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14. ABSTRACT
The purpose of this project is to investigate the functional and mechanistic roles of a neural enzyme monoamine oxidase A (MAOA) and its downstream neuronal effectors in mediating prostate cancer-nerve reciprocal interactions and prostate cancer metastasis. Throughout the Year 2 of this study, we demonstrated that MAOA promotes perineural invasion of prostate cancer cells in 3D prostate cancer-nerve cell co-cultures and that MAOA inhibitor clorgyline suppresses prostate cancer cell perineural invasion in vitro. By analyzing orthotopic xenograft tumor samples generated from the Year-1 study, we also showed that MAOA promotes tumor cell proliferation and nerve density, which could be inhibited by the MAOA inhibitor clorgyline, in xenograft tumors.

15. SUBJECT TERMS
Prostate cancer, monoamine oxidase A, perineural invasion, metastasis, clorgyline, xenograft mouse models

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1. INTRODUCTION: Monoamine oxidase A (MAOA), **the subject of the present study**, is a key neural enzyme that metabolizes monoamine neurotransmitters and regulates neurotransmission, neural circuits and brain function. We found that MAOA is increased in high-grade and metastatic prostate cancer (PC), particularly associated with perineural invasion (PNI), which may be mediated via MAOA's downstream neurotrophic and axon guidance factors, such as semaphorin 3C (SEMA3C), plexin A2 (PLXNA2) and neuropilin 1 (NRP1). **The purpose of this research** is twofold: 1) to define the functional and mechanistic roles of MAOA and its downstream effectors in mediating PC-nerve reciprocal interactions and PC metastasis, and 2) to evaluate the effectiveness of targeting MAOA and its associated neuronal genes by pharmacological approaches on disrupting tumor-nerve interactions and abrogating PC invasion and metastasis in PC-nerve cell co-culture and prostate tumor xenograft mouse models. **The scope of this research** involves experiments and assays to study the roles of MAOA and its associated neuronal genes in PC progression and metastasis in vitro (PC-3, LAPC4, LNCaP and 22Rv1 cells) and in vivo (PNI and nerve-facilitated metastasis to distant organs).

2. KEYWORDS: prostate cancer (PC), monoamine oxidase A (MAOA), perineural invasion, metastasis, clorgyline, xenograft mouse models.

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
  The progress during the Year 2 of this project in accordance with the detailed task assignments as presented in SOW, with slight adjustments, is described below:

  Major Task 1 (**Specific Aim 1**): To determine whether and how the MAOA/NRP1 axis mediates PC-nerve interactions in cell co-culture models.

Subtask 2: Set up 2D and 3D PC cell (PC-3, LAPC4, LNCaP and 22Rv1)-DRG neuron (PC-12 and 50B11) co-culture models and study the role of MAOA in mediating the reciprocal interactions between cancer cells and neurons by a series of functional cell assays and brightfield/fluorescence microscopy.	Months 21-24	Dr. Wu

  Major Task 2 (**Specific Aim 1**): To determine the effect of the MAOA/NRP1 axis on tumor-nerve interactions and PC metastasis in orthotopic xenograft mouse models.

Subtask 2: Tumor sample analyses examining tumor histopathology, tumor cell proliferation/apoptosis, tumor expression of MAOA/NRP1/NGF/SEMA3C, and nerve density by IHC staining, TUNEL assay and ELISA assay.	Months 13-16	Dr. Wu

  Major Task 3 (**Specific Aim 2**): To determine the effect of the MAOA small molecule inhibitor clorgyline on PC-nerve interactions in an in vitro 3D cell co-culture model.

Subtask 1: Examine the effect of MAOA inhibitor clorgyline on PC cell (LNCaP and 22Rv1)-DRG neuron (PC-12 and 50B11) interactions in a 3D co-culture model by functional cell assays and brightfield/fluorescence microscopy.	Months 33-36	Dr. Wu

Major Task 4 (**Specific Aim 2**): To evaluate the effectiveness of MAOA inhibitor clorgyline on abrogating prostate tumor invasion and metastasis in orthotopic tumor xenograft mouse models.

Subtask 2: Tumor sample analyses examining tumor histopathology, tumor cell proliferation/apoptosis, tumor expression of MAOA/NRP1/NGF/SEMA3C, and nerve density by IHC staining, TUNEL assay and ELISA assay.	Months 17-20	Dr. Wu

Major Task 2, Subtask 2 and Major Task 4, Subtask 2 were performed partially by focusing on MAOA's effect on tumor cell proliferation and nerve density in xenograft samples during Year 2. The remaining analyses in these two subtasks will be completed after we confirm the mediating roles of NGF, SEMA3C and NRP1 in MAOA-dependent PC PNI as proposed in Major Task 1, Subtasks 3 and 4 (Months 25-32). To compensate this change, Major Task 3, Subtask 1 was performed ahead of schedule as outlined in SOW. Please see detailed explanations in CHANGES/PROBLEMS section below.

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

1) Major activities:

Major Task 1, **Subtask 2**: We established a 3D PC-nerve cell co-culture model following a published protocol with modifications. Briefly, the rat pheochromocytoma PC-12 and immortalized rat DRG neuron 50B11 cells were seeded in a 2-µl drop of growth factor-reduced Matrigel (2×10^4 cell/drop), which were treated by NGF (100 ng/ml, 4 days) and forskolin (75 µM, 24 hours) respectively to allow cell differentiation into a neuronal phenotype with neurite outgrowth. Subsequently, PC cells were labeled with 25 uM fluorescent CellTracker Green CMFDA for 1 hour at 37^{o}C, and seeded (1.2×10^5 cells/drop) for co-culturing with induced neuronal cells for 4 days. Image acquisition was performed by a Leica DM IL microscope equipped with a MC190 HD camera. Nerve-invading PC cells, defined as fluorescent cancer cells in association Matrigel-embedded neurites, were outlined and quantified by ImageJ software, which were further subjected to subtraction of the intrinsic ability of PC cells to invade a 2-µl drop of blank Matrigel under the same experimental conditions in parallel for comparison of PNI between different groups. We co-cultured MAOA-manipulated PC cells [MAOA-overexpressing (OE) PC-3 and LAPC4, and MAOA-knockdown (KD) LNCaP and 22Rv1] with PC-12 or 50B11 cells, and determined the ability of MAOA to mediate PNI of PC cells in vitro (Figures 1 and 2, see below).

Major Task 2, **Subtask 2**: We analyzed tumor samples obtained from mice harboring orthotopic xenograft tumors, including a) control and MAOA-OE PC-3, and b) control and MAOA-KD ARCaP$_M$. We specifically examined MAOA's effects on tumor cell proliferation and nerve density by multiplexed quantum dot labeling (mQDL) analysis using Ki-67 and NF-H/NF-L as markers respectively, and assessed staining intensity at the single-cell level by inForm software (Figure 3, see below).

Major Task 3, **Subtask 1**: We evaluated the effect of the MAOA inhibitor clorgyline on PC cell PNI in a 3D PC-nerve cell co-culture model. We co-cultured LNCaP and 22Rv1 PC cells, which both have abundant expression levels of MAOA, with induced neuron-like/neuronal PC-12 or 50B11 cells as described above (Figure 4, see below).

Major Task 4, **Subtask 2**: We analyzed tumor samples obtained from $ARCaP_M$ or 22Rv1 orthotopic tumor-bearing mice that received clorgyline or saline treatment. We specifically examined MAOA's influence on tumor cell proliferation and nerve density by mQDL assays using Ki-67 and NF-H/NF-L as markers respectively, and assessed staining intensity at the single-cell level (Figure 5, see below).

2) Specific objectives:

Major Task 1, **Subtask 2**: To characterize MAOA's role in mediating PNI of PC cells in vitro.

Major Task 2, **Subtask 2**: To determine MAOA's effect on tumor cell proliferation and nerve density in vivo.

Major Task 3, **Subtask 1**: To evaluate the effectiveness of MAOA inhibitor clorgyline on suppressing PNI in PC-nerve cell co-culture models.

Major Task 4, **Subtask 2**: To determine the effects of MAOA inhibitor clorgyline on tumor cell proliferation and nerve density in vivo.

3) Significant results or key outcomes:

Major Task 1, **Subtask 2**: We successfully established a 3D PC-nerve cell co-culture model to mimic clinical observations of PNI in PC. Using this model, we demonstrated that OE of MAOA in PC-3 and LAPC4 cells promoted PNI by up to 2-fold (Figure 1). Conversely, stable KD of MAOA in LNCaP and 22Rv1 cells reduced PNI by 28-87% (Figure 2).

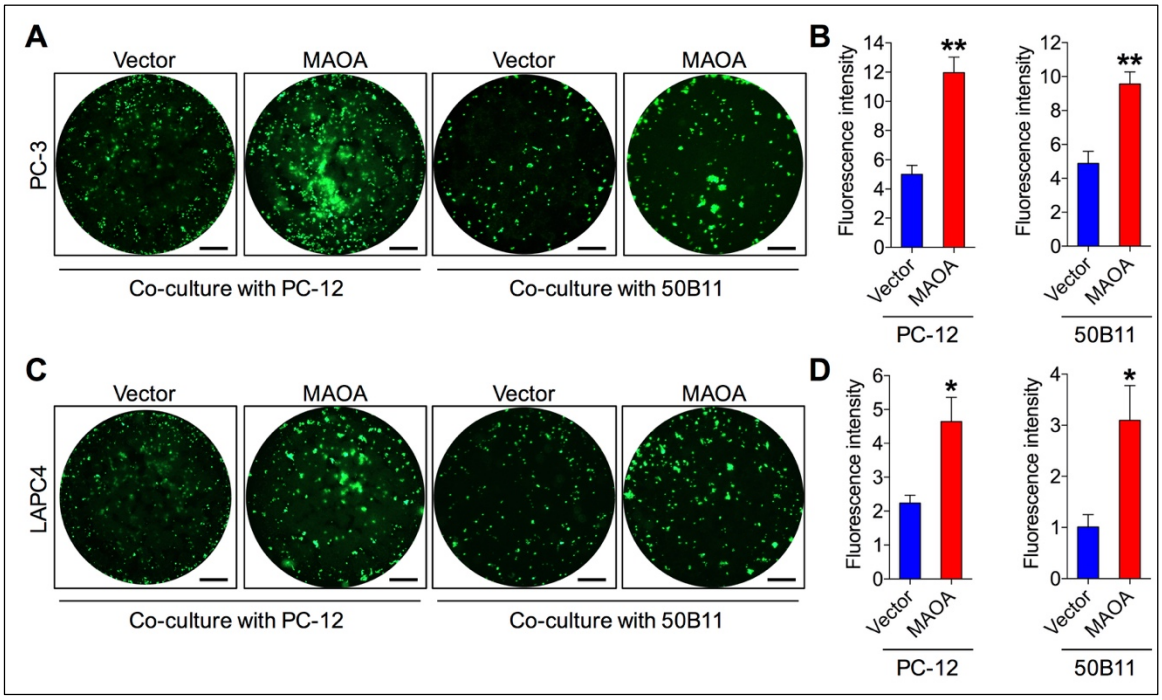

Figure 1. Enforced expression of MAOA promoted PNI of PC cells in vitro. **(A, C)** Representative images of fluorescent dye-labeled, nerve-invading PC-3 (A) and LAPC4 (C) cells in a 3D cell co-culture model with induced neuron-like/neuronal PC-12 or 50B11 cells. Scale bars: 50 μm. **(B, D)** Quantitative analysis of nerve-invading PC-3 (B, n=4 or 8) and LAPC4 (D, n=4) cells by fluorescence intensity after subtracting intrinsic invasion of PC cells alone in different groups of cell co-cultures as indicated. Vector, control for MAOA OE; MAOA, MAOA OE. Data represent the mean ± SEM. * p<0.05, ** p<0.01.

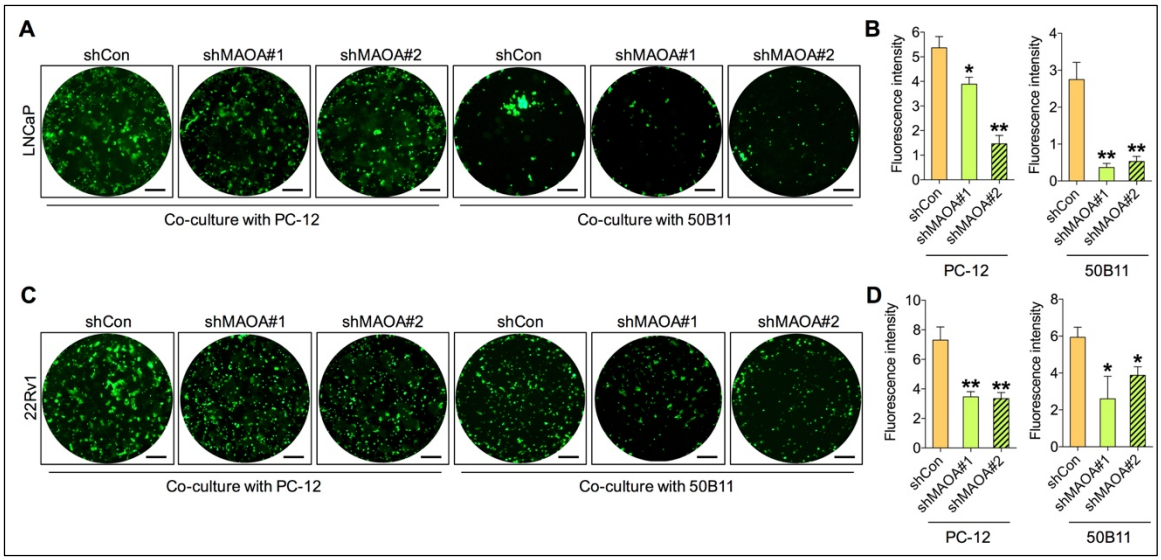

Figure 2. shRNA-mediated stable KD of MAOA reduced PNI of PC cells in vitro. **(A, C)** Representative images of fluorescent dye-labeled, nerve-invading LNCaP (A) and 22Rv1 (C) cells in a 3D cell co-culture model with induced neuron like/neuronal PC-12 or 50B11 cells. Two independent MAOA-KD sublines (shMAOa#1 and shMAOA#2) with hairpins targeting 2 separate, non-overlapping regions were used for each cell line. Scale bars: 50 μm. **(B, D)** Quantitative analysis of nerve-invading LNCaP (B, n=5 or 8) and 22Rv1 (D, n=5 or 8) cells by fluorescence intensity after subtracting intrinsic invasion of PC cells alone in different groups of cell co-cultures as indicated. shCon, control for MAOA KD; shMAOA, MAOA KD. Data represent the mean ± SEM. * p<0.05, ** p<0.01.

Major Task 2, **Subtask 2**: We showed that OE of MAOA significantly increased average cell-based staining intensity of Ki-67 and NF-H/NF-L, which are indicative of tumor cell proliferation and nerve density respectively, in PC-3 xenograft tumors. Conversely, we found that MAOA KD led to a reduction in tumor cell proliferation and nerve density as evidenced by lower Ki-67 and NF-H/NF-L staining intensity respectively in ARCaP$_M$ xenograft tumors.

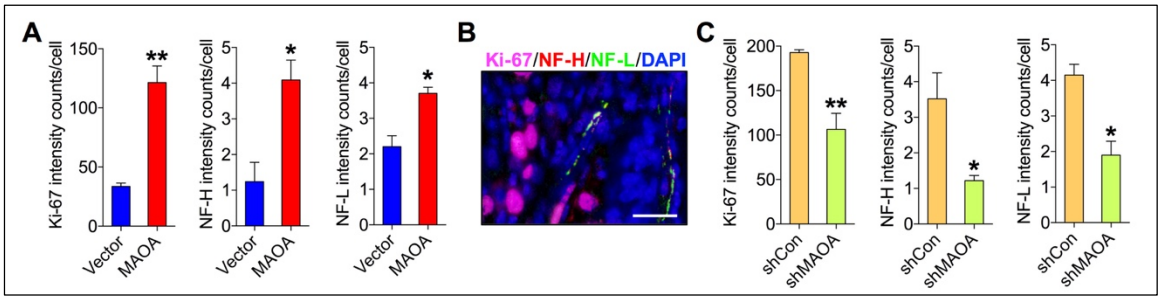

Figure 3. MAOA upregulated tumor cell proliferation and nerve density in xenograft tumors. **(A)** Average cell-based staining intensity counts of Ki-67 (left, n=3), NF-H (middle, n=3) and NF-L (right, n=3) in control and

MAOA-OE PC-3 orthotopic xenograft tumors. (B) mQDL analysis of Ki-67 (pink), NF-H (red) and NF-L (green) in a representative $ARCaP_M$ control xenograft tumor. Scale bar: 5 μm. **(C)** Average cell-based staining intensity counts of Ki-67 (left, n=3), NF-H (middle, n=3) and NF-L (right, n=3) in control and MAOA-KD $ARCaP_M$ orthotopic xenograft tumors. Vector, control for MAOA OE; MAOA, MAOA OE; shCon, control for MAOA KD; shMAOA, MAOA KD. Data represent the mean ± SEM. * p<0.05, ** p<0.01.

Major Task 3, **Subtask 1**: We demonstrated that the MAOA inhibitor clorgyline effectively suppressed PNI of LNCaP and 22Rv1 by 41% and 50% respectively in 3D PC-nerve cell co-culture models (Figure 4).

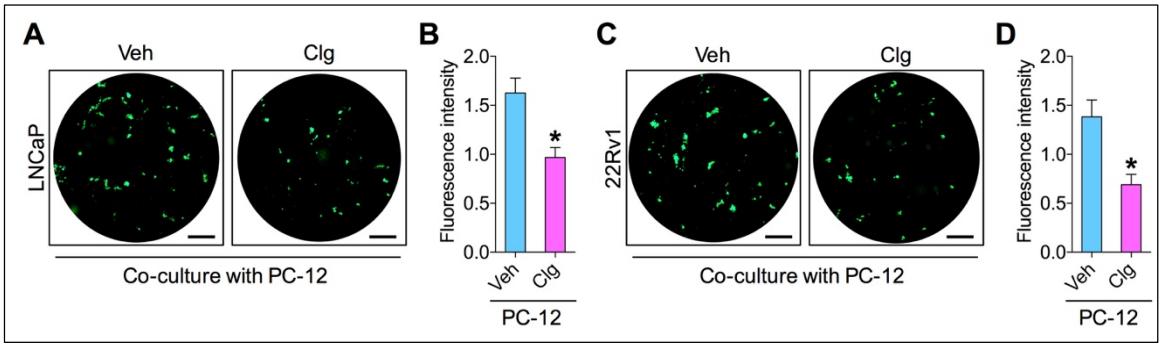

Figure 4. Clorgyline treatment reduced PNI of PC cells in vitro. **(A, C)** Representative images of fluorescent dye-labeled, nerve-invading LNCaP (A) and 22Rv1 (C) cells in a 3D cell co-culture model with induced PC-12 cells in response to clorgyline treatment (1 μM, 4 days) where PBS was used as a vehicle (Veh). Scale bars: 50 μm. **(B, D)** Quantitative analysis of nerve-invading LNCaP (B, n=4) and 22Rv1 (D, n=4) cells by fluorescence intensity after subtracting intrinsic invasion of PC cells alone in different groups of cell co-cultures as indicated. Data represent the mean ± SEM. * p<0.05.

Major Task 4, **Subtask 2**: We demonstrated that clorgyline treatment effectively reduced tumor cell proliferation and nerve density as evidenced by lower Ki-67 and NF-H/NF-L staining respectively in LNCaP and 22Rv1 xenograft tumors.

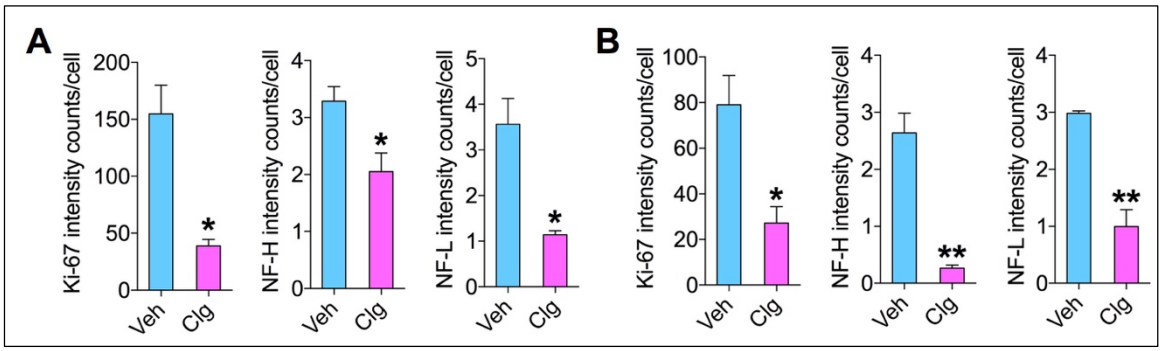

Figure 5. MAOA inhibitor clorgyline suppressed tumor cell proliferation and nerve density in xenograft tumors. **(A, B)** Average cell-based staining intensity counts of Ki-67 (left, n=3), NF-H (middle, n=3) and NF-L (right, n=3) in control and clorgyline-treated $ARCaP_M$ (A) and 22Rv1 (B) orthotopic xenograft tumors. Veh, vehicle, which is saline as used in control tumors; Clg, clorgyline. Data represent the mean ± SEM. * p<0.05, ** p<0.01.

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 communicates of interest?**
  Nothing to Report.

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

  As outlined in SOW, I will continue to work on the remaining subtasks next year, with special emphasis on two areas: 1) to determine whether and how MAOA-downstream NGF, SEMA3C, PLXNA2 and NRP1 mediate MAOA's effect on PNI in the established 3D PC-nerve cell co-culture models (Months 25-32); and 2) to continue to analyze xenograft tumor samples for tumor expression of MAOA/NGF/SEMA3C/PLXNA2/NRP1 and other indices by IHC and other biochemical assays (Months 33-36). These studies will provide mechanistic insights into MAOA-driven PNI and nerve-facilitated progression/metastasis of PC.

4. IMPACT:

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

  Our findings have so far clearly demonstrated MAOA's role in promoting PNI in vitro using a 3D PC-nerve cell co-culture model and PC progression/metastasis in vivo using orthotopic xenograft models, which was supported by pharmacological experimental evidence where a MAOA inhibitor effectively reduced PNI in vitro and prostate tumor growth/progression in mice. Collectively, these results provide evidence on MAOA's function in mediating extraprostatic extension of PC cells, accompanied by nerve- and other route-facilitated PC cell spread, and lay foundations for further pursuit of the underlying molecular mechanisms.

- **What was the impact on other disciplines?**
  Nothing to Report.

- **What was the impact on technology transfer?**
  Nothing to Report.

- **What was the impact on society beyond science and technology?**
  Nothing to Report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change:** We performed partially the proposed tumor sample analyses (Major Task 2, Subtask 1 and Major Task 4, Subtask 1) by mQDL assays of Ki-67 and NF-H/NF-L expression, which are indicative of tumor cell proliferation and nerve density respectively, in Year 2. We plan to complete the remaining proposed experiments, including IHC analysis of NGF/SEMA3C/PLXNA2/NRP1 expression, in these subtasks after we confirm the mediating roles of these molecules in MAOA-dependent PNI of PC cells (Major Task 1, Subtasks 3 and 4, Months 25-32) in Months 33-36 of Year 3. To compensate this change in the SOW timeline, we showed that MAOA inhibitor clorgyline effectively reduced PNI of PC cells in vitro (Major Task 3, Subtask 1) ahead of schedule as outlined in SOW. We expect to complete all the proposed experiments in this project as described in SOW by the end of Year 3.

- **Actual or anticipated problems or delays and actions or plans to resolve them:** Nothing to Report.

- **Changes that had a significant impact on expenditures:** Nothing to Report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  - **Journal publications:**
    Yin L, Li J, Liao CP, Wu BJ*. (2018) Monoamine oxidase deficiency causes prostate atrophy and reduces prostate progenitor cell activity. (*corresponding author) *Stem Cells*, 36(8):1249-58.

    Lin TP, Li J, Li Q, Li X, Liu C, Zeng N, Huang JM, Chu GC, Lin CH, Zhau HE, Chung LW, Wu BJ*, Shih JC*. (2018) R1 regulates prostate tumor growth and progression by transcriptional suppression of the E3 ligase HUWE1 to stabilize c-Myc. (*co-corresponding author) *Molecular Cancer Research*, 16(12):1940-51.

  - **Books or other non-periodical, one-time publications:** None.

  - **Other publications, conference papers, and presentations:**
    Presentations:
    Wu BJ. "Exploring and Exploiting Neuromimicry in Antiandrogen Drug Resistance of Prostate Cancer". Washington State University, Spokane, WA. (10/2018)

    Wu BJ. "Exploring and Exploiting Neuromimicry in Prostate Cancer". The Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China. (12/2018)

- **Website(s) or other Internet site(s):** None.

- **Technologies or techniques:** None.

- **Inventions, patent applications, and/or licenses:** None.

- **Other Products:** None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Boyang (Jason) Wu, Ph.D.
Project Role:	PI
Research Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	1.2

Contribution to Project:	Dr. Wu coordinated and performed work on analyzing tumor samples as described above.
Funding Support	DOD PCRP W81XWH-15-1-0493, WSU Start-up Fund

Name:	Jingjing Li
Project Role:	Postdoctoral Research Associate
Research Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	6
Contribution to Project:	Dr. Li performed work on establishing 3D PC-nerve cell co-cultures and characterizing MAOA's roles in PNI of PC cells in vitro.
Funding Support:	WSU Start-up fund

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

  WSU New Faculty Seed Grant (Wu) 05/16/17-08/15/18 0.36 Calendar Months
  WSU Office of Research $30,000
  "Deciphering the Stromal-Tumor Cell Interaction in Prostate Cancer"
  The goal of this project is to dissect inflammatory signals in stromal-tumor interactions of prostate cancer.
  OVERLAP: No scientific, budgetary and commitment/effort overlap with this project.

  CONquer canCER Now Award (Wu) 07/01/18-06/30/20 0.72 Calendar Months
  Concern Foundation $60,000/year
  "Dissecting Stromal Signals to Target Prostate Cancer Microenvironment"
  The goal of this project is to investigate the role of MAOA in stromal reprogramming and stromal-epithelial crosstalk of prostate cancer.
  OVERLAP: No scientific, budgetary and commitment/effort overlap with this project.

- **What other organizations were involved as partners?**
  Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not Applicable.
- **QUAD CHARTS:** Not Applicable.

9. APPENDICES: Not Applicable.