboda, K., Mehra, R., Wang, L., Li, X., Keller, E.T., and Miranti, C.K. 2018. Notch3 Promotes Prostate Cancer-Induced Bone Lesion Development via MMP-3. Oncogene, under review.

## 7. Participants and other collaborating organizations:

I initiated a collaboration with Dr. Evan Keller at University of Michigan to interrogate a 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 TMA-170 to stain for Notch3 and MMP3.

I initiated a collaboration with Dr. Kathylynn Saboda at University of Arizona to support the biostatistical analysis of the TMA.
**Organization name**: University of Arizona
**Location**: Tucson, Arizona
**Partner's Contribution to project**: Biostatistical analysis of TMA data

## 8. Special Reporting Requirements

I have nothing to report.

## 9. Appendices

Society of Basic Urologic Research (SBUR) Annual Meeting, Tampa, FL Nov 9-12, 2017

**Notch3 promotes Prostate Cancer-Induced Bone Metastasis in a MMP3-dependent manner**

**Ganguly, S.[1], Li, X.[1], and Miranti, C.K.[1,2]**
**[1]Van Andel Research Institute, Grand Rapids, MI**
**[2]University of Arizona Cancer Center, Tucson, AZ**

***Background:***
Notch signaling is known to be dysregulated in PCa, but its role in PCa-induced bone metastasis is not known. Currently approved therapies are focused on the targeting of events occurring inside the tumor and do not fully consider the contributions of the host microenvironment. Thus, better understanding of the host/tumor interactions that trigger and drive metastatic processes could provide additional avenues for therapeutic intervention.

***Methods:***
PCa cells, in which Notch3 expression was manipulated, were injected into the tibiae of SCID mice. Development of bone lesions was monitored by x-ray and measured using Metamorph software. The tibiae were harvested at end time points for histological analyses, qRT-PCR, or western blots. qRT-PCR and western blotting were performed on the homogenized tibiae to evaluate the molecular signaling. Cultured bone marrow from naïve mice was used for *in vitro* differentiation of osteoblasts or osteoclasts in the presence or absence of conditioned medium from cancer cells in which Notch3 expression was manipulated. The proliferations of osteoblasts or osteoclasts were read out by Crystal violet staining or MTT assay and the differentiations were read out by ALP or TRAP staining, respectively.

***Results:***
PCa cell lines that promote mixed osteoblastic bone lesions (C42B and 22RV1) express more Notch3 relative to cell lines, which promote osteolytic bone lesions (PC3). 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. Conditioned medium from PC3-NICD3 cells also decreased osteoclasts and inhibited osteoclastgenesis. 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 decreased human specific MMP3. PCa cells expressing Notch3sh promoted increased osteoclasts number *in vivo* and *in vitro.* Our results also indicate that NICD3 inhibits lytic lesion development and *in vitro* osteoclast differentiation in a cancer cell secreted MMP3-dependent manner.

***Conclusions:***
Notch signaling in PCa tumors 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.

Cancer and Bone Society (CABS) Meeting, Indianapolis, IN, May 4-6, 2017

# Notch3 Promotes Prostate Cancer-Induced Osteoblastic Bone Metastasis

**Ganguly, S.[1], Li, X.[1], and Miranti, C.K.[1,2]**
**[1]Van Andel Research Institute, Grand Rapids, MI**
**[2]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 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 osteoclastgenesis, 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.