Award Number: W81XWH-12-1-0212

TITLE: Wnt/Beta-Catenin, Foxa2, and CXCR4 Axis Controls Prostate Cancer Progressionn

PRINCIPAL INVESTIGATOR: Xiuping Yu

CONTRACTING ORGANIZATION: Louisiana State University Health Sciences Center
SHREVEPORT, LA 71103

REPORT DATE: SEPTEMBER 2019

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEPTEMBER 2019	Final	7/1/2012 – 6/30/2019

4. TITLE AND SUBTITLE

Wnt/Beta-Catenin, Foxa2, and CXCR4 Axis Controls

Prostate Cancer Progression

5a. CONTRACT NUMBER
W81XWH-12-1-0212

5b. GRANT NUMBER
PC111074

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Xiuping Yu, Ph.D.

E-Mail: xyu@lsuhsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Louisiana State University Health Sciences Center
SHREVEPORT, LA 71103

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

The development of metastasis to the bone is the most dangerous complication of advanced prostate cancer (PCa), frequently resulting in significant pain. Androgen deprivation therapy is the gold standard treatment for metastatic PCa patients. However, prostate tumors become resistant to hormone ablation therapy and tumors begin to grow again. The purpose of this research is to identify mechanisms that promote metastatic castrate-resistant prostate cancer progression with the ultimate goal of finding new ways to treat patients with advanced PCa. The Wnt/beta-Catenin signaling pathway is a mechanism cancer cells use to communicate with their environment and to grow after androgen deprivation. Our previous research indicates the existence of an axis of Wnt/beta-Catenin, FOXA2, and CXCR4. In this study, we investigated their functional implication in PCa. We found that Wnt/beta-Catenin signaling is active in advanced PCa. FOXA2, a downstream target of Wnt/beta-Catenin, is important for the establishment of PCa bone metastasis. Further, we found that FOXA2 regulates the expression of PTHrP and integrin alpha 1, promoting PCa bone colonization. Furthermore, we found FOXA2 sustains AR signaling after androgen deprivation, providing a mechanism for castrate-resistant growth of PCa cells. Taken together, our study revealed a critical role of Wnt/beta-Catenin signaling and FOXA2 in PCa bone metastases.

15. SUBJECT TERMS

Wnt beta-Catenin, Foxa2, CXCR4, prostate cancer, metastasis, castrate resistant, integrin, PTHrP, AR, bone

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
U	U	U	UU	154	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction

Castration resistant prostate cancer (CRPCa) and metastasis to the bone are the two major causes of mortality in PCa patients. Understanding the mechanisms by which the PCa cells grow in the bone and relapse after hormone ablation is critical for the development of new therapeutic approaches for the treatment of advanced PCa. Wnt/beta-Catenin signaling is implicated in the establishment of bone metastasis and in the development of CRPCa. We have previously reported that activation of Wnt/beta-Catenin enables the murine prostate to continuously grow following castration. In this DOD-supported project, we studied the involvement of Wnt/beta-Catenin signaling and its targets FOXA2 and CXCR4 in CRPCa progression as well as in the establishment of PCa bone metastases. Our objectives are 1) to determine if Wnt/beta-Catenin signaling induces FOXA2 and CXCR4 to promote CRPCa growth; 2) to determine if the expression of FOXA2 facilitates castration resistant PCa growth in the bone; and 3) to determine the suitability of pharmacological inhibition of Wnt-FOXA2-CXCR4 axis in conjunction with hormone deprivation to inhibit PCa growth and CR relapse in the bone. In this study, we identified molecular mechanisms that promote PCa bone metastasis and progression to CRPCa.

2. Keywords:

prostate cancer, Wnt/beta-Catenin, Foxa2, CXCR4, integrin, PTHrP, AR, bone, castrate resistance, metastasis

3. Accomplishments

The major goal is to study the involvement of Wnt/beta-Catenin signaling, Foxa2, and CXCR4 in promoting castrate-resistant growth and in controlling prostate cancer's ability to stay in the bone and colonize the bone. We approached this goal through the following tasks:

Task 1: To determine if Wnt/beta-Catenin signaling induces Foxa2 and CXCR4 to promote castration resistant prostate cancer growth.

Task 1a. To determine if active Wnt/beta-Catenin signaling induces Foxa2 and CXCR4 to promote androgen independent prostate cancer cell growth ***in vitro*.** We have completed this task, see report submitted in 2013, p4-6 and attachments 1,2, 5, 6 & 7.

Task1b. To determine if active Wnt/beta-Catenin signaling and the expression of FOXA2 promote castration resistant prostate cancer growth in vivo. We have completed this task, see report submitted in 2014, p4-6 and attachments 1, 2 & 3

Using cultured stromal and epithelial cells, we examined the expression of Wnts and assessed their ability to activate Wnt/beta-Catenin signaling. We found that cancer-associated fibroblasts produce canonical Wnts and activate Wnt/beta-Catenin signaling in PCa cells. Also, we over-expressed FOXA2 in PCa cells and found that Foxa2 over-expression enables castrate-resistant PCa cell growth. However, knocking down Foxa2 in PCa cells did not affect cell proliferation *in vitro*. Overexpression of FOXA2 decreased the levels of AR but activated AR signaling after androgen deprivation. Inversely, knocking down Foxa2 induced both AR and AR signaling. When the FOXA2-overexpression cells were grafted *in vivo*, different from the *in vitro* results, over-expression of FOXA2 did not confer growth advantage in PCa cells. Knocking down FOXA2 did not affect tumor growth when they were grafted subcutaneously or under kidney capsule but decreased tumor growth when they were grafted in the bone (tumor growth in the bone will be further discussed in task 2). Similarly, blocking CXCR4 signaling inhibited CRPCa growth *in vitro*, but exhibited opposite effect *in vivo*. Taken together, these data indicate that

stromal/epithelial interaction plays an important role in the activation of Wnt/beta-Catenin signaling in PCa, and that FOXA2's and CXCR4's involvement in PCa is different *in vitro* and *in vivo*. Similarly, co-supported with this grant and other funding, we found that the expression of AR also confers a differential function in PCa *in vitro* and *in vivo*. Ectopic expression of AR inhibited cell proliferation *in vitro* but promoted carcinogenesis *in vivo*. This finding is novel because it not only indicates the differential function of these genes in different environment, it also re-assured the importance of considering tumor microenvironment in cancer research. Additionally, co-supported with this grant and other funding, we found activation of Wnt/beta-Catenin signaling induced the expression of SOX2 and that SOX2 is expressed in neuroendocrine PCa (NEPCa). We are the first one reporting the involvement of SOX2 in NEPCa. This has stimulated lot of research interest on SOX2 in PCa research field. Furthermore, co-supported with this grant and other funding, we identified a novel mechanism that activates Wnt/beta-Catenin signaling. We found the expression of YAP1 is lost in NEPCa and that loss of YAP1 augments Wnt/beta-Catenin signaling. This mechanism is novel because it is an addition to the conventional Wnt-component genes that modulate this signaling pathway. Also, our study raised the concern if YAP1 inhibitors could be safely used as therapeutic adjuvants for treating advanced PCa.

Task 2. To determine if the expression of Foxa2 facilitates castration resistant prostate cancer growth in the bone.

Task 2a. To determine if FOXA2 is involved in the interaction between PCa cells and the bone microenvironment by controlling the expression of osteoclastogenesis related genes in vitro. We have completed this task, see report submitted in 2014, p7, report submitted in 2017, p4, report submitted in 2018, p4-10, and attachments 3, 4 & 5.
Task 2b. To determine if over-expression of FOXA2 facilitates prostate cancer growth in the bone and progression to castration resistant prostate cancer. We have completed this task, see report submitted in 2014, p7-8 and attachments 3 & 5.
Task 2c. To determine if knocking down Foxa2 in prostate cancer cells impairs their growth in the bone. We have completed this task, see report submitted in 2013, p6-7 and attachments 3, 4 & 8.
For this task, we firstly conducted *in vitro* co-culture of PCa cells with bone cells. We found that overexpression of FOXA2 in PCa cells did not change osteoclastogenesis when bone-derived monocyte (preosteoclasts) were co-cultured with PCa cells. However, FOXA2 knockdown decreased the expression of TRAP and PTHrP in pre-osteoblasts MC-3T3 cells when they were co-cultured with PCa cells. Both TRAP and PTHrP are involved in bone remodeling. FOXA2 knockdown also decreased the expression of multiple integrins, which are important factors that are involved in mediating PCa cells interaction with bone matrix proteins. But FOXA2 knockdown did not affect the expression of RANKL, which is another important factor that regulates bone remodeling. Also, we conducted TRAP staining on monocyte cells that were treated with conditioned medium collected from PCa cells with or without FOXA2 over-expression. Consistent with the lack of induction of osteoclastogenesis, the over-expression of FOXA2 did not induce monocyte to differentiate into TRAP-positive cells. However, knocking down FOXA2 not only decreased the production of TRAP, PTHrP, and integrins, but also decreased PCa-mediated PCa/bone interaction. These data indicate that FOXA2 is not sufficient but is essential for mediating the communication between PCa and bone cells.
For *in vivo* functional study, we inoculated Foxa2 over-expressing PCa cells into mouse tibias and analyzed the bone lesions mediated by these cells. We found, surprisingly, that the

overexpression of FOXA2 reduced bone lesions. Additionally, we assessed how androgen deprivation would affect PCa-mediated bone lesions. We found that the expression of FOXA2 decreased PCa-mediated bone lesions in both intact and castrated mice. And surprisingly, when AR-positive PCa 22Rv1 cells were inoculated into the bones, castration even increased lytic bone lesions, although the osteoblastic bone lesions decreased after castration. The increased lytic bone lesions could result from the weakened bone condition after androgen deprivation. However, knocking down FOXA2 in PCa cells decreased bone destruction when these cancer cells were injected into bones.
Taken together, these data indicate that the expression of FOXA2 is not sufficient to induce PCa-mediated bone lesions, but this gene is required for PCa cells to interact with bone microenvironment and promote PCa bone colonization.

Task 3. To determine the suitability of pharmacological inhibition of Wnt-Foxa2-CXCR4 axis in conjunction with hormone deprivation to inhibit prostate cancer growth and castration resistant relapse in the bone and to prepare manuscript for publication. We have completed this task, see report submitted in 2013, p7-8, report submitted in 2017, p4, and new data presented below in page 8 of this report.
For this task, AR-positive PCa 22Rv1 cells were inoculated into the tibiae of SCID mice. The host mice were treated with CXCR4 inhibitor (AMD3100) or Wnt/beta-Catenin inhibitor (ICG-001). The tumor growth and bone lesions were monitor by x-ray imaging. We found 22Rv1 cells generated mixed osteoclastic/osteoblastic bone lesions. Growing of these PCa cells caused lytic destruction to the cortical bone but stimulated new bone formation outside of cortical bones. The lytic bone lesions and the new bone formation were analyzed by using microCT scanning. Our analysis indicated that castration increased lytic bone lesions. CXCR4 inhibitor and Wnt inhibitor did not decreased lytic bone destruction in intact mice (CXCR4 inhibitor even increased lytic lesions). These inhibitors decrease lytic lesions in castrated mice, but not sufficient to overcome the effect resulted from castration. Overall, these inhibitors did not affect the overall bone destruction compared to intact non-treated mice. A possible explanation for the increased lytic bone lesions in CXCR4 inhibitor treated mice is that AMD3100 liberated PCa cells in the bone, resulting in more metastatic sites/lesions. This raised the concern whether CXCR4 inhibitors could be safely used as therapeutic adjuvants for treating PCa bone metastasis.

Major Accomplishments:

- Accomplishments in research that are proposed in the DOD grant:

3.1 Wnt/beta-Catenin signaling is activated in advanced PCa
This research is **partially supported by DOD grant**. A manuscript is in preparation and attached (**attachment 1**). Briefly, using mouse models (TRAMP/Wnt-reporter mice), we determined if Wnt/β-Catenin signaling is active in neuroendocrine PCa (NEPCa, the most aggressive form of PCa). We found Wnt/β-Catenin pathway is active in a subset of NEPCa. Further, we explored the mechanisms that activate Wnt/beta-Catenin signaling. We found that castration induced the expression of canonical Wnt as well as endogenous Wnt inhibitors in wild type prostates. However, Wnt inhibitors were not induced in PCa after castration. The lack of induction of Wnt inhibitors permits the activation of Wnt/beta-Catenin signaling in PCa cells. Furthermore, we found stromal/epithelial interaction is involved in the activation of Wnt/beta-Catenin signaling, promoting NE differentiation of PCa cells. Data collected from this research has led to a NIH R01grant funding. We are currently expanding this research.

3.2 Loss of YAP1 activates Wnt/beta-Catenin signaling in PCa

This research is **partially supported by DOD grant**. A manuscript is in preparation and attached (**attachment 2**). Briefly, in this study, we found the expression of YAP1 is lost in both human and mouse NEPCa. Also, our data indicate that decreased expression of YAP1 enhances Wnt/beta-Catenin signaling in PCa cells. This manuscript will be submitted for publication soon.

3.3 FOXA2 is important for the establishment of PCa bone metastasis

This research is **mostly supported by DOD grant**. A manuscript is under review for the journal of Cancer Letters (**attachment 3**). Briefly, in this study, we found that FOXA2 is expressed in a subset of PCa bone metastasis specimens. To determine the functional role of FOXA2 in PCa metastasis, we knocked down the expression of FOXA2 in aggressive PCa PC3 cells. The PC3/FOXA2-knockdown cells generated fewer bone lesions following intra-tibial injection compared to control cells. Further, we found that FOXA2 knockdown decreased the expression of PTHLH, which encodes PTHrP, a well-established factor that regulates bone remodeling. These results indicate that FOXA2 is involved in PCa growth in bone.

3.4 FOXA2 regulates the expression of integrin α1, promoting PCa bone colonization

This research is **mostly supported by DOD grant**. It is a continuation of the research presented in attachment 3. A manuscript is in preparation and attached (**attachment 4**). It will be submitted for publication once the manuscript in attachment 3 is published. We found FOXA2 regulates the expression of integrin α1, a collagen I-binding integrin, in PCa cells. FOXA2-knockdown (KD) cells exhibited reduced adhesion, spreading, and integrin signaling on a cell culture surface coated with collagen I, the major component of bone extracellular matrix. Blocking integrin α1 signaling with a neutralizing antibody reproduced the decreased adhesion phenotype of PC3/FOXA2-KD cells. Inversely, overexpression of integrin α1 rescued adherence properties. Taken together, these results show that FOXA2 upregulates expression of integrin α1 in PCa cells, promoting adherence to collagen I, the major components of the extracellular matrix in bone tissues. These results support the existence of FOXA2/ITGA1 axis in regulating PCa bone colonization.

3.5 FOXA2 sustains AR signaling after androgen deprivation, providing a mechanism for castrate-resistant growth of PCa cells

This research is **mostly supported by DOD grant**. A paper is published on Am J Clin Exp Urol (**attachment 5**). Briefly, in this study, we identified a mechanism that retains AR signaling after androgen deprivation using TRAMP SV40 T antigen transgenic mice, which is a mouse model for PCa. We found that Foxa2 is co-expressed in T-antigen positive cells. Ectopic expression of Foxa2 drives the T-antigen expression regardless of the presence of androgens. Using this model system, we further explored the mechanism that activates AR-responsive promoters in the absence of androgens. Chromatin immunoprecipitation revealed the occupancy of both H3K27Ac, an epigenetic mark of an active transcription, and Foxa2 at the known AR target promoters, Probasin and FKBP5, in the absence of androgen stimulation. In conclusion, we have identified a mechanism that enables PCa to retain the AR signaling pathway after androgen ablation.

- Accomplishments in research that are not proposed in but related to the DOD grant

3.6 SOX2 is expressed in NEPCa

This research is **partially supported by DOD grant (see** report submitted in 2013, p8**)**. A paper is published on Prostate Cancer and Prostatic Disease (**attachment 6**). Briefly, in this study, we found SOX2, a Wnt/beta-Catenin target gene, is expressed in developing prostate as well as NEPCa. This is the first report on the involvement of SOX2 in NEPCa. The results in this paper are well accepted in the field and have stimulated lot of research interest on the functional role of SOX2 in NEPCa progression.

3.7 AR signaling differentially regulates prostate cancer growth
This research is **partially supported by DOD grant**. A paper is published on Oncotarget (**attachment 7**). Briefly, in this study, we found ectopic expression of AR in normal prostate epithelial cells inhibits cell proliferation *in vitro* but promotes carcinogenesis and cancer growth *in vivo*. We also identified an involvement of IL6/STAT3 in AR-mediated carcinogenesis.

-New data since last report

We finished analyzing data from previous animal experiment (the data analysis is presented in **attachment 8**). We found that when PCa 22Rv1 cells were injected into tibiae of SCID mice, they generated mixed osteoclastic and osteoblastic bone lesions. Surprisingly, castration increased lytic bone lesions. Treatment with CXCR4 inhibitor (AMD3100) and Wnt inhibitor (ICG-001) did not decreased lytic bone destruction in intact mice (CXCR4 inhibitor even increased lytic lesions). These inhibitors decreased lytic lesion in castrated mice, but the reduction was not big enough to overcome the castration-induced lytic reaction. Overall, combination of castration and inhibitor treatment did not decrease bone destruction compared to intact non-treated mice. For details, please see attachment 8.

Training opportunity:
This project provides the training and professional development opportunities for graduate student, Zachary Connelly, who is the major person who works on this project. Under PI's guidance, Zac did the Foxa2 knockdown experiments and characterized how knockdown of Foxa2 affects the expression of integrins and PCa cells's ability to interact with bone cells, to adhere and spread on collagen I, the major components of bone matrix, and to form the focal adhesion complex. Zac also characterized the NeoTag1 cells that overexpress Foxa2. Zac has presented his work in multiple international and national conferences including a podium presentation at the American Society for Biochemistry and Molecular Biology annual meeting, San Diego, CA. Zac has passed his PhD. dissertation defense in July 1, 2019.

Results disseminated to communities of interest:

PI (Dr. Yu) and PhD student (Zachary Connelly) participated in Family Health Day at LSUHSC-Shreveport to help people from local community understand the basics about prostate cancer.

Plan to do during the next reporting period:
Nothing to report for next period.

4. IMPACT:
-impact on the development of the principal discipline of the project
For prostate cancer research field: PCa preferentially metastasizes to the bone. However, our understanding on the mechanisms that drive PCa bone colonization is still limited. In our study,

we found Wnt/beta-Catenin signaling is activated in NEPCa. Active Wnt/beta-Catenin signaling induces the expression of FOXA2. FOXA2 promotes castrate-resistant PCa growth. Foxa2 also promotes PCa's ability to colonize bone through regulation of PTHrP as well as bone-matrix interacting proteins, integrin α1. This study highlighted the importance of targeting Wnt/beta-Catenin signaling. It also revealed important mechanisms that promote PCa cells growth in the bone. Furthermore, in his study, we identified Wnt/beta-Catenin as well as two additional drug-able targets, PTHrP and integrins, for the treatment of FOXA2-positive PCa bone metastases. However, our study on the CXCR4 inhibitor revealed an unexpected result that blocking CXCR4 signaling can have the unwanted opposite effect on PCa bone metastasis. This suggests that we shall be cautious in using CXCR4 inhibitors in clinic for the treatment of PCa bone metastasis.

-impact on other disciplines

Our findings on the involvement of Wnt/beta-Catenin signaling, FOXA2, CXCR4, PTHrP, and integrins in prostate cancer bone metastasis will also have an impact on the bone research as well as integrin research .

- impact on technology transfer

Nothing to Report

- impact on society beyond science and technology

The findings from this study provide important information about the involvement of Wnt/beta-Catenin signaling, FOXA2, CXCR4, integrins, and PTHrP in prostate cancer progression. These discoveries have the potential to be translated into targeted therapy and influence clinical practice.

5, CHANGES/PROBLEMS:

Changes in approach and reasons for change: Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them: this research was delayed because PI moved from Vanderbilt University at Nashville, TN to LSU Health Sciences center at Shreveport, LA. It was granted no-cost-extension.

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

-Results different from what expected:

In task 3, we determined the suitability of pharmacological inhibition of Wnt-FOXA2-CXCR4 axis in conjunction with hormone deprivation to inhibit prostate cancer growth and castration resistant relapse in the bone. In our research, we injected Pca cells into the tibias of SCID mice and treated the host mice with Wnt inhibitor (ICG-001) and CXCR4 inhibitor (AMD3100, also called Plerixafor). The tumor growth in the tibias was monitored by X-ray imaging and the bone lesions were evaluated by micro CT scanning. We found that the results are a little different from what we expected. Firstly, castration of host mice did not decrease bone lesions. On the contrary, castration even increased lytic bone lesions, although reduced the osteoblastic bone lesions. Secondly, treatment with plerixafor (AMD3100) did not decrease PCa-mediated bone lesions either. A possible explanation is that androgen deprivation weakened the bone condition. Also, plerixafor may have liberated prostate cancer cells in the bone, resulting in more metastatic

sites/lesions. This raised the alarm if CXCR4 inhibitors could be safely used as therapeutic adjuvants for treating PCa bone metastasis.

6, PRODUCTS

Journal publications

1. **Yu X***, Cates JM, Morrissey C, You C, Grabowska MM, Zhang J, DeGraff DJ, Strand DW, Franco OE, Lin-Tsai O, Hayward SW, Matusik RJ*. SOX2 expression in the developing and adult prostate, as well as in benign and malignant pathological states, *corresponding authors, *Prostate Cancer and Prostatic Dis*, 2014 Dec;17(4):301-9. PMID: 25091041

2. Yang S, Jiang M, Grabowska MM, Li J, Connelly Z, Zhang J, Hayward SW, Cates JM, Han G, **Yu X*** Androgen receptor differentially regulates the proliferation of prostatic epithelial cells *in vitro* and *in vivo* *corresponding author, *Oncotarget.* 2016 Oct 25;7(43):70404-70419 PMID: 27611945

3. Connelly Z, Yang S, Chen F, Yeh Y, Khater N, Jin R, Matusik R, **Yu X*** Foxa2 Activates the Transcription of Androgen Receptor Target Genes in Castrate Resistant Prostatic Tumors *corresponding author. *American Journal of Clinical and Experimental Urology*, 2018 Oct 20;6(5):172-181

Books or other non-periodical, one-time publications: Nothing to report

Other publications, conference papers and presentations

-Podium presentation:

1. Yu, X. Activation of Wnt/beta-Catenin promotes castrate-resistant prostate cancer progression, Prostate Cancer 2015 conference, Orlando, FL, June 2015
2. Yu, X. Wnt/beta-Catenin signaling in NEPCa, Augusta University, Augusta, GA, January 2016
3. Yu, X. Wnt/beta-Catenin signaling in NEPCa, Texas A&M, College Station, TX, February 2016
4. Yu, X. Wnt/beta-Catenin signaling in PCa progression, LSU-S, Shreveport, LA, November 2016
5. Yu, X. Wnt/beta-Catenin signaling in PCa progression, LA Tech, Ruston, February 2017
6. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. Graduate Research Day. LSUHSC-Shreveport, LA. 26, April 2019
7. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. Grand Rounds Department of Urology. LSUHSC-Shreveport, LA 5, February 2019.
8. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. Feist-Weiller Cancer Center Seminar Series. LSUHSC-Shreveport. Shreveport, LA. 13, June 2018.
9. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. American Society for Biochemistry and Molecular Biology. Physiological Regulation by Cell Signaling. San Diego, Ca. 22, April 2018
10. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. Eastern Atlantic Research Forum. The University of Miami Miller School of Medicine. Miami, FL. 22, February 2018.
11. Connelly, Z. FOXA2 Promotes Prostate Cancer Bone Colonization. Grand Rounds Department of Urology. LSUHSC-Shreveport, LA. 29, Janurary 2018.
12. Connelly, Z. Wnt/β-Catenin downstream target, FOXA2, plays a major role in CRPCa metastasis. Feist-Weiller Cancer Center Seminar Series. LSUHSC-Shreveport, LA. 6, June 2017.

13. Connelly, Z. FOXA2 Activates the AR Responsive Promoters in the Ablation of Androgen. ArkLaTex Interdisciplinary Cell Conference. LSU Shreveport, LA. 5, November 2016.

-Poster Presentation:

1. Tian, X., Yang, S., and Yu X. AR signaling differentially regulates the growth of prostate epithelial cells. SBUR Symposium. Fort Lauderdale, FL, November 2015.
2. Connelly ZM, Yang S, Yu X. Foxa2 Activates the AR Responsive Promoters after Androgen Deprivation. Industry Day. Shreveport, LA, October 2016.
3. Connelly ZM, Yang S, Li J, Matusik R, Yu X. Foxa2 activates the AR responsive promoters in the absence of androgen. SBUR Symposium. Scottsdale, AZ. November 2016.
4. Connelly ZM, Yang S, Li J, Yu X. Wnt/beta-catenin signaling in neuroendocrine prostate cancer. SBUR Symposium. Scottsdale, AZ, November 2016.
5. Yang, S., Jiang, M., Graboska, M., Connelly, Z., Cates, J., Han, G., and Yu Xiuping. AR signaling differentially regulates prostate epithelial in vitro and in vivo. AACR Annual Meeting. New Orleans, LA, April 2016.
6. Connelly ZM, Yang S, Orr W, Jin R, DeGraff D, Zhang X, Morrissey C, Corey E, Matusik R, Yu X. FOXA2 Promotes Prostate Cancer Progression. SBUR Symposia. Tampa, FL, November 2017.
7. Connelly ZM, Yang S, Li J, Matusik R, Yu Xiuping. Wnt/beta-catenin and Foxa2 axis activates AR signaling in castration resistant prostate cancer. AACR annual meeting. Washington, DC, April 2017.
8. Connelly ZM, Yang S, Blankenship A, Yu X. Role of Polycomb proteins in neuroendocrine prostate cancer. AACR annual meeting. Chicago, IL, April 2018.
9. Shi M, Connelly ZM, Yang S, Zhang X, Morrissey C, Corey E, Shi R, Yu X. FOXA2 protein expression is associated with neuroendocrine phenotype and androgen receptor status in the metastatic castration-resistant prostate cancer. The United States and Canadian Academy of Pathology 2018 Annual Meeting. Vancouver, Canada, March 2018.
10. Connelly ZM, Yang S, Yu X. FOXA2 Promotes Prostate Cancer Bone Colonization. SBUR annual meeting, Rancho Mirage, CA, November 2018.
11. Siyuan Cheng, Shu Yang, Zachary M. Connelly, Fenghua Chen, Xiuping Yu AR signaling promotes prostate carcinogenesis. AACR annual meeting, Atlanta, GA, April 2019
12. Zachary M. Connelly, Renjie Jin, Shu Yang, Siyuan Cheng, Jianghong Zhang, Nazih Khater, A. Wayne Orr, Robert J. Matusik, Xiuping Yu FOXA2 Promotes Prostate Cancer Bone Colonization. AACR annual meeting, Atlanta, GA, April 2019

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

LSU Health Sciences Center at Shreveport, LA:

Xiuping Yu: PI.	eRACommons Identifier: Yux123
Zachary Connelly: graduate student.	eRACommons Identifier: ZConnelly
Shu Yang: research associate.	eRACommons Identifier: N/A
Siyuan Cheng: graduate student.	eRACommons Identifier: SIYUUANCHENG

Vanderbilt University at Nashville, TN (when PI was working at Vanderbilt):
Jianghong Zhang: research associate eRACommons Identifier: N/A

Collaborators:
Wayne Orr: professor, unpaid consultant, LSU Health Sciences Center at Shreveport, LA
Supported by NIH grant HL098435, HL133497, and HL141155

Robert Matusik: professor, unpaid consultant, Vanderbilt University at Nashville, TN
Supported by Vanderbilt University, Department of Urology

Renjie Jin: research faculty, unpaid collaborator, Vanderbilt University at Nashville, TN
Supported by Vanderbilt University, Department of Urology

Eva Corey: professor, unpaid consultant, University of Washington at Seattle, WA
Supported by Prostate Cancer Biorepository Network grant W81XWH-14-2-0183

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period: yes, PI obtained two NIH grants and is co-investigator of another DOD grant. Please see attachment 9

8, Special Reporting Requirements N/A
9, Appendices
attachment 1: manuscript, Wnt/β-Catenin signaling is active in neuroendocrine prostate cancer
attachment 2: manuscript, The expression of YAP1 is lost in neuroendocrine prostate cancer
attachment 3: manuscript, FOXA2 Promotes Prostate Cancer Bone Metastasis (under review)
attachment 4: manuscript, FOXA2 Regulates Integrin α1 Expression Facilitating Prostate Cancer Bone Colonization
attachment 5: publication, Foxa2 activates the transcription of androgen receptor target genes in castrate resistant prostatic tumors
attachment 6: publication, SOX2 expression in the developing, adult, as well as diseased prostate
attachment 7: publication, Androgen receptor differentially regulates the proliferation of prostatic epithelial cells in vitro and in vivo
attachment 8: X-ray and microCT analysis of PCa-mediated bone lesions
attachment 9: updated research support
attachment 10: PI's updated CV

Wnt/β-Catenin signaling is active in neuroendocrine prostate cancer

Running title: Wnt/β-Catenin signaling in neuroendocrine prostate cancer

Key words: Wnt/β-Catenin, neuroendocrine, prostate cancer

Abstract

Neuroendocrine (NE) differentiation commonly occurs in late stage prostate cancer (PCa)[1-19]. Acquiring an NE phenotype is one proposed mechanism of resistance to contemporary AR-targeted treatments, and thought to represent up to 25% of lethal PCa. Currently, there is no effective treatment for PCa with prominent NE phenotype that presents as "therapy-related" NEPCa[5-9]. Understanding how the NE phenotype arises is important for identifying therapeutic targets for the treatment of advanced PCa. We have previously shown that activation of Wnt/β-Catenin signaling promotes the development of castrate-resistant PCa with increased NE phenotype. Our study has also shown that activation of Wnt/β-Catenin signaling induces the expression of FOXA2, a member of the family of forkhead transcription factors that is expressed in both human and mouse NEPCa. The expression of FOXA2, a Wnt/β-Catenin signaling downstream target gene, in NEPCa suggests that Wnt/β-Catenin pathway is active in NEPCa. To experimentally determine if Wnt/β-Catenin signaling is active in NEPCa, we bred TRAMP mice (NEPCa mouse model[20-22]) with Wnt-reporter mice to directly monitor the activity of Wnt/β-Catenin signaling during the development of NEPCa. We found that the Wnt/β-Catenin pathway is, indeed, active in NEPCa tumors. Further, we explored the possible mechanisms that activate Wnt/β-Catenin signaling in NEPCa. We examined the expression of canonical Wnts and Wnt inhibitory genes in TRAMP tumors. We found the expression of Wnt7a was elevated, but the expression of Dkk1, an endogenous Wnt inhibitor, was decreased in TRAMP NEPCa tumors.

Elevated expression of WNT7a, coupled with decreased Wnt inhibitor Dkk1, could activate Wnt/β-Catenin signaling in NEPCa. In conclusion, Wnt/β-Catenin signaling is activated in NEPCa.

Introduction

Androgen deprivation therapy is the gold standard treatment for advanced prostate cancer (PCa). Patients initially respond well, resulting in tumor regression, but ultimately progress to castrate-resistant PCa. New anti-androgens have shown significant improvement; however, these new therapies eventually fail[23, 24]. Moreover, after PCa fails androgen deprivation therapy, an aggressive neuroendocrine (NE) phenotype ensues with high morbidity and an average survival of less than 1.5 years[1-19] . There is currently no effective treatment for PCa with a prominent NE phenotype[25]. Identifying the mechanism(s) through which the NE phenotype arises subsequent to androgen deprivation therapy is critical for the development of novel therapeutics against PCa.

Although it was observed long ago that castration accelerates the emergence of a NE phenotype in PCa, the underlying mechanisms by which androgen deprivation promotes NE differentiation remain unclear. We have shown that activation of Wnt/β-Catenin signaling promotes the development of castrate-resistant PCa with an increased NE phenotype[26]. Our studies also demonstrated that activation of Wnt/β-Catenin signaling induced the expression of Foxa2[26], a transcription factor expressed in both human and mouse NEPCa[27, 28], suggesting that Wnt/β-Catenin signaling is active in NEPCa.

Wnts are a family of secreted glycoproteins that act in a short-range paracrine/autocrine manner[29]. Activation of the Wnt pathway is known to involve both the canonical Wnt/β-Catenin and the non-canonical pathways[29]. Canonical Wnts stabilize β-Catenin and result in the

cytoplasmic/nuclear accumulation of β-Catenin[29]. Human histology studies have shown that active Wnt/β-Catenin signaling is strongly associated with advanced stage PCa and recurrence[30-37]. In addition, studies have shown that Wnt antagonists (WIF1, DKKs, or sFRPs) are often down regulated in PCa[38-41]. Activating mutations in exon 3 of the β-Catenin gene occur in only 5% of primary PCa[42]; however, new data on samples from patients who fail androgen deprivation therapy demonstrate that mutations in the Wnt/β-Catenin pathway occur in 18% castrate-resistant PCa, whereas mutations in this pathway are virtually undetectable in hormone naïve PCa[30, 31]. In accordance with the enrichment of Wnt/β-Catenin mutations in castrate-resistant PCa, nuclear β-Catenin, an indicator of active Wnt/β-Catenin signaling, is associated with metastatic castrate-resistant PCa[32-35], suggesting that active Wnt/β-Catenin signaling would endow PCa cells with a selective advantage during tumor progression.

A hallmark of active Wnt/β-Catenin signaling is the nuclear staining of β-Catenin[29]. However, because β-Catenin is strongly expressed on cell membranes and nuclear β-Catenin is relatively low, it is often difficult to detect nuclear β-Catenin by IHC staining on tissue sections. Additionally, the Wnt/β-Catenin pathway is complex. There are 19 Wnts and many more factors that modulate Wnt/β-Catenin signaling in a positive and/or negative manner[29]. Thus, it would be impossible to conclusively determine whether this pathway is active in NEPCa tumors by assessing only the expression levels of the component genes. In this study, using TRAMP/Wnt-reporter mouse models, we examined if the Wnt/β-Catenin pathway is active in NEPCa.

TRAMP is a SV40 T-antigen transgenic mouse model for NEPCa research[43]. T-antigen inactivates p53 and RB, two proteins whose loss of function is not only associated with but also functionally involved in human NEPCa[44-47]. The Wnt-reporter mouse line we selected for this research is a transgenic mouse line in which GFP is fused to histone H2B and placed under the

control of a Wnt/β-Catenin responsive promoter[48]. Active Wnt/β-Catenin signaling turns on the expression of GFP-histone, which is readily detectable in nuclei. The establishment of TRAMP/Wnt-reporter mice enabled us to directly monitor the Wnt/β-Catenin activity in NEPCa tumors. As for PCa progression in human, castration accelerates the development of NEPCa tumors in TRAMP mice[20-22]. Therefore, we explored how androgen deprivation regulates the expression of Wnt-related genes, resulting in the activation of this pathway in NEPCa.

Results:

1. Wnt/β-Catenin signaling is active in NEPCa (we are still collecting data from more mice).

To collect direct evidence that the Wnt/β-Catenin pathway is active in NEPCa, we bred TRAMP mice (mouse model of NEPCa[20-22]) with Wnt-reporter mice, in which GFP expression is under the control of a Wnt/β-Catenin-responsive promoter[48]. Because of the higher prevalence of NEPCa, TRAMP mice with mixed FVB/BL6 genetic background were used for this research and bred with Wnt-reporter mice. We found that a subset of TRAMP/Wnt-reporter mice developed NEPCa, which is defined by both histology and IHC staining of marker genes, Foxa2 and chromogranin A. Consistent with previous report, Foxa2 was detected in all the chromogranin A-expressing NEPCa tumors. Also, we found a subset of TRAMP/Wnt-reporter NEPCa tumors displayed positive staining of GFP (Fig. 1) while the others have little to none GFP expression, even though they are positive for Foxa2, a downstream target of Wnt/β-Catenin signaling. One of the TRAMP/Wnt-reporter mice developed cancer metastasis in lymph nodes, liver, and lung. GFP and Foxa2 as well as NEPCa marker chromogranin A are highly expressed in the metastatic tumors (Fig.1).

2. The expression of Wnt ligands and Wnt inhibitory genes is altered in NEPCa, contributing to the activation of the signaling pathway.

To explore the mechanism(s) that activate the Wnt/β-Catenin signaling in NEPCa, using quantitative (q)RT-PCR, we screened for the differential expression of Wnt-related genes in normal prostates, prostate intraepithelial neoplasia (PIN), and NEPCa. Prostatic tissues collected from TRAMP mice were all histologically confirmed. Also, we assessed the expression of Chromogranin A and Synaptophysin in the specimens and used these data to confirm the NED phenotype in the samples. We found that the expression of Wnt7a, a canonical Wnt ligand that can activate the Wnt/β-Catenin signaling,[49] was elevated in NEPCa tumors (Fig. 2). Additionally, we found the expression of Dkk1 was decreased in NEPCa, which is in line with the activation of the Wnt/β-Catenin signaling in NEPCa, while the expression of other secretory Wnt inhibitory genes (other Dkks and sFRPs) did not show consistent changes.

Previous studies suggest that androgen deprivation accelerates the emergence of NEPCa. To determine if and how castration alters the expression of Wnt-related genes contributing to the activation of the pathway, we analyzed the association between the expression of Wnt-related genes and NE phenotype in tumors derived from both intact and castrated TRAMP. We found castration induced Wnt7a level in both normal prostate and TRAMP tumors (Fig. 2). Castration induced Dkk1 expression in wild-type prostates but Dkk1 levels were low in TRAMP tumors and even lower in the TRAMP tumors that were derived from castrated mice (Fig. 2). These data suggest that castration induces Dkk1 expression in normal prostate, and this may function to suppress the Wnt/β-Catenin signaling; however, this Wnt-inhibitory mechanism is lost in PCa.

3. Stromal/epithelial interaction is involved in regulating NE differentiation of PCa cells

Stromal cells play an important role in modulating PCa behaviors. Therefore, we examined whether stromal/epithelial interactions regulate the expression of Wnt-related genes in PCa. The expression of WNT7A and DKKs were analyzed by qRT-PCR. As shown in Fig. 3, WNT7A was preferentially expressed in LNCaP but barely detectable in normal prostate fibroblasts (NPFs). Normal prostate fibroblasts (NPFs) express DKK1 at high levels (Fig. 3). However, DKK1 expression is significantly lower when the NPFs are co-cultured with PCa LNCaP, regardless of androgen presence (Fig. 3). Concomitant with the elevated expression of WNT7A and the decreased expression of DKK1, NEPCa marker CD56 was robustly induced by androgen deprivation in the LNCaP/NPFs co-cultures (Fig. 3); whereas CD56 was only marginally induced by androgen deprivation in LNCaP cells that were cultured alone. Taken together, these data suggest that the altered expression of WNT7A and DKK1 provides a mechanism to activate the Wnt/β-Catenin signaling in PCa cells, resulting in NE differentiation and castrate-resistant progression.

4. The expression of DKK1 decreased in advanced PCa.

To determine whether DKK1 expression is lost in late-stage PCa[50], we analyzed microarray data extracted from GDS1439[51]. As shown in Fig. 4, DKK1 expression was decreased/lost in all the castrate-resistant human PCa samples examined. Also, 5 out of the 6 DKK1-decreased PCa display positive expression of FOXA2, a NEPCa marker. Given that FOXA2 is a Wnt/β-Catenin target gene, these data suggest that loss of DKK1 enables Wnt/β-Catenin signaling and the NE phenotype.

Furthermore, promoter methylation is a key mechanism to silence the DKK1 expression in several types of cancer[52, 53]. We conducted methylation-specific PCR analysis of DKK1

promoter and found that this promoter was methylated in cancer-associated fibroblasts but not in normal fibroblasts. Co-culture with PCa LNCaP cells increased DKK1 promoter methylation in stromal cells (**Fig. 4**).

Discussion

NE cells represent only a minor population in the prostate epithelia and primary NEPCa is rare. However, NE differentiation of prostatic adenocarcinoma is commonly reported and associated with the advanced stage of PCa[1-13]. The NE components in prostatic adenocarcinoma express NE markers, such as Synaptophysin and Chromogranin A, often accompanied with little or no AR expression[11-13]. Studies have found that tumors with prominent NE components are typically hormone refractory and highly aggressive[1-13]. After long-term hormone therapy, an increase in NE differentiation is often observed in PCa[1-13]. NE differentiation is not only associated with castrate-resistant PCa, accumulating evidence suggests that androgen withdrawal even induces NE differentiation in human PCa[3-6]. A similar phenomenon was also observed in cell culture and animal models. For example, studies showed that androgen depletion or knocking down AR induced NE differentiation in androgen-dependent LNCaP cells[54-56], and castration accelerated the emergence of NE tumors in TRAMP mice[20-22]. Additionally, androgen withdrawal by castration of host mice induced NE differentiation in human PCa xenografts[57-59]. Taken together, these findings suggest that androgen deprivation selects for, or even accelerates, the process of NE differentiation. Our study identified a signaling pathway, Wnt/beta-Catenin signaling, that is involved in NE differentiation. We also found castration induced the expression of Wnt7a but failed to induce the Wnt inhibitor, DKK1, providing a mechanism for the activation of Wnt/beta-Catenin signaling and cancer progression.

GRANT SUPPORT

This research was supported by NIH R03 CA212567 and R01 CA226285, DOD grant W81XWH-12-1-0212, and LSUHSC FWCC and Office of Research funding to XY.

REFERENCES

[1] Abrahamsson PA: Neuroendocrine differentiation in prostatic carcinoma. The Prostate 1999, 39:135-48.
[2] Ahlgren G, Pedersen K, Lundberg S, Aus G, Hugosson J, Abrahamsson PA: Regressive changes and neuroendocrine differentiation in prostate cancer after neoadjuvant hormonal treatment. The Prostate 2000, 42:274-9.
[3] Hirano D, Okada Y, Minei S, Takimoto Y, Nemoto N: Neuroendocrine differentiation in hormone refractory prostate cancer following androgen deprivation therapy. European urology 2004, 45:586-92; discussion 92.
[4] Jiborn T, Bjartell A, Abrahamsson PA: Neuroendocrine differentiation in prostatic carcinoma during hormonal treatment. Urology 1998, 51:585-9.
[5] Beltran H, Tomlins S, Aparicio A, Arora V, Rickman D, Ayala G, Huang J, True L, Gleave ME, Soule H, Logothetis C, Rubin MA: Aggressive variants of castration-resistant prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2014, 20:2846-50.
[6] Terry S, Beltran H: The many faces of neuroendocrine differentiation in prostate cancer progression. Front Oncol 2014, 4:60.
[7] Epstein JI, Amin MB, Beltran H, Lotan TL, Mosquera JM, Reuter VE, Robinson BD, Troncoso P, Rubin MA: Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am J Surg Pathol 2014, 38:756-67.
[8] Nadal R, Schweizer M, Kryvenko ON, Epstein JI, Eisenberger MA: Small cell carcinoma of the prostate. Nature reviews Urology 2014, 11:213-9.
[9] Beltran H, Tagawa ST, Park K, MacDonald T, Milowsky MI, Mosquera JM, Rubin MA, Nanus DM: Challenges in recognizing treatment-related neuroendocrine prostate cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2012, 30:e386-9.
[10] Mosquera JM, Beltran H, Park K, MacDonald TY, Robinson BD, Tagawa ST, Perner S, Bismar TA, Erbersdobler A, Dhir R, Nelson JB, Nanus DM, Rubin MA: Concurrent AURKA and MYCN gene amplifications are harbingers of lethal treatment-related neuroendocrine prostate cancer. Neoplasia 2013, 15:1-10.
[11] Huang J, Yao JL, di Sant'Agnese PA, Yang Q, Bourne PA, Na Y: Immunohistochemical characterization of neuroendocrine cells in prostate cancer. The Prostate 2006, 66:1399-406.
[12] Tzelepi V, Zhang J, Lu JF, Kleb B, Wu G, Wan X, Hoang A, Efstathiou E, Sircar K, Navone NM, Troncoso P, Liang S, Logothetis CJ, Maity SN, Aparicio AM: Modeling a lethal prostate cancer variant with small-cell carcinoma features. Clinical cancer research : an official journal of the American Association for Cancer Research 2012, 18:666-77.
[13] Yao JL, Madeb R, Bourne P, Lei J, Yang X, Tickoo S, Liu Z, Tan D, Cheng L, Hatem F, Huang J, Anthony di Sant'Agnese P: Small cell carcinoma of the prostate: an immunohistochemical study. Am J Surg Pathol 2006, 30:705-12.

[14] Parimi V, Goyal R, Poropatich K, Yang XJ: Neuroendocrine differentiation of prostate cancer: a review. Am J Clin Exp Urol 2014, 2:273-85.
[15] Vlachostergios PJ, Puca L, Beltran H: Emerging Variants of Castration-Resistant Prostate Cancer. Curr Oncol Rep 2017, 19:32.
[16] Priemer DS, Montironi R, Wang L, Williamson SR, Lopez-Beltran A, Cheng L: Neuroendocrine Tumors of the Prostate: Emerging Insights from Molecular Data and Updates to the 2016 World Health Organization Classification. Endocr Pathol 2016, 27:123-35.
[17] Klimstra DS, Beltran H, Lilenbaum R, Bergsland E: The spectrum of neuroendocrine tumors: histologic classification, unique features and areas of overlap. Am Soc Clin Oncol Educ Book 2015:92-103.
[18] Dang Q, Li L, Xie H, He D, Chen J, Song W, Chang LS, Chang HC, Yeh S, Chang C: Anti-androgen enzalutamide enhances prostate cancer neuroendocrine (NE) differentiation via altering the infiltrated mast cells --> androgen receptor (AR) --> miRNA32 signals. Mol Oncol 2015, 9:1241-51.
[19] Roubaud G, Liaw BC, Oh WK, Mulholland DJ: Strategies to avoid treatment-induced lineage crisis in advanced prostate cancer. Nat Rev Clin Oncol 2017, 14:269-83.
[20] Huss WJ, Gray DR, Tavakoli K, Marmillion ME, Durham LE, Johnson MA, Greenberg NM, Smith GJ: Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model. Neoplasia 2007, 9:938-50.
[21] Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM: Androgen-independent prostate cancer progression in the TRAMP model. Cancer research 1997, 57:4687-91.
[22] Johnson MA, Iversen P, Schwier P, Corn AL, Sandusky G, Graff J, Neubauer BL: Castration triggers growth of previously static androgen-independent lesions in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. The Prostate 2005, 62:322-38.
[23] Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL: Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 2009, 324:787-90.
[24] Reid AH, Attard G, Barrie E, de Bono JS: CYP17 inhibition as a hormonal strategy for prostate cancer. Nat Clin Pract Urol 2008, 5:610-20.
[25] Debes JD, Tindall DJ: Mechanisms of androgen-refractory prostate cancer. N Engl J Med 2004, 351:1488-90.
[26] Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ: Wnt/beta-catenin activation promotes prostate tumor progression in a mouse model. Oncogene 2011, 30:1868-79.
[27] Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R, Matusik RJ: Expression and role of Foxa proteins in prostate cancer. The Prostate 2006, 66:1013-28.
[28] Yu X, Gupta A, Wang Y, Suzuki K, Mirosevich J, Orgebin-Crist MC, Matusik RJ: Foxa1 and Foxa2 interact with the androgen receptor to regulate prostate and epididymal genes differentially. Ann N Y Acad Sci 2005, 1061:77-93.
[29] Miller JR: The Wnts. Genome Biol 2002, 3:REVIEWS3001.
[30] Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, Quist MJ, Jing X, Lonigro RJ, Brenner JC, Asangani IA, Ateeq B, Chun SY, Siddiqui J, Sam L, Anstett M, Mehra R, Prensner JR, Palanisamy N, Ryslik GA, Vandin F, Raphael BJ, Kunju LP, Rhodes DR, Pienta KJ, Chinnaiyan AM, Tomlins SA: The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012, 487:239-43.
[31] Rajan P, Sudbery IM, Villasevil ME, Mui E, Fleming J, Davis M, Ahmad I, Edwards J, Sansom OJ, Sims D, Ponting CP, Heger A, McMenemin RM, Pedley ID, Leung HY: Next-generation sequencing of advanced prostate cancer treated with androgen-deprivation therapy. European urology 2014, 66:32-9.

[32] Chen G, Shukeir N, Potti A, Sircar K, Aprikian A, Goltzman D, Rabbani SA: Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. Cancer 2004, 101:1345-56.
[33] Yokoyama NN, Shao S, Hoang BH, Mercola D, Zi X: Wnt signaling in castration-resistant prostate cancer: implications for therapy. Am J Clin Exp Urol 2014, 2:27-44.
[34] Wan X, Liu J, Lu JF, Tzelepi V, Yang J, Starbuck MW, Diao L, Wang J, Efstathiou E, Vazquez ES, Troncoso P, Maity SN, Navone NM: Activation of beta-catenin signaling in androgen receptor-negative prostate cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research 2012, 18:726-36.
[35] de la Taille A, Rubin MA, Chen MW, Vacherot F, de Medina SG, Burchardt M, Buttyan R, Chopin D: Beta-catenin-related anomalies in apoptosis-resistant and hormone-refractory prostate cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research 2003, 9:1801-7.
[36] Yardy GW, Brewster SF: Wnt signalling and prostate cancer. Prostate Cancer Prostatic Dis 2005, 8:119-26.
[37] Chesire DR, Ewing CM, Gage WR, Isaacs WB: In vitro evidence for complex modes of nuclear beta-catenin signaling during prostate growth and tumorigenesis. Oncogene 2002, 21:2679-94.
[38] Wissmann C, Wild PJ, Kaiser S, Roepcke S, Stoehr R, Woenckhaus M, Kristiansen G, Hsieh JC, Hofstaedter F, Hartmann A, Knuechel R, Rosenthal A, Pilarsky C: WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. The Journal of pathology 2003, 201:204-12.
[39] Horvath LG, Henshall SM, Kench JG, Saunders DN, Lee CS, Golovsky D, Brenner PC, O'Neill GF, Kooner R, Stricker PD, Grygiel JJ, Sutherland RL: Membranous expression of secreted frizzled-related protein 4 predicts for good prognosis in localized prostate cancer and inhibits PC3 cellular proliferation in vitro. Clinical cancer research : an official journal of the American Association for Cancer Research 2004, 10:615-25.
[40] Kawano Y, Kitaoka M, Hamada Y, Walker MM, Waxman J, Kypta RM: Regulation of prostate cell growth and morphogenesis by Dickkopf-3. Oncogene 2006, 25:6528-37.
[41] Perry AS, O'Hurley G, Raheem OA, Brennan K, Wong S, O'Grady A, Kennedy AM, Marignol L, Murphy TM, Sullivan L, Barrett C, Loftus B, Thornhill J, Hewitt SM, Lawler M, Kay E, Lynch T, Hollywood D: Gene expression and epigenetic discovery screen reveal methylation of SFRP2 in prostate cancer. Int J Cancer 2013, 132:1771-80.
[42] Chesire DR, Ewing CM, Sauvageot J, Bova GS, Isaacs WB: Detection and analysis of beta-catenin mutations in prostate cancer. The Prostate 2000, 45:323-34.
[43] Chiaverotti T, Couto SS, Donjacour A, Mao JH, Nagase H, Cardiff RD, Cunha GR, Balmain A: Dissociation of epithelial and neuroendocrine carcinoma lineages in the transgenic adenocarcinoma of mouse prostate model of prostate cancer. Am J Pathol 2008, 172:236-46.
[44] Tan HL, Sood A, Rahimi HA, Wang W, Gupta N, Hicks J, Mosier S, Gocke CD, Epstein JI, Netto GJ, Liu W, Isaacs WB, De Marzo AM, Lotan TL: Rb loss is characteristic of prostatic small cell neuroendocrine carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research 2014, 20:890-903.
[45] Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, Montgomery B, Taplin ME, Pritchard CC, Attard G, Beltran H, Abida W, Bradley RK, Vinson J, Cao X, Vats P, Kunju LP, Hussain M, Feng FY, Tomlins SA, Cooney KA, Smith DC, Brennan C, Siddiqui J, Mehra R, Chen Y, Rathkopf DE, Morris MJ, Solomon SB, Durack JC, Reuter VE, Gopalan A, Gao J, Loda M, Lis RT, Bowden M, Balk SP, Gaviola G, Sougnez C, Gupta M, Yu EY, Mostaghel EA, Cheng HH, Mulcahy H, True LD, Plymate SR, Dvinge H, Ferraldeschi R, Flohr P, Miranda S, Zafeiriou Z, Tunariu N, Mateo J, Perez-Lopez R, Demichelis F, Robinson BD, Schiffman M, Nanus DM, Tagawa ST, Sigaras A, Eng KW, Elemento O, Sboner A, Heath EI,

Scher HI, Pienta KJ, Kantoff P, de Bono JS, Rubin MA, Nelson PS, Garraway LA, Sawyers CL, Chinnaiyan AM: Integrative clinical genomics of advanced prostate cancer. Cell 2015, 161:1215-28.
[46] Beltran H, Yelensky R, Frampton GM, Park K, Downing SR, MacDonald TY, Jarosz M, Lipson D, Tagawa ST, Nanus DM, Stephens PJ, Mosquera JM, Cronin MT, Rubin MA: Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. European urology 2013, 63:920-6.
[47] Zhou Z, Flesken-Nikitin A, Corney DC, Wang W, Goodrich DW, Roy-Burman P, Nikitin AY: Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer. Cancer research 2006, 66:7889-98.
[48] Ferrer-Vaquer A, Piliszek A, Tian G, Aho RJ, Dufort D, Hadjantonakis AK: A sensitive and bright single-cell resolution live imaging reporter of Wnt/ss-catenin signaling in the mouse. BMC Dev Biol 2010, 10:121.
[49] David MD, Canti C, Herreros J: Wnt-3a and Wnt-3 differently stimulate proliferation and neurogenesis of spinal neural precursors and promote neurite outgrowth by canonical signaling. J Neurosci Res 2010, 88:3011-23.
[50] Hall CL, Daignault SD, Shah RB, Pienta KJ, Keller ET: Dickkopf-1 expression increases early in prostate cancer development and decreases during progression from primary tumor to metastasis. Prostate 2008, 68:1396-404.
[51] Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, Shah RB, Chandran U, Monzon FA, Becich MJ, Wei JT, Pienta KJ, Ghosh D, Rubin MA, Chinnaiyan AM: Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer cell 2005, 8:393-406.
[52] Aguilera O, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM: Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. Oncogene 2006, 25:4116-21.
[53] Kocemba KA, Groen RW, van Andel H, Kersten MJ, Mahtouk K, Spaargaren M, Pals ST: Transcriptional silencing of the Wnt-antagonist DKK1 by promoter methylation is associated with enhanced Wnt signaling in advanced multiple myeloma. PLoS One 2012, 7:e30359.
[54] Yuan TC, Veeramani S, Lin MF: Neuroendocrine-like prostate cancer cells: neuroendocrine transdifferentiation of prostate adenocarcinoma cells. Endocrine-related cancer 2007, 14:531-47.
[55] Wright ME, Tsai MJ, Aebersold R: Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Molecular endocrinology 2003, 17:1726-37.
[56] Shen R, Dorai T, Szaboles M, Katz AE, Olsson CA, Buttyan R: Transdifferentiation of cultured human prostate cancer cells to a neuroendocrine cell phenotype in a hormone-depleted medium. Urol Oncol 1997, 3:67-75.
[57] Lin D, Wyatt AW, Xue H, Wang Y, Dong X, Haegert A, Wu R, Brahmbhatt S, Mo F, Jong L, Bell RH, Anderson S, Hurtado-Coll A, Fazli L, Sharma M, Beltran H, Rubin M, Cox M, Gout PW, Morris J, Goldenberg L, Volik SV, Gleave ME, Collins CC, Wang Y: High fidelity patient-derived xenografts for accelerating prostate cancer discovery and drug development. Cancer research 2014, 74:1272-83.
[58] Noordzij MA, van Weerden WM, de Ridder CM, van der Kwast TH, Schroder FH, van Steenbrugge GJ: Neuroendocrine differentiation in human prostatic tumor models. Am J Pathol 1996, 149:859-71.
[59] Jongsma J, Oomen MH, Noordzij MA, Van Weerden WM, Martens GJ, van der Kwast TH, Schroder FH, van Steenbrugge GJ: Kinetics of neuroendocrine differentiation in an androgen-dependent human prostate xenograft model. Am J Pathol 1999, 154:543-51.
[60] Suzuki H, Toyota M, Carraway H, Gabrielson E, Ohmura T, Fujikane T, Nishikawa N, Sogabe Y, Nojima M, Sonoda T, Mori M, Hirata K, Imai K, Shinomura Y, Baylin SB, Tokino T: Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer 2008, 98:1147-56.

Figure legends

Fig. 1 Wnt/β-Catenin pathway is active in NEPCa. Upper panels: IHC staining for GFP and Chromogranin A on serial sections derived from TRAMP/Wnt-reporter prostate. GFP and Chromogranin A (Chr A) were highly expressed in NEPCa but not in the PIN areas. Lower panels: IHC staining of T-antigen and GFP on serial sections derived from PCa lung metastasis of a TRAMP/Wnt-reporter mouse.

Fig. 2 Castration induces the expression of Wnt7a, whereas Dkk1 expression is lower in NEPCa. qRT-PCR was used to assess the expression of Wnt7a and Dkk1 in prostates derived from intact or castrated (CX) wild type (WT) and TRAMP mice. It is noteworthy that the castrated TRAMP tumors are NEPCa and the intact TRAMP tumors have PIN histology.

Fig. 3 stromal/epithelial interaction is involved in the activation of Wnt signaling. qRT-PCR was conducted to assess the expression of WNT7A and CD56 (NEPCa marker) in PCa. LNCaP cells were cultured alone or co-cultured with normal prostate fibroblasts (NPFs) in the presence or absence of androgens. Androgen-deprivation significantly induced the expression of WNT7A and CD56 in LNCaP cells when they were co-cultured with NPFs. DKK1 was highly expressed in NPFs, but co-culture with PCa (LNCaP) cells silenced the expression of DKK1. (*, $p<0.05$. **, $p<0.01$, t-test)

Fig. 4 DKK1 expression is decreased in NEPCa (data extracted from microarray data GDS1439). Upper panels: DKK1 expression was decreased in all the 6 castrate-resistant PCa, 5 of which demonstrating elevated expressions of FOXA2 and 3 demonstrating increased chromogranin A (CHGA). Benign: benign prostates; localized: localized PCa; CRPC: castrate-resistant PCa. lower panels: methylation-specific PCR[60] analysis of CAFs, NPFs, or NPFs that

were co-cultured with LNCaP, with or without enzalutamide (ENZ). ‘M’ lanes are PCR products with methylation-specific primers; ‘U’ lanes are with unmethylated-specific primers.

Fig. 1

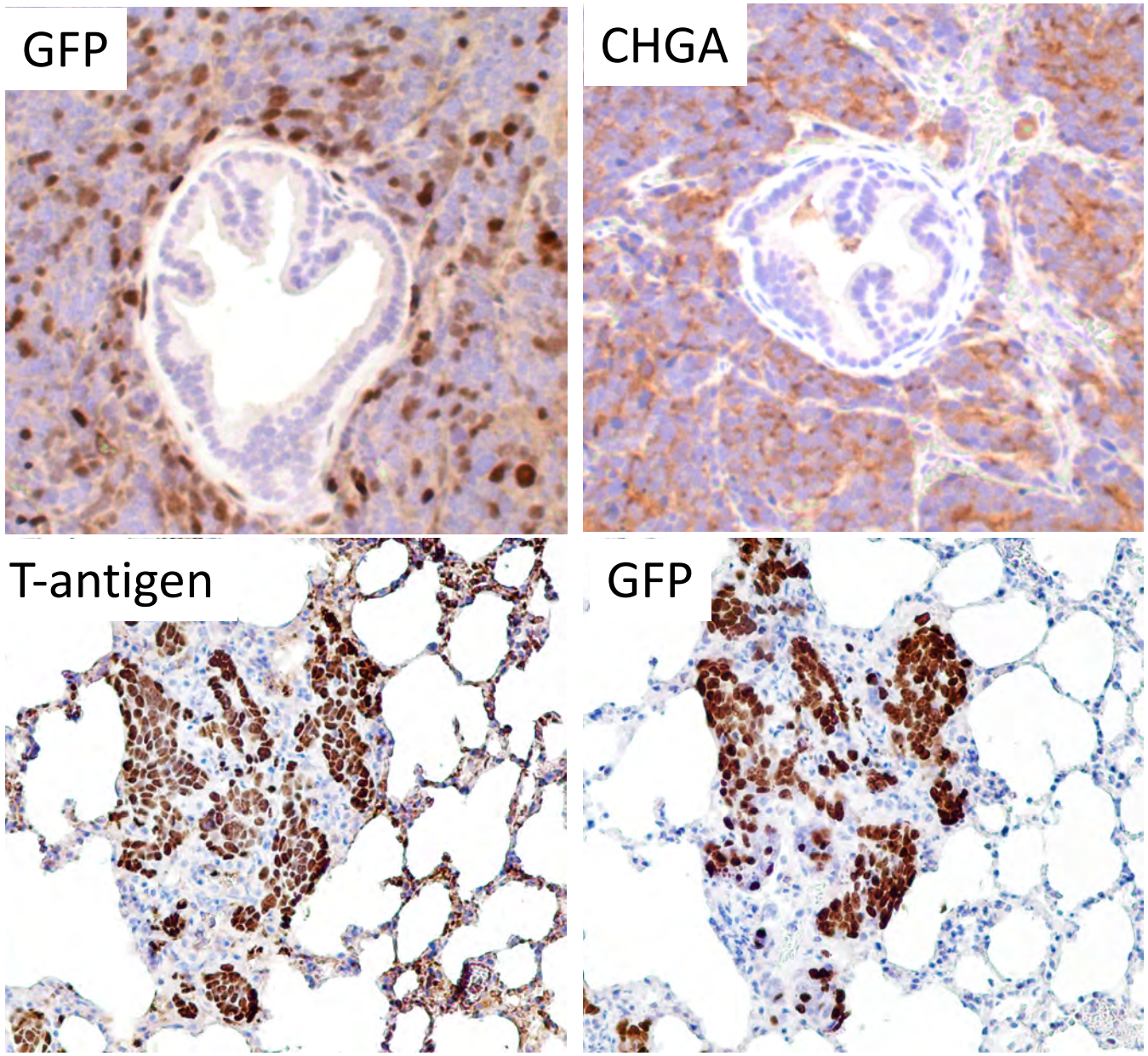


Fig. 2

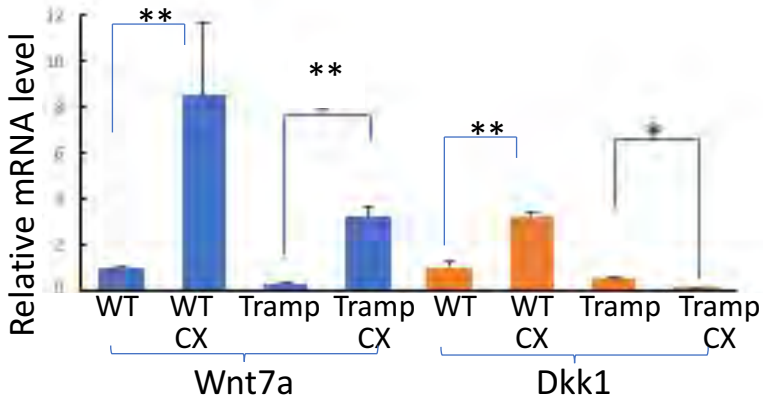

Relative mRNA level
**
**
**
*
WT
WT CX
Tramp
Tramp CX
WT
WT CX
Tramp
Tramp CX
Wnt7a
Dkk1

Fig. 3

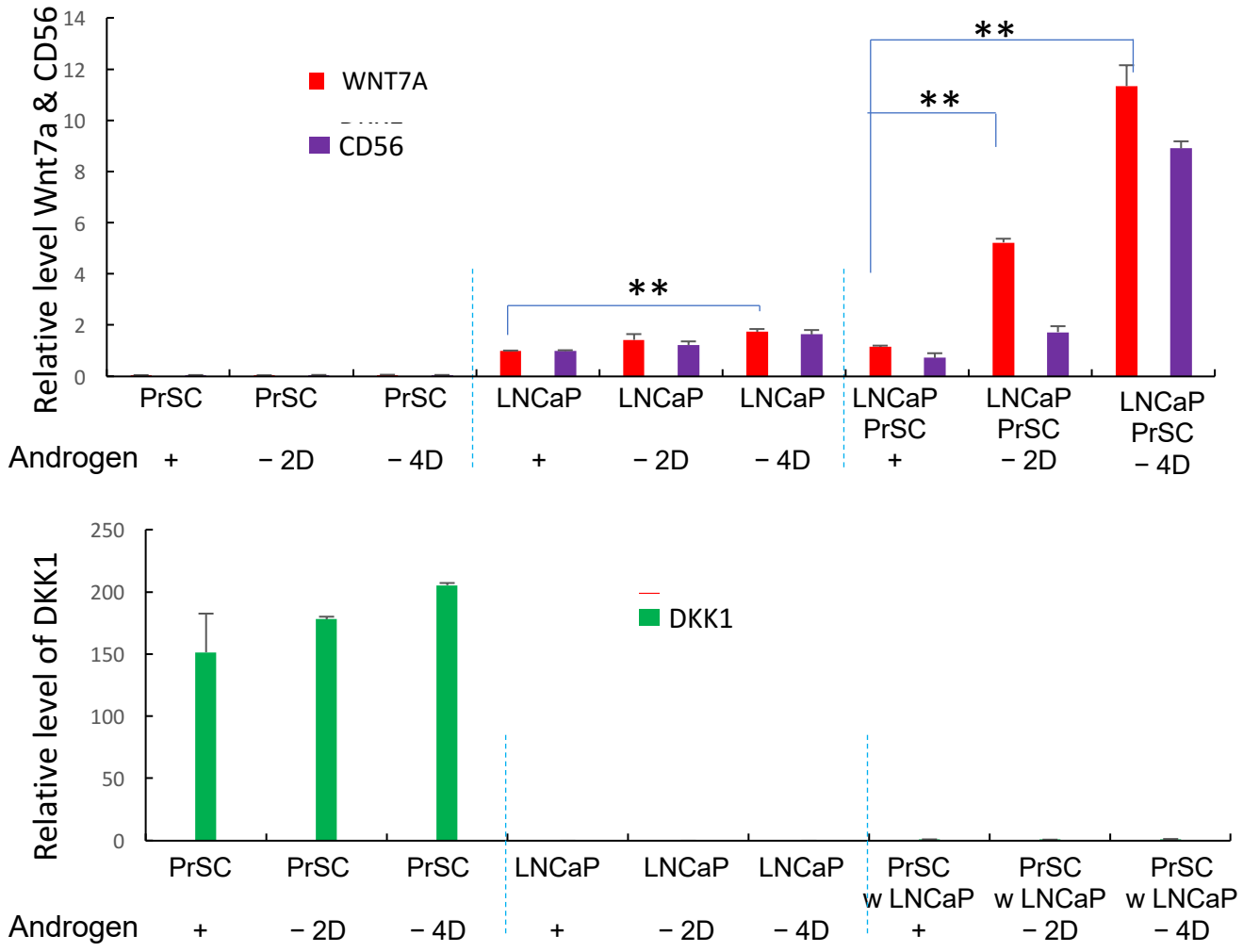
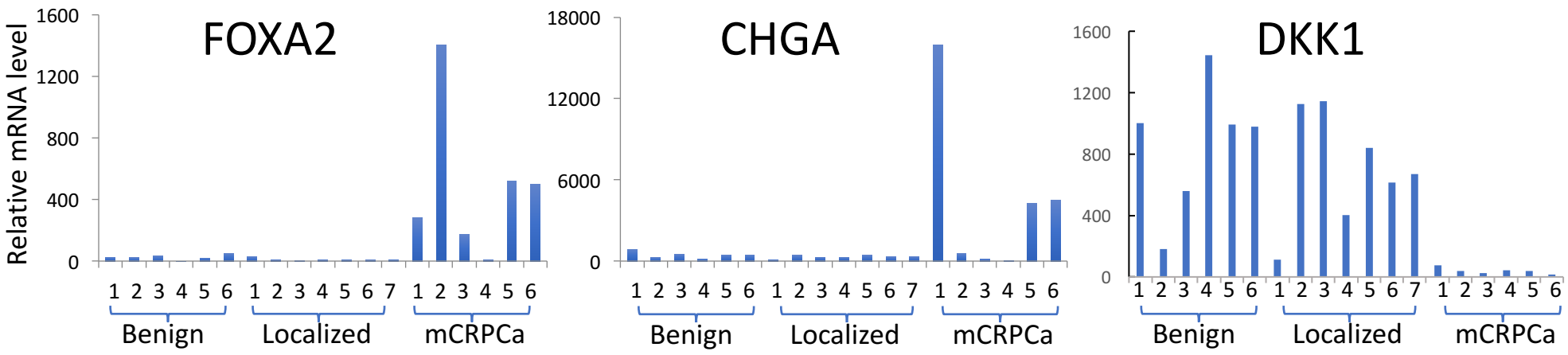
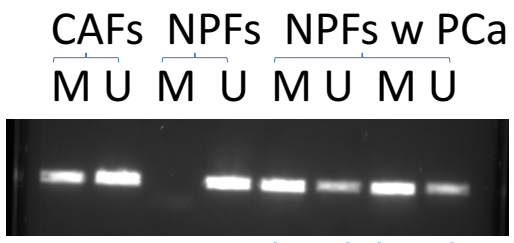

Relative level Wnt7a & CD56
14
12
10
8
6
4
2
0
WNT7A
CD56
**
**
**
PrSC
PrSC
PrSC
LNCaP
LNCaP
LNCaP
LNCaP PrSC
LNCaP PrSC
LNCaP PrSC
Androgen
+
− 2D
− 4D
+
− 2D
− 4D
+
− 2D
− 4D
Relative level of DKK1
250
200
150
100
50
0
DKK1
PrSC
PrSC
PrSC
LNCaP
LNCaP
LNCaP
PrSC w LNCaP
PrSC w LNCaP
PrSC w LNCaP
Androgen
+
− 2D
− 4D
+
− 2D
− 4D
+
− 2D
− 4D

Fig. 4





Methylation-specific PCR

The expression of YAP1 is lost in neuroendocrine prostate cancer

Siyuan Cheng[1*], Nestor Prieto-Dominguez[1*], Shu Yang[1*], Zachary M. Connelly[1], Samantha StPierre[1], Bryce Rushing[1], Andy Watkins[1], Lawrance Shi[1], Yong Teng[2], Meredith Lakey[3], Lyndsey Buckner Baiamonte[3,] Colm Morrissey[4], Eva Corey[4], Runhua Shi[5], Mingxia Shi[6], Yunshin Yeh[7#], Xiuping Yu[1, 8#]

[1]Department of Biochemistry & Molecular Biology, [5]Department of Medicine, [6]Department of Pathology, [8]Department of Urology, LSU Health Sciences Center, Shreveport, LA

[2]Department of Oral Biology and Diagnostic Sciences, Augusta University, Augusta, GA

[3]Ochsner Clinic Foundation, New Orleans, LA

[4]Dept of Urology, University of Washington, Seattle, WA

[7]Department of Pathology, Overton Brooks Medical Center, Shreveport, LA

*Cheng S, Prieto-Dominguez N, and Yang S contribute equally

Correspondence to: Yunshin Yeh, email: Yunshin Yeh, Yunshin.Yeh@va.gov mailing address: 510 E Stoner Ave, Shreveport, LA 71101. Xiuping Yu, email: xyu@lsuhsc.edu mailing address: 1501 Kings Hwy, LSU Health Sciences Center, Shreveport, LA 71103

ABSTRACT

Background After long term androgen deprivation therapy, prostate cancer (PCa) acquires an aggressive neuroendocrine (NE)-like phenotype. This process reduces PCa patients' survival by enhancing tumor malignancy and cell resistance to therapies. YAP1 deregulation has been related to the cancer progression. However, its role in PCa NE differentiation has not been assessed yet.

Methods In this study, we analyzed by immunohistochemistry whether YAP1 levels change during PCa initiation, progression and NE differentiation. Moreover, we evaluated the ability of YAP1 to modulate Wnt/β-catenin signaling by luciferase reporter assays.

Results YAP1 expression was present in the basal epithelial cells in benign prostate tissues. YAP1 expression was lost in low grade PCa but was detected in high grade prostate adenocarcinomas. Interestingly, the expression of YAP1 was abolished in both human and mouse NEPCa, suggesting that this loss is implicated in the acquisition of NE phenotype. Finally, knocking down YAP1 promoted the activity of Wnt/β-catenin signaling in PCa cells.

Conclusions The expression of YAP1 is lost in NEPCa and reduced YAP1 augments Wnt/β-catenin signaling in PCa cells.

INTRODUCTION

Prostate cancer (PCa) is the most diagnosed cancer among American men [1]. Although androgen deprivation therapy (ADT) is effective in the treatment of advanced PCa, most of the prostate tumors eventually become resistant to the treatment, progressing into castrate-resistant prostate cancer (CRPCa) [2]. During this process, some PCa cells loses their prostate features and trans-differentiate into neuroendocrine prostate cancer (NEPCa) [3]. This phenotype enhances the aggressiveness of prostatic tumors and reduces their sensitivity to therapies, diminishing drastically patients' survival [3]. Understanding the molecular mechanisms that drive PCa NE differentiation is critical for preventing the arising of this lethal phenotype.

YES-associated Protein (YAP1) and Transcriptional Coactivator with PDZ-binding Motif (TAZ) are key transcriptional coactivators in the modulation of several processes in mammalian cells, such as glucose uptake, proliferation, spreading, apoptosis and differentiation [4]. Despite the similarities that YAP1/TAZ present in domain structure and activation mechanism, neither their protein-protein interactions nor their gene activation patterns overlap, even inducing opposite responses during certain cellular processes [5,6]. The activity of these transcriptional coactivators is mainly modulated by the core kinases of the Hippo pathway [4]. Indeed, the activation of Mammalian Sterile 20-like Kinase 1/2 (MST1/2) by upstream signals induces the binding of Salvador Homolog 1 (SAV1) and the phosphorylation and activation of Large Tumor Suppressor Homolog 1 (LATS1/2) and MOB Kinase Activator 1A and 1B (MOB1A/1B) [4]. Subsequently, the dimerization of these two activated proteins induces the phosphorylation of YAP1/TAZ [4]. As a result of this process, their translocation to the nucleus is impaired, promoting their degradation through the ubiquitin-proteasome system [4]. Oppositely, when the Hippo pathway is inactivated, YAP1/TAZ can cross the nuclear membrane, bind to the TEA domain proteins 1-4 (TEAD1-4) proteins, and activate the transcription of target genes [4]. Additionally, their translocation to the nucleus can be modulated by Wnt/β-catenin signaling in a Hippo-independent manner [7]. They are sequestered in the β-catenin destruction complex, which impairs their nuclear translocation and promotes their proteasomal degradation by recruiting E3 ubiquitin ligase, β-transducin repeat containing protein (β-TrCP) [7]. Moreover, the loss of these transcriptional coactivators can induce β-catenin-dependent gene expression, suggesting that the Hippo and the Wnt/β-catenin pathways are interconnected to develop an integrated response in mammalians [8].

Given their implication in promoting cell proliferation and spreading, deregulation of YAP1/TAZ has been related to cancer initiation and progression with either oncogenic or tumor suppressant roles [9,10]. In PCa, the overexpression of YAP1/TAZ has been associated with cancer proliferation and invasiveness [11]. However, these effects are mainly detectable in CRPCa because androgen signaling can restrain epigenetically the expression of these proteins [12]. Although their function in NEPCa has not been assessed yet, it has recently been suggested that YAP1 is implicated in the NE differentiation of lung cancer cells [10]. In the present study, we found that the expression of YAP1 was down regulated in NEPCa. We also showed that a downregulation of YAP1 activated Wnt/β-catenin signaling, which could promote NE differentiation of PCa. As far as known, this is the first study that analyzes the involvement of YAP1 in NEPCa.

MATERIAL AND METHODS

Sample obtaining

De-identified human prostate tissue specimens were obtained from the Overton Brooks VA Medical Center, LSU Health Sciences Center Biorepository Core, Ochsner Health System Biorepository, and Tissue for Research at UK. All the tissues were used in accordance with LSU Health Sciences Center-Shreveport IRB protocols. Archived tissues derived from TRAMP, 12T-7 LADY, 12T-10 LADY, and NE10 mice were used for this study.

Immunohistochemistry and Hematoxylin and eosin (H&E) staining.

Immunostaining was performed as described previously [13]. Antigen retrieval was conducted using a pressure cooker with an antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 10 minutes. Subsequently, endogenous peroxidases were blocked using 0.3% hydrogen peroxidase in methanol and slices were incubated overnight at 4 °C with the primary antibodies for YAP1, chromogranin A (CHGA) (sc-101199 and sc-1488 respectively, Santa Cruz biotechnology, Santa Cruz, CA), synaptophysin (SYP) (611880, BD biosciences, San Jose, CA), p63, FOXA2 and TAZ (ab735, ab108422 and ab110239 respectively, Abcam, Cambridge, MA). The antibody-antigen interaction was detected by the Vectastain elite ABC peroxidase kit and visualized with the ImmPACT™ DAB peroxidase substrate kit (Vector Laboratories). Afterward,

the slices were counterstained with hematoxylin, dehydrated, mounted with Cytoseal™ 60 (Thermo Fisher Scientific, Waltham, MA, USA) and imaged with a Zeiss microscope (Pleasanton, CA).

For H&E staining, rehydrated slices were sequentially dyed with eosin and hematoxylin, dehydrated and cover-slipped before being imaged with a Zeiss microscope.

Immunofluorescence

Immunofluorescence staining was conducted as described previously [13]. After antigen retrieval, tissue sections were incubated overnight at 4 °C with the primary antibodies against cytokeratin 5 (CK5) or 14 (CK14) (904801 and 905501 respectively, BioLegend, San Diego, CA) and YAP (Santa Cruz biotechnology). Subsequently, samples were incubated with fluorescent secondary anti-rabbit and anti-mouse antibodies (DI-2594 and DI-1088 respectively, Vector Laboratories) for 1 hour, cover-slipped with Vectashield® mounting media (Vector Laboratories) and imaged at 60x magnification with a Nikon fluorescence microscope (Melville, NY).

Bioinformatics

RNA-seq datasets deposited in either cBioportal or in the Gene expression omnibus (GEO) tool of the National Center of Biotechnology Information (Bethesda, MD) were analyzed by RStudio software [14]. To represent the data, heat maps and boxplots were made in R by the pheatmap and ggplot2 packages respectively [15,16].

Cell culture, gene silencing and Luciferase assay

The human PCa cell line PC3 was purchased from American type culture collection (ATCC) and routinely grown in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. All these reagents were purchased from Genesee Scientific (El Cajon, CA). To perform gene silencing, cells were transfected with siRNAs against

YAP1 or a non-coding sequence (sc-38673 and sc-37007 respectively, Santa Cruz Biotechnology) by using Lipofectamine® LTX & PLUS™ Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. For luciferase assay cells were co-transfected with siRNA and the TOPflash plasmid, which is a β-catenin-responsive luciferase reporter construct [17]. After 36 hours of incubation, luciferase activities were measured by using Promega Luciferase Assay kit (Maddison, WI) following the manufacturer's instructions.

Western blotting

Right after treatments, cells were lysed with passive lysis buffer (Promega), sonicated for 5 pulses of 10 seconds at 30% amplitude and centrifuged for 30 minutes at 12,000g. Equal amounts of protein were subjected to SDS-PAGE, transferred to a PDVF membrane (Bio-Rad, Hercules, CA), blocked with 5% non-fat milk for 1 hour and incubated overnight with YAP1 (Santa Cruz biotechnology) or TAZ (Abcam) primary antibodies. β-actin (3700, Cell signaling) was used as the loading control. Subsequently, membranes were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (Healthcare, Pittsburg, PA). Proteins were revealed by using ProSignal® Dura ECL Reagent (Genesee Scientific) and visualized in a Chemidoc™ Touch Imaging System (Bio-Rad).

RESULTS

YAP1/TAZ are expressed in benign prostate basal cells

Firstly, we validated the specificity of the YAP1 antibody by Western blot using cell lysate of PC3 cells, which highly express YAP1, and PC3/YAP1 knock down cells. As shown in suppl. Fig. 1, the level of YAP1 was knocked down in PCa PC3 cells. We then assessed the expression of YAP1/TAZ in benign prostate tissues. YAP1 was clearly expressed in the nucleus of the prostate p63-positive basal epithelial cells, as well as stromal cells, but it was absent in the p63-negative luminal epithelial cells (Figs. 1A & 1B). TAZ expression followed the same pattern in non-cancerous prostate epithelium (Fig. 1C). Additionally, dual immunofluorescence staining of YAP1 and CK5 or CK14, which specifically mark prostate basal cells, was performed to corroborate the previous YAP1 expression patterns in prostate epithelium. As expected, YAP1 immunostaining was mainly present in the nucleus of the cells that were positive for CK5 and CK14 (Figs. 1D to

1K). These results indicate that the expression of YAP1 and TAZ is limited to the basal epithelial cells in non-cancerous prostate.

The expression of YAP1 increased in PIN but lost in NEPCa of mouse prostate tumors.

Changes in the Yap1/Taz expression levels during PCa progression were analyzed by immunohistochemistry using tissues derived from both TRAMP and LADY mice, the two widely used PCa mouse models [18,19]. TRAMP mice develop prostate intraepithelial neoplasia (PIN) and a subset progress into NEPCa [20,21]. As shown in Figs. 2A and 2B, Yap1 expression levels were slightly elevated in the PIN tumors of TRAMP mice (Fig. 2C) compared to wild type prostates (Fig. 2A). In the TRAMP mice that developed NEPCa, Yap1 level was even higher in the focal PIN areas in NEPCa tumors (Fig. 2E). However, this protein was undetectable in the NEPCa areas (Fig. 2E). Taz displayed a similar expression pattern. Its level was low in wild type prostate (Fig. 2B), increased in PIN (Fig. 2D), but decreased in NEPCa (Fig. 2F).

12T-7 LADY mice develop PIN [19]. Yap1 expression was detected in the PIN tumors in the 12T-7 LADY mice (Fig. 3A). However, the 12T-10 LADY mice, which develops NEPCa [22], exhibited high levels of this protein in the focal PIN areas, but a complete loss of Yap1 immunostaining in the FOXA2-positive NEPCa areas of these tumors (Figs 3C & 3D). The Yap1 expression was also lost in NE10 tumors (Figs 3E & 3F), which is a xenograft line derived from the 12T-10 NEPCa [23]. Additionally, Yap1 immunostaining was absent in FOXA2-positive, NEPCa metastases to the liver and lung (Figs 4A to 4D). Immunohistochemistry of Taz on LADY tissues generated lot of non-specific staining and the results were not conclusive, possibly due to the age of these specimens. While the archived TRAMP tissues were collected in less than 4 years ago, the LADY tissues were collected more than 15 years ago. Nonetheless, these results indicate that mouse-derived NEPCa tumors exhibit a marked reduction in Yap1 expression compared to the non-NE tumors.

The expression of YAP1 increased in human high grade prostate adenocarcinomas but decreased in NEPCa

YAP1 expression levels were also evaluated in human prostate tissues including benign prostate, low grade adenocarcinoma (AdPCa), high grade AdPCa, and NEPCa. YAP1 expression was detected in stromal cells in most cases. However, the expression of this protein in epithelial cells was altered during PCa progression (Figs 5A to 5O). Specifically, YAP1 was expressed in the basal epithelial cells as well as stromal cells in benign prostatic tissues (Fig. 5A). In low grade AdPCa with Gleason score (GS) less than or equal to 7, YAP1 expression was detected in the basal epithelia and stromal cells but absent in most cancer cells (Fig. 5D). High grade AdPCa with GS greater than or equal to 8 exhibited higher YAP1 immunostaining than low-grade tumors. YAP1 expression was detected in cancer cells in GS8, GS9, and GS10 cases but absent in one GS GS8 case (Fig. 5G). However, YAP1 expression was lost in NEPCa, including both prostate adenocarcinoma with NE differentiation (Fig. 5J) and small cell NEPCa (Fig. 5M). In the adenocarcinomas with NE differentiation (Fig. 5J), YAP1 expression was still present in the adenocarcinoma cells but absent in NEPCa cells. FOXA2 (Fig. 5K), chromogranin A (CHGA, Fig. 5L) and synaptophysin (SYP, Fig. 5O) were used as markers of NEPCa. These results indicate that human NEPCa lacks YAP1 expression.

To further validate the reduced YAP1 expression in NEPCa, we analyzed the mRNA levels of YAP1, TAZ, AR and NEPCa markers including FOXA2, SOX2, SYP and CHGA in RNA-seq dataset of 46 LuCaP PCa patient-derived xenografts (PDXs). As shown in Fig. 6A, YAP1 expression was lost in all the nine $NE^{positive}$ LuCaP PDXs including eight $AR^{negative}$ and one $AR^{positive}$ NEPCa. However, loss of YAP1 expression only occurred in one out of thirty-five $AR^{positive}$ AdPCa. YAP1 expression was detectable in the double negative LuCaP 173.2 as well as the $AR^{low}/NE^{negative}$ LuCaP 176 PDX. The expression of TAZ in these LuCaP PDXs did not show any consistent patterns (Fig. 6A). Additionally, the RNA-seq data published by Beltran *et al* [25] corroborated that NEPCa tumors exhibited reduced YAP1 expression. However, this trend was not followed by TAZ [25] (Fig. 6B). Additionally, we analyzed the mRNA expression of YAP1 and TAZ in several PCa cell lines using publicly available RNA-seq data [24]. The expression level of YAP1 was the lowest in H660 cells, the only NEPCa cell line, compared to all the other PCa cell lines tested (Fig. 6C). Oppositely, the mRNA levels of YAP1 and TAZ remained high in the other cell lines [24] (Fig. 6C). Taken together, these data indicate that NEPCa cells present a reduction or a loss of YAP1 expression.

YAP1 loss in AdPCa cells induces Wnt/β-catenin activity.

After demonstrating that YAP1 expression was reduced in NEPCa cells, we decided to analyze if the loss of this protein has any functional implications in PCa. Previous studies have linked the activation of Wnt/β-catenin signaling with NE differentiation of PCa [26]. Moreover, the loss of YAP1/TAZ seems to stimulate Wnt/β-catenin signaling [7,8]. Therefore, we assessed how reduced YAP1 expression would affect the Wnt/β-catenin activity by knocking down the expression of YAP1 followed by luciferase-reporter assays. As shown in Fig. 7, the activity of Wnt/β-catenin increased after YAP1 expression was reduced in AdPCa cells, indicating that the downregulation of YAP1 augments Wnt/β-catenin signaling, which could lead to NE differentiation.

DISCUSSION

The arising of neuroendocrine-like cells in advanced PCa is a harmful event that usually occurs after ADT, because it leads to cancer progression and reduced sensitivity to conventional therapies [3]. Identifying the mechanisms that drive this NE differentiation process is fundamental to improve patients' survival [3]. Here, we have discovered for the first time that the Hippo pathway effector YAP1 is reduced in NEPCa.

YAP1 and its homologous protein TAZ are mainly implicated in the regulation of tissue homeostasis and organ size [4]. Their dysregulation could promote massive overgrowth and tumorigenesis [27]. In cancer, YAP1/TAZ usually work as oncogenes since they promote cell proliferation, spreading and mobility; as well as prevent programmed cell death through apoptosis and anoikis (cell-detachment-induced apoptosis) [28]. However, YAP1/TAZ have also been demonstrated to exert tumor suppressive roles in certain cellular contexts [28]. For instance, YAP1 overexpression reduces small cell lung carcinoma (SCLC) growth by inducing cell apoptosis [29]. Inversely, its loss stimulates breast cancer cell proliferation and mobility, protecting them from anoikis [30]. These data suggest that YAP1 and TAZ exert a dual role during tumorigenesis, mainly because of their ability to interact with different proteins according to the cellular context

[28]. Therefore, it would be interesting to monitor the changes in the levels of these proteins during PCa progression.

Firstly, we discovered that the expression of these proteins was restrained to the basal cells in benign prostate epithelia, being nearly absent in the luminal cells. Our results support the use of these proteins as markers for prostate basal epithelial cells, as it has been previously enunciated by Liu *et al* [31]. Besides, this epithelial expression signature is not limited only to the prostate tissue, but is also present in other epithelial tissues, such as the cornea, the airway epithelium, the skin and the breast ductal lining [32-35]. It has been suggested that YAP1/TAZ proteins are necessary to maintain endothelial homeostasis, probably through promoting tissue renewal [36].

On the other hand, YAP1 expression was absent in the cancer cells of early AdPCa tumors where basal cells are lost. However, its expression increased in more advanced stages of this malignant disease. These results corroborate the existence of a positive trend between the expression levels of this protein and AdPCa malignancy, where YAP1-positive tumors present higher Gleason scores and tumor-node-metastasis (TNM) stages [37,38]. Additionally, the inhibition of this protein in high-grade PCa cells not only impair their proliferation, but also enhances cell sensitiveness to ADT, suggesting that this protein might inhibit AR signaling in advanced PCa [11,12]. Curiously, it has been recently proven that AR signaling inhibits YAP1 transcription by promoting the methylation of its promoter, which could explain its low expression levels in both luminal cells and low-grade tumors, where this receptor is expressed [12,39]. In summary, these findings suggest that the inhibition of YAP1 could control AdPCa proliferation and spreading.

Despite the ability of YAP1 to modulate cell differentiation [4], its' involvement in PCa NE differentiation has not been assessed yet. Prior studies have not only demonstrated that the expression of this protein is reduced in SCLC cells, but have also proven that this downregulation is necessary for the induction of NE differentiation [10,40]. In this paper, we have demonstrated that the expression of this transcriptional coactivator was clearly diminished in both human and mouse NEPCa tumor samples, as well as cell lines. These findings were consistent with those obtained in SCLC cells [10,40], suggesting that the lack of this protein could be essential for NE differentiation. However, the molecular mechanism that relates the loss of YAP1 with the expression of the mediators that drive NE differentiation has not been clearly elucidated yet. Prior

studies in lung cancer have demonstrated that this transcriptional coactivator directly induces the expression of several neuroendocrine markers, such as Rab3A to achieve NE differentiation [10]. Here, we decided to study how this downregulation affects the Wnt/β-catenin signaling since its activation has been related to the induction of NE differentiation [26]. Besides, YAP1/TAZ have been shown to inhibit the activation of this pathway by recruiting β-TrCP to the β-catenin destruction complex, inducing its degradation by the ubiquitin-proteasome system [7]. Consequently, the downregulation of YAP1 could induce the nuclear translocation of β-catenin even in the absence of Wnt signaling [8]. Our results corroborate the existence of this interrelationship in PCa cells, demonstrating that Wnt/β-catenin activity increased after YAP1 knockdown. These results are consistent with previous findings in literature [41,42], supporting the idea that the activity of Wnt/β-catenin is partially dependent on YAP1 levels. Taken together, these results indicate that the loss of YAP1 promotes Wnt/β-catenin activity in AdPCa cells, which could be partially responsible for inducing NE differentiation of PCa.

Taken together, our results highlighted the importance of YAP1 expression in the modulation of PCa progression. Its overexpression promotes AdPCa malignancy, but its downregulation could induce NE differentiation by activating the Wnt/β-catenin signaling. Although we have established for the first time that the main effectors of the Hippo pathway are downregulated in NEPCa cells, more studies would be necessary to determine how the loss of YAP1 affects NE differentiation, as well as if YAP1 inhibitors could be safely used as therapeutic adjuvants for treating AdPCa.

CONFLICT OF INTEREST

The authors disclosure no conflict of interest

ACKNOWLEDGEMENT

This research was supported by NIH R03 CA212567 and R01 CA226285, DOD grant W81XWH-12-1-0212, and LSUHSC FWCC and Office of Research funding to XY.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; **69**(1)**:** 7-34.
2. Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. *N Engl J Med* 2004; **351**(15)**:** 1488-1490.
3. Beltran H, Tomlins S, Aparicio A, Arora V, Rickman D, Ayala G *et al*. Aggressive variants of castration-resistant prostate cancer. *Clin Cancer Res* 2014; **20**(11)**:** 2846-2850.
4. Ma S, Meng Z, Chen R, Guan KL. The Hippo pathway: Biology and pathophysiology. *Annu Rev Biochem* 2019; **88:** 577-604.
5. Plouffe SW, Lin KC, Moore JL, 3rd, Tan FE, Ma S, Ye Z *et al*. The Hippo pathway effector proteins YAP and TAZ have both distinct and overlapping functions in the cell. *J Biol Chem* 2018; **293**(28)**:** 11230-11240.
6. Callus BA, Finch-Edmondson ML, Fletcher S, Wilton SD. YAPping about and not forgetting TAZ. *FEBS Lett* 2019; **593**(3)**:** 253-276.
7. Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S *et al*. YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the wnt response. *Cell* 2014; **158**(1)**:** 157-170.
8. Varelas X, Miller BW, Sopko R, Song S, Gregorieff A, Fellouse FA *et al*. The Hippo pathway regulates Wnt/β-catenin signaling. *Dev Cell* 2010; **18**(4)**:** 579-591.
9. Yu FX, Zhao B, Guan KL. Hippo pathway in organ size control, tissue homeostasis, and cancer. *Cell* 2015; **163**(4)**:** 811-828.
10. Ito T, Matsubara D, Tanaka I, Makiya K, Tanei ZI, Kumagai Y *et al*. Loss of *YAP1* defines neuroendocrine differentiation of lung tumors. *Cancer Sci* 2016; **107**(10)**:** 1527-1538.
11. Zhang L, Yang S, Chen X, Stauffer S, Yu F, Lele SM *et al*. The Hippo pathway effector YAP regulates motility, invasion, and castration-resistant growth of prostate cancer cells. *Mol Cell Biol* 2015; **35**(8)**:** 1350-1362.
12. Jiang N, Ke B, Hjort-Jensen K, Iglesias-Gato D, Wang Z, Chang P *et al*. YAP1 regulates prostate cancer stem cell-like characteristics to promote castration resistant growth. *Oncotarget* 2017; **8**(70)**:** 115054-115067.
13. Connelly ZM, Yang S, Chen F, Yeh Y, Khater N, Jin R *et al*. Foxa2 activates the transcription of androgen receptor target genes in castrate resistant prostatic tumors. *Am J Clin Exp Urol* 2018; **6**(5)**:** 172-181.
14. RStudio Team. RStudio: Integrated Development for R. Boston, MA, 2015 URL: http://www.rstudio.com/ (7/8/19).
15. Wickham H. *Ggplot2. Elegant graphics for data analysis*. Springer International Publishing: New York, NY, 2016.
16. Kolde R. Package 'pheatmap', 2015. URL: https://docplayer.net/36920782-Package-pheatmap-december-11-index-7-pretty-heatmaps.html (7/8/19).
17. Yao L, Zhao H, Tang H, Xiong J, Zhao W, Liu L *et al*. Blockade of β-catenin signaling attenuates toluene diisocyanate-induced experimental asthma. *Allergy* 2017; **72**(4)**:** 579-589.
18. Gingrich JR, Barrios RJ, Morton RA, Boyce BF, DeMayo FJ, Finegold MJ *et al*. Metastatic prostate cancer in a transgenic mouse. *Cancer Res* 1996; **56**(18)**:** 4096-4102.
19. Kasper S, Sheppard PC, Yan Y, Pettigrew N, Borowsky AD, Prins GS *et al*. Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: A model for prostate cancer. *Lab Invest* 1998; **78**(3)**:** 319-333.
20. Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM. Androgen-independent prostate cancer progression in the TRAMP model. *Cancer Res* 1997; **57**(21)**:** 4687-4691.
21. Huss WJ, Gray DR, Tavakoli K, Marmillion ME, Durham LE, Johnson MA *et al*. Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model. *Neoplasia* 2007; **9**(11)**:** 938-950.

22. Masumori N, Thomas TZ, Chaurand P, Case T, Paul M, Kasper S *et al*. A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential. *Cancer Res* 2001; **61**(5)**:** 2239-2249.
23. Masumori N, Tsuchiya K, Tu WH, Lee C, Kasper S, Tsukamoto T *et al*. An allograft model of androgen independent prostatic neuroendocrine carcinoma derived from a large probasin promoter-T antigen transgenic mouse line. *J Urol* 2004; **171**(1)**:** 439-442.
24. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S *et al*. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 2012; **483**(7391)**:** 603-607.
25. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J *et al*. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nat Med* 2016; **22**(3)**:** 298-305.
26. Yang X, Chen MW, Terry S, Vacherot F, Chopin DK, Bemis DL *et al*. A human- and male-specific protocadherin that acts through the Wnt signaling pathway to induce neuroendocrine transdifferentiation of prostate cancer cells. *Cancer Res* 2005; **65**(12)**:** 5263-5271.
27. Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R *et al*. YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr Biol* 2007; **17**(23)**:** 2054-2060.
28. Moroishi T, Hansen CG, Guan KL. The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer* 2015; **15**(2)**:** 73-79.
29. Nishikawa E, Osada H, Okazaki Y, Arima C, Tomida S, Tatematsu Y *et al*. *miR-375* is activated by ASH1 and inhibits YAP1 in a lineage-dependent manner in lung cancer. *Cancer Res* 2011; **71**(19)**:** 6165-6173.
30. Yuan M, Tomlinson V, Lara R, Holliday D, Chelala C, Harada T *et al*. Yes-associated protein (YAP) functions as a tumor suppressor in breast. *Cell Death Differ* 2008; **15**(11)**:** 1752-1759.
31. Liu CY, Yu T, Huang Y, Cui L, Hong W. ETS (E26 transformation-specific) up-regulation of the transcriptional co-activator TAZ promotes cell migration and metastasis in prostate cancer. *J Biol Chem* 2017; **292**(22)**:** 9420-9430.
32. Jaramillo-Rodriguez Y, Cerda-Flores RM, Ruiz-Ramos R, Lopez-Marquez FC, Calderon-Garciduenas AL. YAP expression in normal and neoplastic breast tissue: An immunohistochemical study. *Arch Med Res* 2014; **45**(3)**:** 223-228.
33. Foster JW, Jones RR, Bippes CA, Gouveia RM, Connon CJ. Differential nuclear expression of YAP in basal epithelial cells across the cornea and substrates of differing stiffness. *Exp Eye Res* 2014; **127:** 37-41.
34. Schlegelmilch K, Mohseni M, Kirak O, Pruszak J, Rodriguez JR, Zhou D *et al*. Yap1 acts downstream of α-catenin to control epidermal proliferation. *Cell* 2011; **144**(5)**:** 782-795.
35. Zhao R, Fallon TR, Saladi SV, Pardo-Saganta A, Villoria J, Mou H *et al*. Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. *Dev Cell* 2014; **30**(2)**:** 151-165.
36. Boopathy GTK, Hong W. Role of Hippo pathway-YAP/TAZ signaling in angiogenesis. *Front Cell Dev Biol* 2019; **7:** 49.
37. Feng J, Ren P, Gou J, Li Z. Prognostic significance of TAZ expression in various cancers: A meta-analysis. *Onco Targets Ther* 2016; **9:** 5235-5244.
38. Sun Z, Xu R, Li X, Ren W, Ou C, Wang Q *et al*. Prognostic value of yes-associated protein 1 (YAP1) in various cancers: A meta-analysis. *PLoS One* 2015; **10**(8)**:** e0135119.
39. Jiang N, Hjorth-Jensen K, Hekmat O, Iglesias-Gato D, Kruse T, Wang C *et al*. *In vivo* quantitative phosphoproteomic profiling identifies novel regulators of castration-resistant prostate cancer growth. *Oncogene* 2015; **34**(21)**:** 2764-2776.
40. Horie M, Saito A, Ohshima M, Suzuki HI, Nagase T. YAP and TAZ modulate cell phenotype in a subset of small cell lung cancer. *Cancer Sci* 2016; **107**(12)**:** 1755-1766.
41. Imajo M, Miyatake K, Limura A, Miyamoto A, Nishida E. A molecular mechanism that links Hippo signalling to the inhibition of Wnt/β-catenin signalling. *EMBO J* 2012; **31**(5)**:** 1109-1122.

42. Barry ER, Morikawa T, Butler BL, Shrestha K, de la Rosa R, Yan KS *et al*. Restriction of intestinal stem cell expansion and the regenerative response by YAP. *Nature* 2013; **493**(7430)**:** 106-110.

FIGURE LEGENDS

Fig. 1 The expression of YAP1 and TAZ in benign prostatic glandular and stromal hyperplasia. Immunohistochemical staining of p63 highlights basal cells in the periphery of the prostatic gland (A). YAP1 displays positive staining of basal cells (positive nuclear staining) and negative staining of the luminal epithelial cells (B). TAZ immunostaining reveals faint stain of the basal cells (C). D to K show dual immunofluorescence staining of YAP1 and cytokeratin 5 (CK5, D to G) or cytokeratin 14 (CK14, H to K) to confirm the presence of YAP1 expression in prostate basal epithelia.

Fig. 2 Microscopic examination of the prostate of TRAMP mice reveals that the expression of Yap1 and Taz increase in PIN but decrease in NEPCa. A & B are serial sections of a wild type prostate demonstrating positive staining of Yap1 and Taz in the occasional basal epithelia. C & D are serial sections of a PIN tumor demonstrating diffuse positive staining of Yap1 and Taz in PIN. E to H are serial sections of a NEPCa demonstrating diffuse positive staining of Yap1 in PIN components but negative in NEPCa cells (E). Taz immunostaining is weakly positive in PIN but negative in NEPCa (F). Foxa2 (G) and Chga (H) immunostaining were positive in NEPCa cells but negative in PIN.

Fig. 3 Microscopic examination of the prostate excised from 12T-7, 12T-10, and NE10 LADY mice. 12T-7 mice develop PIN, 12T-10 mice develop NEPCa with focal PIN, and NE10 is a xenograft line derived from 12T-10 NEPCa. A & B are serial sections of a 12T-7 tumor. Immunohistochemical staining of Yap1 shows positive nuclear staining in PIN (A). Foxa2 immunostaining is negative (B). C & D are serial sections of a 12T-10 NEPCa tumor showing negativity of Yap1 in NEPCa cells in contrast to the positive staining in focal PIN (C). Positive staining of Foxa2 is seen in the NEPCa cells (D). E & F are serial sections of a NE10 tumor showing positive staining of Foxa2 (F), whereas the Yap1 immunostaining is negative (E).

Fig. 4 Sections of the liver and lung with metastasis of NEPCa shows positive staining of Foxa2 (right panels). In contrast, Yap1 immunostain is negative in NEPCa metastases.

Fig. 5 The expression of YAP1 in human prostatic tissues. A to C are serial sections of a benign prostate tissue. YAP1 expression is present in the stroma cells and basal cells (positive for p63). D to F are serial sections of a low grade prostate adenocarcinoma. YAP1 expression is present in stromal cells and occasional basal cells but lost in adenocarcinomas with a Gleason score of 7 or less. G to I are serial sections of a high grade prostate adenocarcinoma. YAP1 was expressed in higher grade prostatic adenocarcinomas with a Gleason score of 8 or higher. J to L are serial sections of a prostate adenocarcinoma with NE

differentiation. YAP1 protein is expressed in high-grade adenocarcinoma cells (Gleason score 8), whereas the expression is lost in NEPCa components. FOXA2 and CHGA proteins are diffusely expressed in NEPCa cells. M to O are serial sections of a small cell carcinoma. YAP1 expression is absent in NEPCa cells.

Fig. 6 The expression of YAP1 in publicly available human PCa datasets. A. The expression of YAP1 and TAZ in patient-derived LuCaP tumors. YAP1 expression was detected in adenocarcinomas but not in NEPCa. B. The expression of YAP decreased in NEPCa compared to adeno-CRPCa in Beltran-NEPCa dataset. C. Heatmap to show the expression of YAP1 decreased in the only NEPCa cell line, H660, compared to other PCa cell lines.

Fig. 7 YAP1 knockdown augments Wnt/β-catenin activity. PCa 22Rv1 and PC3 cells were co-transfected with Top-Flash plasmids and siYAP1. A reduction of YAP1 activated Wnt/β-catenin activity.

Fig. 1

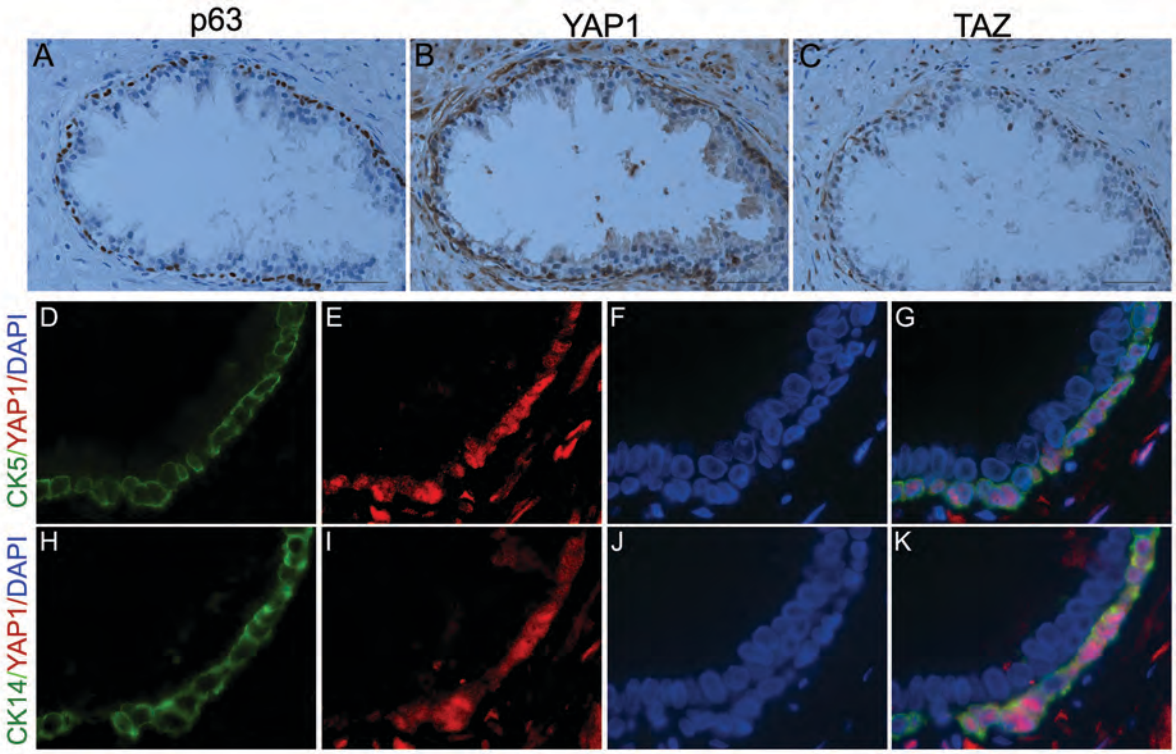


Fig. 2

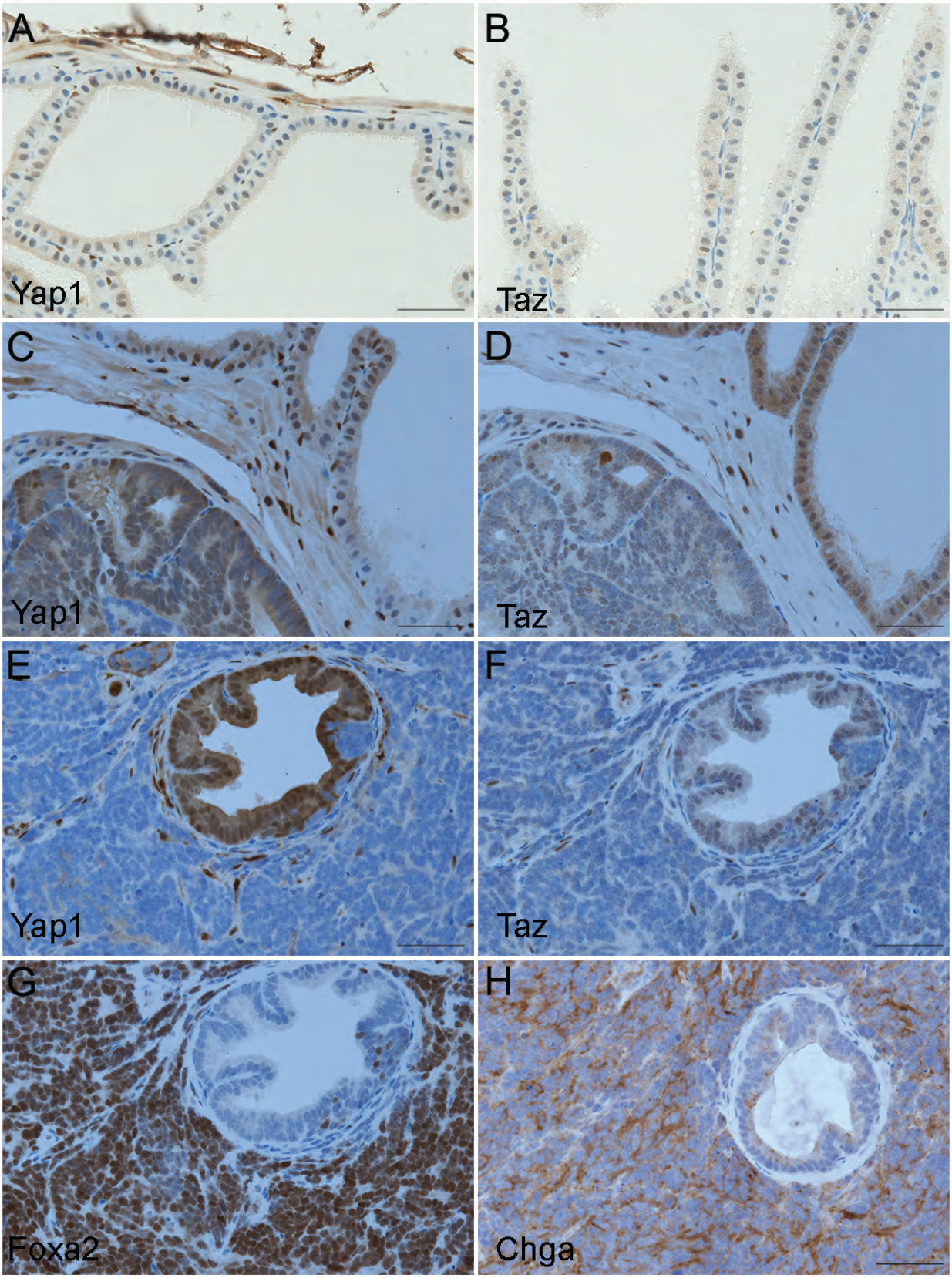


Fig. 3

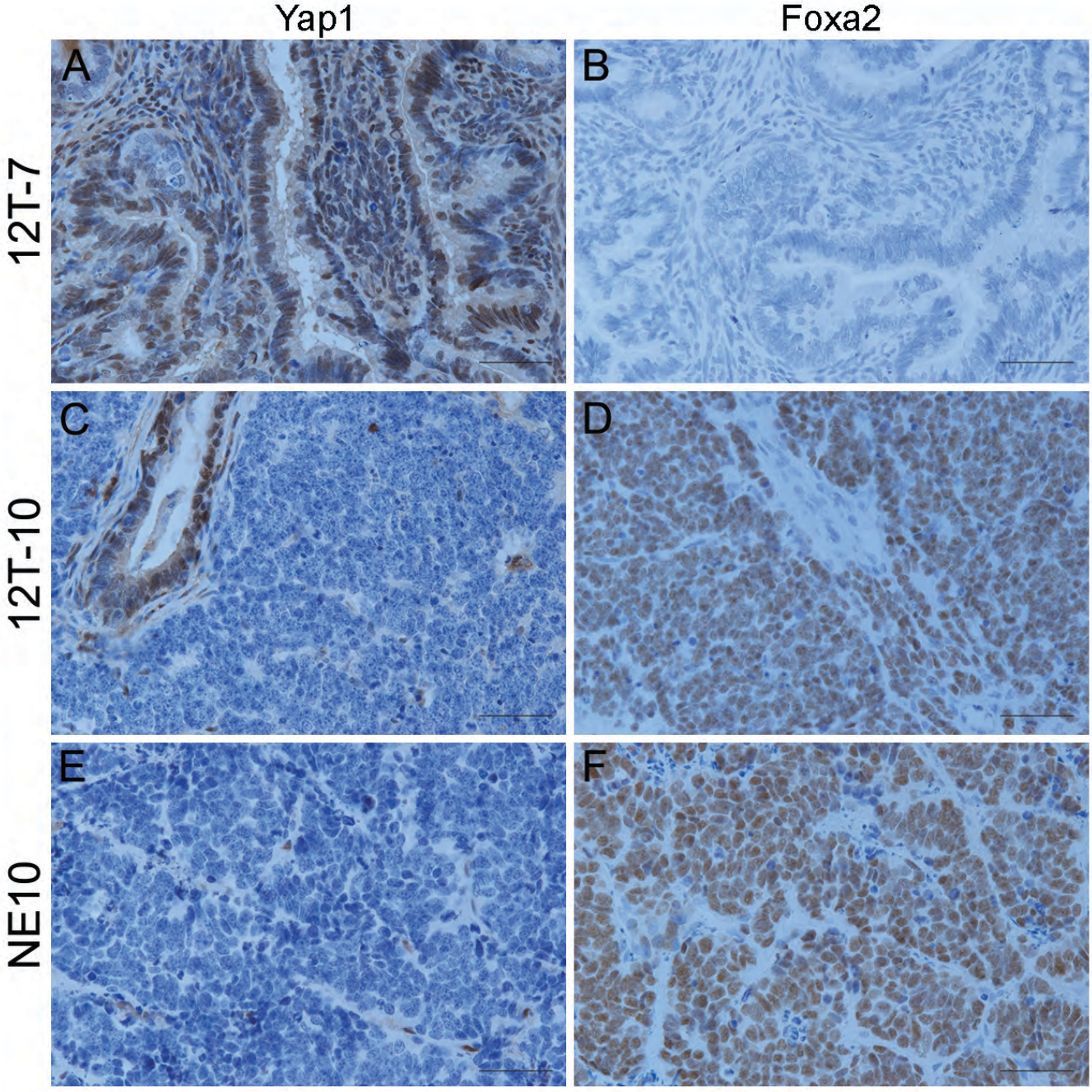


Fig. 4

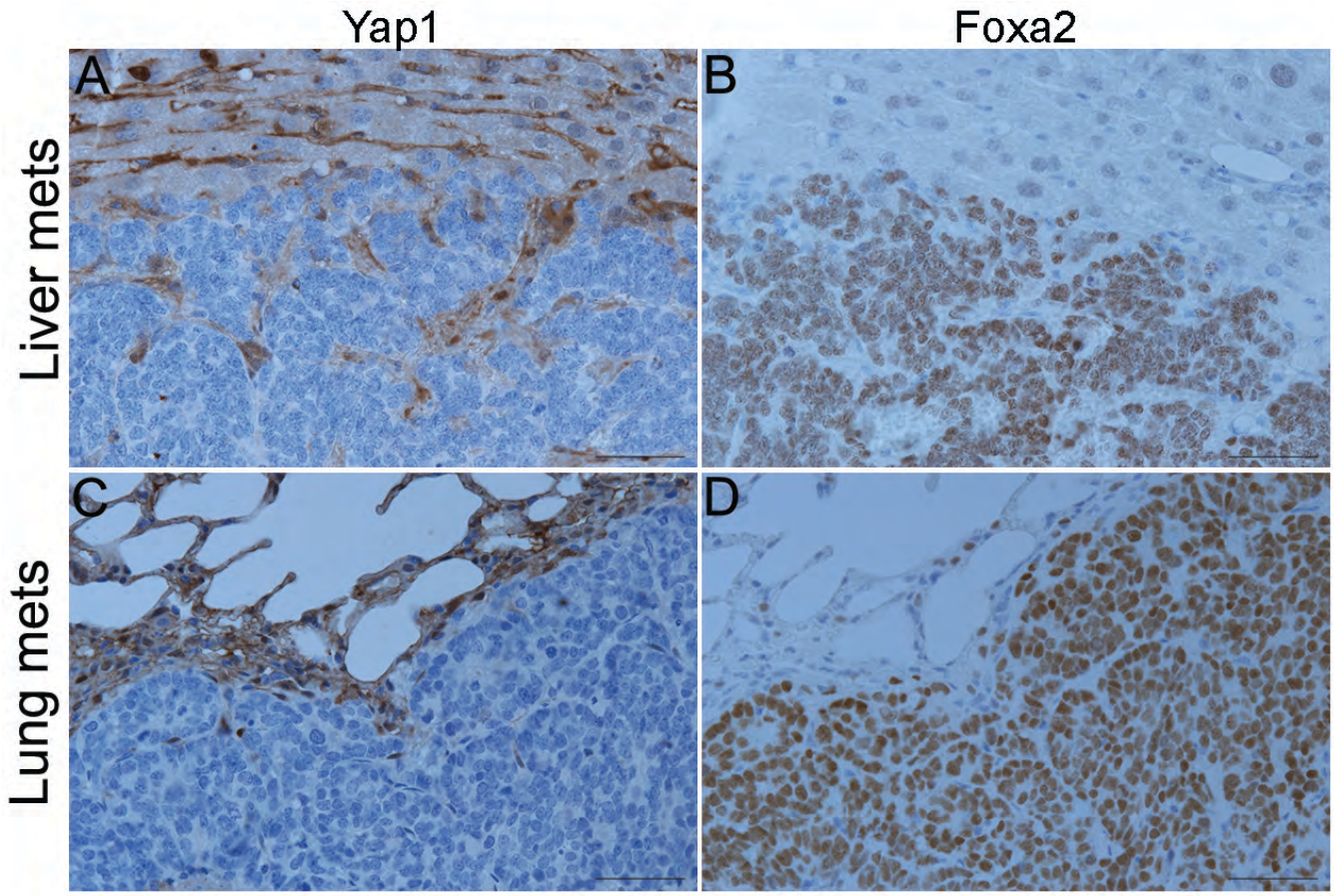


Fig. 5

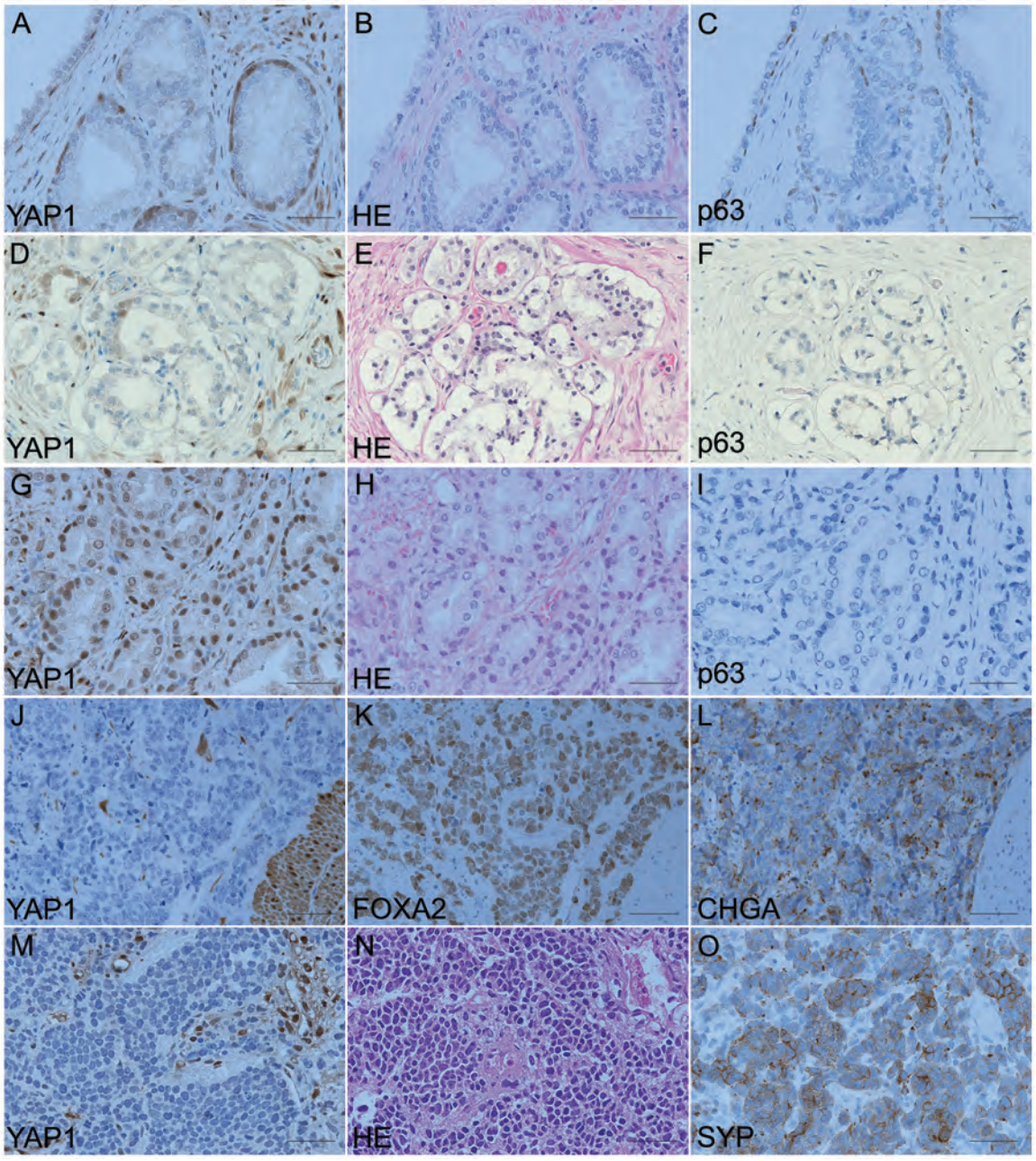

A
YAP1
B
HE
C
p63
D
YAP1
E
HE
F
p63
G
YAP1
H
HE
I
p63
J
YAP1
K
FOXA2
L
CHGA
M
YAP1
N
HE
O
SYP

Fig. 6

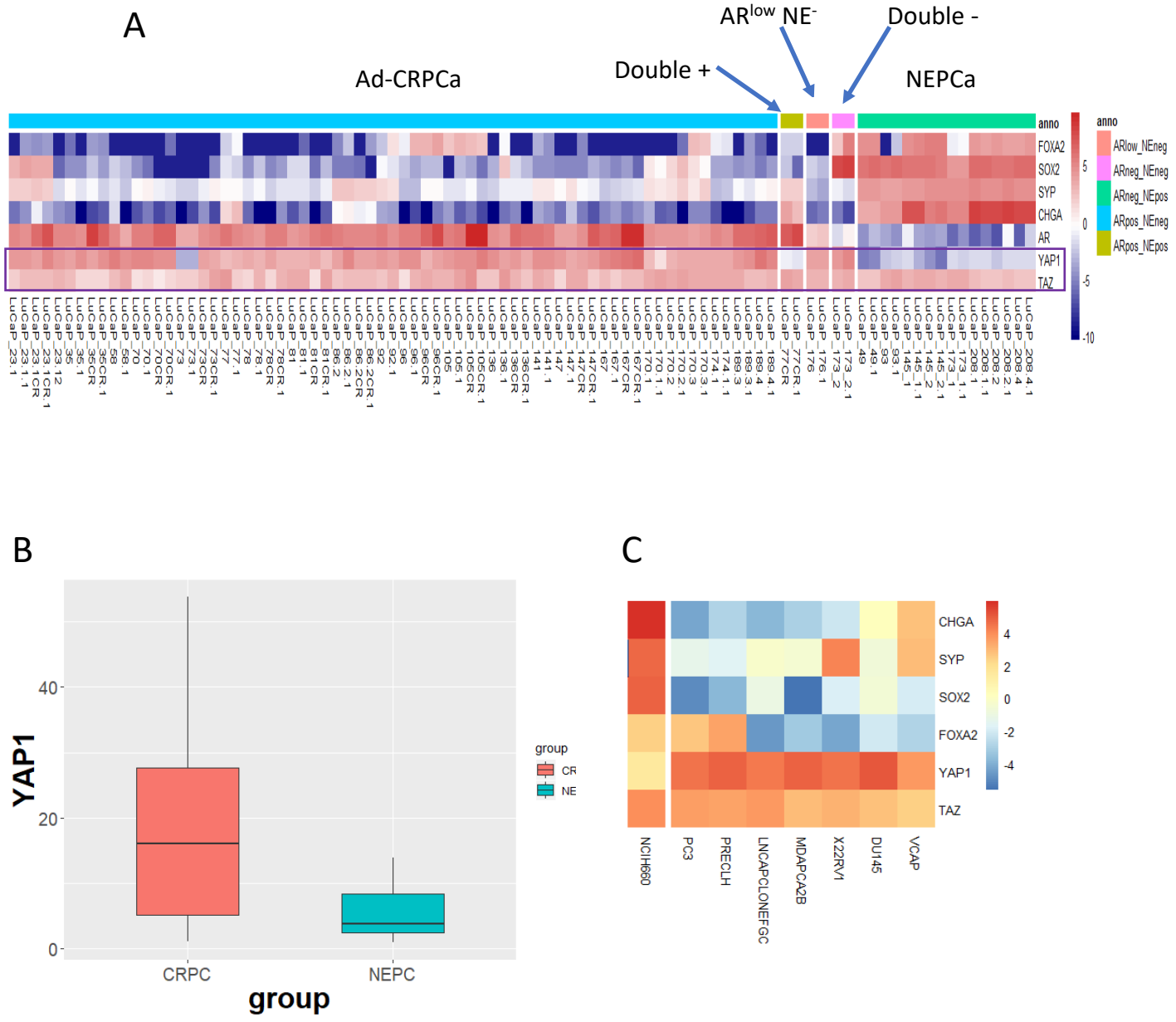
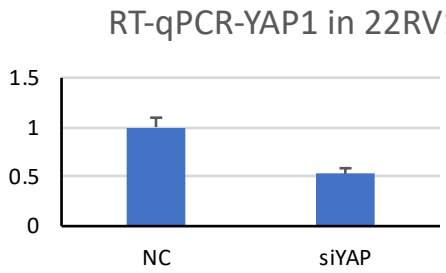
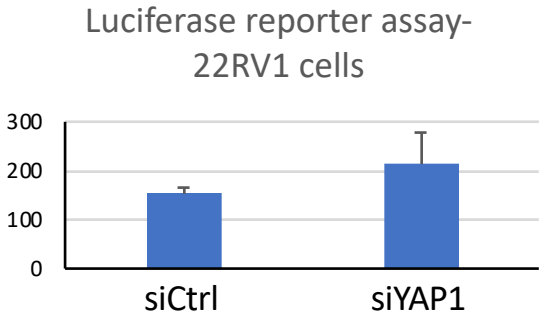
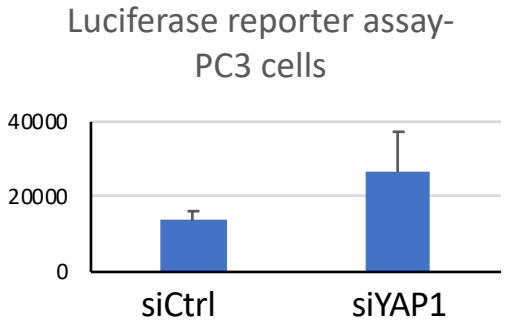

A
AR^low NE^-
Double -
Double +
Ad-CRPCa
NEPCa
anno
FOXA2
SOX2
SYP
CHGA
AR
YAP1
TAZ
ARlow_NEneg
ARneg_NEneg
ARneg_NEpos
ARpos_NEneg
ARpos_NEpos
B
YAP1
group
CR
NE
CRPC
NEPC
C
CHGA
SYP
SOX2
FOXA2
YAP1
TAZ
NCIH660
PC3
PRECLH
LNCAPCLONEFGC
MDAPCA2B
X22RV1
DU145
VCAP

Fig. 7


RT-qPCR-YAP1 in 22RV1
1.5
1
0.5
0
NC
siYAP


Luciferase reporter assay-
22RV1 cells
300
200
100
0
siCtrl
siYAP1


Luciferase reporter assay-
PC3 cells
40000
20000
0
siCtrl
siYAP1

FOXA2 Promotes Prostate Cancer Bone Metastasis

Zachary M. Connelly[1], Renjie Jin[2], Jianghong Zhang[2], Shu Yang[1], Siyuan Cheng[1], Mingxia Shi[3], Justin Cates[4], Runhua Shi[5], David J. DeGraff[6], Peter S. Nelson[7], Yunlong Liu[8], Colm Morrissey[9], Eva Corey[9], Xiuping Yu[1*]

[1]Dept of Biochemistry and Molecular Biology, [3]Dept of Pathology, [5]Dept of Medicine, LSU Health Sciences Center, Shreveport, LA

[2]Dept of Urology, Vanderbilt University Medical Center, Nashville, TN

[4]Dept of Pathology, Vanderbilt University Medical Center, Nashville, TN

[6]Dept of Pathology, Penn State College of Medicine, Hershey, PA

[7]Fred Hutchinson Cancer Research Center, Seattle, WA

[8]Department of Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN

[9]Dept of Urology, University of Washington, Seattle, WA

* Correspondence to: Xiuping Yu, email: xyu@lsuhsc.edu mailing address: 1501 Kings Hwy, LSU Health Sciences Center, Shreveport, LA 71103

Running Title: FOXA2 in Prostate Cancer Bone Metastasis

Abstract

Bone metastases frequently occur in advanced-stage prostate cancer (PCa) patients. Understanding the mechanisms that promote PCa-mediated bone destruction is important for the identification of therapeutic targets of the lethal disease. We found that forkhead box A2 (FOXA2) is expressed in a subset of PCa bone metastasis specimens. To determine the functional role of FOXA2 in PCa metastasis, we knocked down the expression of FOXA2 in aggressive PCa PC3 cells that can grow in bones and elicit an osteolytic reaction. The PC3/FOXA2-knockdown cells generated fewer bone lesions following intra-tibial injection compared to control cells. Further, we found that FOXA2 knockdown decreased the expression of *PTHLH*, which encodes PTHrP, a well-established factor that regulates bone remodeling. These results indicate that FOXA2 is involved in PCa bone metastasis.

Keywords: FOXA2, PTHrP, Prostate Cancer, Bone

Introduction

Micro/macro metastases in bone occur in approximately 90% of advanced PCa patients, resulting in significantly increased morbidity and mortality (1-4). Studies have shown that 20-30% of heavily treated PCa progresses to neuroendocrine prostate cancer (NEPCa) (5), the most aggressive form of this common disease. Approximately 40-50% of NEPCa patients have bone metastases (6). NEPCa does not express AR and therefore it is not responsive to endocrine therapy. Identifying the genes that control the behavior of NEPCa in the bone microenvironment can help increase our understanding of NEPCa establishment in the bone, leading to the development of new therapeutic approaches to treat bone metastases.

FOXA2 is a forkhead transcription factor that is expressed in NEPCa (7). The FOXA transcription factors were initially identified in liver tissues, where they control liver-specific gene expression (8). In prostate, FOXA2 is expressed at the embryonic stages (9). Its expression diminishes after birth but then it is re-expressed in both human and mouse NEPCa (7, 10). In

addition, it has been shown that FOXA2, in cooperation with HIF-1α, promotes neuroendocrine differentiation of PCa (11). However, the functional role of FOXA2 in NEPCa metastasis is yet to be elucidated.

Different from prostate adenocarcinomas, which stimulate new bone formation, NEPCa cells generate osteolytic lesions in bone. Previous studies have well established that Parathyroid Hormone-related Protein (PTHrP) is a major player in mediating cancer-induced osteoclastic bone resorption (12). It has been shown that several types of cancer including neuroendocrine tumors produce high levels of PTHrP, which binds to its receptors expressed on the osteoblasts and osteoclasts in the bone microenvironment, resulting in increased bone resorption and cancer growth (13, 14).

In this study, we investigated the involvement of FOXA2 in PCa bone metastasis. We used PC3 cells that express high levels of FOXA2 and PTHrP (10, 15), exhibit some features of NEPCa (16), and can grow in bone (17). We established FOXA2 knockdown-PC3 cells and assessed how reduced FOXA2 expression influences PCa-mediated bone lesions. We also examined FOXA2's regulation of PTHrP. This study provides the first evidence on the role of FOXA2 in regulating PTHrP expression and promoting NEPCa bone metastasis.

Material and Methods

Human specimens

Research using human specimens was conducted following protocols approved by the Institutional Review Boards at the University of Washington, Vanderbilt University, and LSU Health-Shreveport. A tissue microarray (UWTMA22) consisting of de-identified human metastatic castrate-resistant PCa (CRPCa) specimens was constructed using tissue samples collected from 24 CRPCa patients who succumbed to disease. In total, the TMA consisted of 128 samples with 2 cores for each sample.

Immunohistochemistry

Immunohistochemistry was conducted as described previously (18). Antibodies against FOXA2 (ab108422) were obtained from Abcam, Cambridge, MA and synaptophysin (SYP) (BD 611880) from BD Biosciences, San Jose, CA. Both the percentage of cells stained and the intensity of nuclear FOXA2 or cytoplasmic SYP staining were evaluated. The intensity of expression was assessed on a scale of 0–3. The percentage of expression was assessed on a 0–10 scale such that 0 represented no staining and 10 represented staining of 100% of tumor cells. An overall expression score (OES) was calculated as the product of the intensity and percentage of stained cells. OES was grouped as 0, 1-2 and ≥3. Statistical analysis was performed by using the Spearman correlation test and Cochran-Armitage Trend Test. P-value of <0.05 was considered statistically significant.

Bioinformatics analysis

The LuCaP patient-derived prostate cancer xenografts were established at the University of Washington. RNA was isolated from 46 LuCaP samples each containing a biological replicate, including 35 AR^+/NE^-, 1 AR^{low}/NE^-, 8 AR^-/NE^+,1 $AR^+/NE^{+,}$ and 1 AR^-/NE^- PDXs. RNA extracted from these PDXs was sequenced. GDS1439 is a set of microarray data collected from 19 prostatic tissues ranging from benign, localized prostate adenocarcinoma, to metastatic castrate-resistant PCa (19). NEPCa-Beltran dataset (20) contain RNA-seq data from both non-NE, castrate-resistant PCa (adeno-CRPCa) and NEPCa. The expression of FOXA2 was extracted and analyzed in all three datasets.

Cell culture and establishment of PC3 FOXA2 knockdown cells

PC3 cells (authenticated by ATCC) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). To generate FOXA2 knockdown cells, PC3 cells were infected with control or shFOXA2 lentiviral particles (Sigma, St. Louis, MO) and then cultured in media containing 10 μg/mL puromycin. Stable knockdown cells were maintained

in 2.5 μg/mL puromycin-containing media. Because FOXA2 knockdown efficiency gradually decreased over long-term cell culture, monoclonal lines were generated from PC3/shFOXA2 cells.

RNA isolation, RNA-seq analysis, and real-time PCR

RNA was extracted using Purelink RNA Mini Kit following manufacturer's protocol (Ambion, Life Technologies, Waltham, MA). Purified RNA was treated with RNase-free DNase (Qiagen, Germantown, MD) to remove DNA contamination. RNA-seq was conducted using RNA extracted from PC3/Control and PC3/FOXA2-KD cells (each containing three biological replicates) at the bioinformatic core of Indiana University School of Medicine. GO enrichment analysis was conducted using the list of genes that are up- or down- regulated in the FOXA2-KD cells. For reverse transcription, 1μg of total RNA was used for cDNA synthesis. Quantitative (q)-PCR was conducted to assess mRNA transcript levels of FOXA2 (Forward: 5'-TGCCATGCACTCGGCTTCCA-3' and Reverse: 5'- CCCAGGCCGGCGTTCATGTT-3') and PTHrP (Forward: 5'-ATCAACTTTCCGGAAGCAACCAGC-3' and Reverse: 5'-CCTTGTCATGGAGGAGCTGATGTT-3'). Gene expression was normalized by GAPDH.

Western blot analysis

Cells were lysed in passive lysis buffer (Promega, Madison, WI) and sonicated. Twenty micrograms of total protein were used for Western blot analyses. All antibodies were used at a 1:1000 dilution. Chemiluminescence signals were detected using x-ray film or processed in a Chemidoc Imaging System (Bio-Rad, Hercules, CA). β-actin was used for normalization.

Cell proliferation assay

IncuCyte ZOOM live cell imaging system (Essen BioScience, Ann Arbor, MI) was used to assess the cell proliferation. PC3/Control or PC3/FOXA2-KD cells were seeded into 96-well plate, 500 cells/well. Images were taken every four hours. Cell culture media were refreshed every two days.

All images were analyzed on the Incucyte ZOOM Software with an appropriate mask applied. Total area for each time point was quantified; mean ±standard deviation is shown.

Chemotaxis assays

The IncuCyte Zoom live cell imaging system (Essen BioScience, Ann Arbor, MI) was used to monitor and quantify the number of migrating cells. PC3/Control or PC3/FOXA2-KD cells ($1 \cdot 10^3$) were seeded in media containing 0.5% FBS on collagen I (50 µg/mL)-coated ClearView migration plate (Essen BioScience). The bottom reservoirs contained cell culture media with 10% FBS. Live cell images of the bottom of the chamber were taken every 2 hr. After 48 hours, cell migration was quantified.

Animal maintenance and intratibial injection

Animal experiments were conducted following protocols approved by the IACUC of LSU Health-Shreveport and Vanderbilt University and in accordance with the NIH guidelines. Pooled PC3/Control and PC3/FOXA2-KD cells were injected into tibiae of male SCID mice using an insulin syringe. Tumor growth was monitored by weekly radiographs for 5 weeks.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation was conducted using the EDM Millipore Magna ChIP kit (Burlington, MA) following the manufacturer's protocol. DNA was sheared using Bioruptor (Diagenode, Denville, NJ) for 40 cycles on high, producing approximately 200bp length fragments. Following reverse-crosslink of protein-DNA complexes, and treatment with proteinase K and RNase, DNA was purified by using Quick DNA Isolation Kit (Qiagen, Germantown, MD). Purified DNA was subjected to SYBR Green qPCR. Primers for ChIP-PCR were designed based on the ChIP-seq results available on the Integrative Genome Browser(21). There are two FOXA2 binding sites in the regulatory region of the *PTHLH* gene, designated as A2BS1 and A2BS2. Primers were designed to cover both FOXA2 binding sites. Primer sequences are A2BS1 (Forward: 5'-

CTTGGAATCCGGTGGCATCT-3' and Reverse: 5'-CTGCGATCGCCAACTGTAAC-3') and A2BS2 (Forward: 5'-AGCCACTTGTAGCGAAACCC-3' and Reverse: 5'-ACGACACACGCACTTGAAAC-3'). FOXA2 enrichment was normalized to IgG.

Statistical analysis

Statistical significance was evaluated using a two-sided Student's t test and a p-value of 0.05 was considered statistically significant.

Results

FOXA2 is expressed in metastatic castrate-resistant PCa and is correlated with synaptophysin expression

Expression of FOXA2 was examined using a set of TMAs consisting of castrate-resistant PCa metastases from 24 patients. Out of the total 128 metastatic sites, 124 sites had sufficient tissue for FOXA2 analysis. FOXA2 expression was detected in 28 sites, including 6/29 lymph node, 3/5 lung, 7/14 liver, 1/1 periaortic, and 11/75 bone metastases (Table 1). Out of the 128 sites, 104 samples had sufficient tissue for both FOXA2 and synaptophysin (SYP, NEPCa marker) staining and were used to evaluate the association of FOXA2 with SYP. FOXA2 and SYP were co-expressed in 14/104 samples (13.46%), while FOXA2 expression alone was detected in 10/104 (9.62%) and SYP in 16/104 (15.38%) samples (Table 2). Statistical analysis showed that FOXA2 expression was significantly correlated with SYP expression (Spearman Correlation Coefficient is 0.40, $p<0.0001$). Examples of the staining were shown in Fig. 1 and the overall expression of each sample in supplementary Table s1.

To further validate the association of FOXA2 expression with neuroendocrine phenotype in PCa, we analyzed the expression of FOXA2, AR, and markers of NEPCa including SOX2, chromogranin A (CHGA), and synaptophysin (SYP) in a RNAseq dataset of 46 LuCaP prostate cancer patient-derived xenografts (PDXs). As shown in Fig. 2A, FOXA2 was detected in 6/8 AR^-/NE^+ LuCaP PDXs, but not in the double positive LuCaP 77CR PDX (AR^+/NE^+). Among the NE^- PDXs, FOXA2 was expressed in the double negative LuCaP 173.2 (AR^-/NE^-) (22) and in 6/35

AR^+/NE^- PDXs albeit at lower levels, but not in the AR^{low}/NE^- LuCaP 176 PDX. Statistical analysis indicated that FOXA2 expression was significantly higher in NE^+ PDXs than NE^- PDXs.

Furthermore, we analyzed the expression of FOXA2 in the NEPCa-Beltran dataset that contains RNAseq data of 15 NEPCa and 34 adeno-CRPCa samples *(19)*. FOXA2 was expressed in 13/15 NEPCa and 2/34 adeno-CRPCa albeit at lower levels (Fig. 2B). Statistical analysis indicated that FOXA2 expression was associated with NE phenotype. Finally, analysis of the GDS1439 microarray data showed that FOXA2 level is low in all benign (n=6) and localized prostate tissues (n=7) but increased in 4 of 6 metastatic CRPCa tissues. The 4 cases that show increased FOXA2 expression have decreased AR and increased NEPCa markers (CHGA, SYP, ENO2, and/or SOX2; Fig. 2C). Taken together, these data indicate that the expression of FOXA2 correlates with NE phenotype in PCa.

Effects of FOXA2 knockdown on cell proliferation and chemotaxis

To study the functional involvement of FOXA2 in PCa, we knocked down FOXA2 expression in PC3 cells that express high levels of endogenous FOXA2 (10). Three single clones were selected and designated as PC3/FOXA2-KD I, -II, and -III. While the knockdown resulted in decreased expression of FOXA2 at both the mRNA and protein levels (Figs. 3A and 3B), we did not observe any significant effects on PC3 cell proliferation (Fig. 3C). However, FOXA2 knockdown inhibited PC3 cells invasion on collagen I matrix (Fig. 3D). Although *in vitro* cell proliferation was not impacted by FOXA2 knockdown, it was still possible that inactivation of FOXA2 could reduce cell proliferation *in vivo*. Therefore, to determine this, we conducted subcutaneous injections of the PC3/Control and PC3/FOXA2-KD cells. In concordance with the *in vitro* data (no effects on proliferation), no significant differences in subcutaneous tumor growth were observed (Fig. 3E). Taken together, these results indicate that FOXA2 is not involved in the regulation of proliferation but regulates the invasion of PCa cells.

FOXA2 knockdown decreases PCa-mediated bone lesions

To evaluate the functional role of FOXA2 in PCa bone metastasis, PC3/Control and PC3/FOXA2-KD cells were injected into the tibiae of SCID mice. Inoculation of PC3/Control cells (n=5 tibiae) resulted in profound osteolytic lesions, whereas injection of PC3/FOXA2-KD cells (n=5 tibiae) resulted in less severe osteolytic bone lesions in fewer tibiae (Fig. 4). Consistent with the existence of lytic bone lesions, TRAP staining revealed the presence of active osteoclasts at the surface of the bone lesions in the tibiae that showed bone destruction but not in the ones without bone destruction (Fig. 5).

FOXA2 knockdown decreases the expression of PTHrP

To explore the mechanisms by which FOXA2 knockdown affects PCa-induced bone lesions, we assessed the expression of *PTHLH* and *TNFSF11* that encodes PTHrP and RANKL, respectively, both of which are well-established factors that regulate bone remodeling (23). We found that the mRNA expression of *PHTLH* decreased in FOXA2-KD cells (Fig. 6A). However, the mRNA levels of *TNFSF11* was low in both control and FOXA2-KD PC3 cells, thus RANKL was not further investigated.

FOXA2 is a pioneer transcription factor; therefore, we conducted chromatin immunoprecipitation assays to evaluate whether FOXA2 is recruited to the regulatory regions of *PTHLH*. The results revealed enrichment of FOXA2 at both predicted FOXA2-binding sites on the *PTHLH* regulatory region in PC3/Control cells, but FOXA2 occupancy was significantly decreased in PC3/FOXA2-KD cells (Fig. 6B). Concurrent with the reduced FOXA2 binding, the occupancy of H3K27ac3 and H3K4me3, the histone marks for active promoters, was also decreased (Figs. 6C & 6D). Together, these results indicate that FOXA2 directly regulates *PTHLH* expression in PC3 cells.

Furthermore, we conducted RNA-Seq experiments followed by GO enrichment analysis to identify the genes that are differentially expressed in the PC3/FOXA2-KD cells. Figs. 7A and 7B showed the top genes and pathways altered by FOXA2 knockdown, respectively. Many of them are involved in cells' communication with their microenvironment.

Discussion

Bone metastasis is the most critical complication of advanced PCa (1-4). In this study, we identified a previously undefined role of FOXA2 in PCa bone metastasis. We found that FOXA2 knockdown caused a significant decrease in PCa PC3-mediated bone destruction *in vivo* following intra-tibial injection. To explore the mechanisms by which FOXA2 promotes the cancer-induced bone lesions, we assessed the expression of bone-remodeling genes and found that the levels of PTHrP decreased following FOXA2 knockdown. ChIP assays indicate that FOXA2 directly regulates the expression of PTHrP. PTHrP is a well-established factor that regulates the turnover of bone tissue in both normal physiology and cancer metastasis. FOXA2's regulation of PTHrP could provide a mechanism to promote PCa cells' interaction with the bone microenvironment and facilitate PCa bone colonization.

The formation of metastatic lesions is an active process involving reciprocal communication between PCa cells and resident cellular elements (mainly osteoclasts and osteoblasts) in the bone matrix (24). PCa cells secrete factors that stimulate bone resorption mediated by osteoclasts (25, 26). As osteoclasts resorb bone matrix, they liberate growth factors to support PCa growth in bones (27). This results in a 'vicious cycle' of bone destruction and tumor growth. This process is important for creating a 'fertile' environment to support metastatic cancer cell survival. While the majority of PCa bone metastases are osteoblastic, it is now recognized that osteoclast activity is also critical for creating a "fertile" environment to allow PCa cells to escape apoptosis and to optimize their survival, even in the case of osteoblastic lesions (1, 28). Furthermore, while prostate adenocarcinomas mostly generate osteoblastic lesions, NEPCa cells cause lytic bone destruction. Our discovery of FOXA2's regulation of PTHrP provides a mechanism by which NEPCa cells stimulate bone resorption, promoting successful bone colonization. Additionally, our chemotaxis data indicated that PC3/FOXA-KD cells lost the ability to migrate on/invade through collagen I, a major component of the bone extracellular matrix (29). This could also contribute to the interactions between PCa cells and the bone microenvironment.

Our research reveals a pro-metastatic function of FOXA2 in PCa. However, FOXA2's involvement in cancer appears to be organ-specific. FOXA2 expression is associated with relapse in breast cancer (30), but FOXA2 has been shown to have anti-cancer and anti-metastasis properties in cancers arising from foregut derivatives (lung, stomach, pancreas, and liver), where FOXA2 is involved in regulating cellular differentiation (31-35). In PCa, FOXA2 is a biomarker of NEPCa (7, 10). It has been shown to promote NE differentiation (11) and sustain AR responsive promoters in SV40 T-antigen driven prostatic tumor cells after androgen deprivation (18). However, it has not been investigated previously whether FOXA2 has a functional role in NEPCa metastases. Our work provides evidence supporting FOXA2's role in regulating PTHrP expression and promoting PCa-induced bone lesions.

FOXA2 is a well-established marker of NEPCa. Our finding that the expression of FOXA2 correlates with that of SYP is consistent with previous reports (7, 10). In this study, we found that approximately 15% of PCa bone metastases express FOXA2 (Table 2). This agrees with a previous report that approximately 14% of PCa bone metastases are NEPCa (36).

In conclusion, in this study, we found that FOXA2 is expressed in a subset of PCa bone metastases. FOXA2 regulates the expression of PTHrP and promotes PCa-induced bone destruction.

Acknowledgements

We would like to thank Dr. Robert Matusik at Vanderbilt University Medical Center and Dr. Michael Cher at Wayne State University for advice on the research. This research is supported by the Department of Defense grant W81XWH-12-1-0212 and Prostate Cancer Biorepository Network grant W81XWH-14-2-0183, NIH R03 CA212567, R01 CA226285, U54 GM104940, the PNW Prostate Cancer SPORE P50 CA097186, P01 CA163227, LSUHSC FWCC and Office of Research funding to XY, and Carroll Feist Pre-doctoral Fellowship to ZC.

Conflict of Interest

We declare no conflicts of interest.

References:

1. Vessella RL, Corey E. Targeting factors involved in bone remodeling as treatment strategies in prostate cancer bone metastasis. Clin Cancer Res. 2006;12(20 Pt 2):6285s-90s.
2. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol. 2000;31(5):578-83.
3. Gandaglia G, Abdollah F, Schiffmann J, Trudeau V, Shariat SF, Kim SP, et al. Distribution of metastatic sites in patients with prostate cancer: A population-based analysis. Prostate. 2014;74(2):210-6.
4. Sottnik JL, Keller ET. Understanding and targeting osteoclastic activity in prostate cancer bone metastases. Curr Mol Med. 2013;13(4):626-39.
5. Epstein JI, Amin MB, Beltran H, Lotan TL, Mosquera JM, Reuter VE, et al. Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am J Surg Pathol. 2014;38(6):756-67.
6. Aprikian AG, Cordon-Cardo C, Fair WR, Zhang ZF, Bazinet M, Hamdy SM, et al. Neuroendocrine differentiation in metastatic prostatic adenocarcinoma. J Urol. 1994;151(4):914-9.
7. Park JW, Lee JK, Witte ON, Huang J. FOXA2 is a sensitive and specific marker for small cell neuroendocrine carcinoma of the prostate. Mod Pathol. 2017.
8. Jackson DA, Rowader KE, Stevens K, Jiang C, Milos P, Zaret KS. Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition. Mol Cell Biol. 1993;13(4):2401-10.
9. Mirosevich J, Gao N, Matusik RJ. Expression of Foxa transcription factors in the developing and adult murine prostate. Prostate. 2005;62(4):339-52.
10. Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R, Matusik RJ. Expression and role of Foxa proteins in prostate cancer. Prostate. 2006;66(10):1013-28.
11. Qi J, Nakayama K, Cardiff RD, Borowsky AD, Kaul K, Williams R, et al. Siah2-dependent concerted activity of HIF and FoxA2 regulates formation of neuroendocrine phenotype and neuroendocrine prostate tumors. Cancer Cell. 2010;18(1):23-38.
12. Martin TJ, Johnson RW. Multiple actions of PTHrP in breast cancer bone metastasis. Br J Pharmacol. 2019.
13. Sterling JA, Edwards JR, Martin TJ, Mundy GR. Advances in the biology of bone metastasis: how the skeleton affects tumor behavior. Bone. 2011;48(1):6-15.
14. Kamp K, Feelders RA, van Adrichem RC, de Rijke YB, van Nederveen FH, Kwekkeboom DJ, et al. Parathyroid hormone-related peptide (PTHrP) secretion by gastroenteropancreatic neuroendocrine tumors (GEP-NETs): clinical features, diagnosis, management, and follow-up. J Clin Endocrinol Metab. 2014;99(9):3060-9.
15. Wu G, Iwamura M, di Sant'Agnese PA, Deftos LJ, Cockett AT, Gershagen S. Characterization of the cell-specific expression of parathyroid hormone-related protein in normal and neoplastic prostate tissue. Urology. 1998;51(5A Suppl):110-20.
16. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, et al. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate. 2011;71(15):1668-79.

17. Corey E, Quinn JE, Bladou F, Brown LG, Roudier MP, Brown JM, et al. Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. Prostate. 2002;52(1):20-33.
18. Connelly ZM, Yang S, Chen F, Yeh Y, Khater N, Jin R, et al. Foxa2 activates the transcription of androgen receptor target genes in castrate resistant prostatic tumors. Am J Clin Exp Urol. 2018;6(5):172-81.
19. Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell. 2005;8(5):393-406.
20. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nature medicine. 2016;22(3):298-305.
21. Freese NH, Norris DC, Loraine AE. Integrated genome browser: visual analytics platform for genomics. Bioinformatics. 2016;32(14):2089-95.
22. Bluemn EG, Coleman IM, Lucas JM, Coleman RT, Hernandez-Lopez S, Tharakan R, et al. Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling. Cancer Cell. 2017;32(4):474-89.e6.
23. Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, et al. Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. Cancer Res. 1990;50(23):7710-6.
24. Roodman GD. Mechanisms of bone metastasis. N Engl J Med. 2004;350(16):1655-64.
25. Chu GC, Zhau HE, Wang R, Rogatko A, Feng X, Zayzafoon M, et al. RANK- and c-Met-mediated signal network promotes prostate cancer metastatic colonization. Endocr Relat Cancer. 2014;21(2):311-26.
26. Morrissey C, Lai JS, Brown LG, Wang YC, Roudier MP, Coleman IM, et al. The Expression of Osteoclastogenesis-Associated Factors and Osteoblast Response to Osteolytic Prostate Cancer Cells. Prostate. 2010;70(4):412-24.
27. Lind M. Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implants fixation. Acta Orthop Scand Suppl. 1998;283:2-37.
28. Cher ML. Mechanisms governing bone metastasis in prostate cancer. Curr Opin Urol. 2001;11(5):483-8.
29. Hall CL, Dubyk CW, Riesenberger TA, Shein D, Keller ET, van Golen KL. Type I collagen receptor (alpha2beta1) signaling promotes prostate cancer invasion through RhoC GTPase. Neoplasia. 2008;10(8):797-803.
30. Perez-Balaguer A, Ortiz-Martinez F, Garcia-Martinez A, Pomares-Navarro C, Lerma E, Peiro G. FOXA2 mRNA expression is associated with relapse in patients with Triple-Negative/Basal-like breast carcinoma. Breast Cancer Res Treat. 2015;153(2):465-74.
31. Vorvis C, Hatziapostolou M, Mahurkar-Joshi S, Koutsioumpa M, Williams J, Donahue TR, et al. Transcriptomic and CRISPR/Cas9 technologies reveal FOXA2 as a tumor suppressor gene in pancreatic cancer. Am J Physiol Gastrointest Liver Physiol. 2016;310(11):G1124-37.
32. Chand V, Pandey A, Kopanja D, Guzman G, Raychaudhuri P. Opposing Roles of the Fork-head box genes FoxM1 and FoxA2 in Liver Cancer. Mol Cancer Res. 2019.
33. Wang J, Zhu CP, Hu PF, Qian H, Ning BF, Zhang Q, et al. FOXA2 suppresses the metastasis of hepatocellular carcinoma partially through matrix metalloproteinase-9 inhibition. Carcinogenesis. 2014;35(11):2576-83.

34. Li CM, Gocheva V, Oudin MJ, Bhutkar A, Wang SY, Date SR, et al. Foxa2 and Cdx2 cooperate with Nkx2-1 to inhibit lung adenocarcinoma metastasis. Genes Dev. 2015;29(17):1850-62.
35. Li C, Lu S, Shi Y. MicroRNA-187 promotes growth and metastasis of gastric cancer by inhibiting FOXA2. Oncol Rep. 2017;37(3):1747-55.
36. Aggarwal R, Huang J, Alumkal JJ, Zhang L, Feng FY, Thomas GV, et al. Clinical and Genomic Characterization of Treatment-Emergent Small-Cell Neuroendocrine Prostate Cancer: A Multi-institutional Prospective Study. J Clin Oncol. 2018;36(24):2492-503.

Figure legends:

Figure 1. FOXA2 expression in metastatic PCa tissues. IHC staining of FOXA2 and synaptophysin (SYP) was conducted on serial sections derived from tissue microarray UWTMA22 which contains PCa specimens collected from 24 metastatic CRPCa patients who succumbed to disease. **A & B:** PCa metastases in liver. **C & D:** PCa metastases in bone. FOXA2 and SYP were co-expressed in 14/104 samples, while FOXA2 expression alone was detected in 10/104 and SYP in 16/104 samples. The expression of FOXA2 is corelated with NEPCa marker SYP.

Figure 2. The expression of FOXA2 mRNA in benign and malignant prostate specimens. A The expression of FOXA2 in LuCaP PDXs. The expression levels of FOXA2, AR, and NEPCa markers CHGA, SYP, and SOX2 were extracted from RNAseq data derived from 46 LuCaP PDXs. The PDXs were catalogued into five groups. Group 1 is AR^{+}/NE^{-}, group 2 AR^{low}/NE^{-}, group 3 AR^{-}/NE^{-}, group 4 AR^{-}/NE^{+}, and group 5 AR^{+}/NE^{+} LuCaP PDXs. FOXA2 expression was detected in 6/8 AR^{-}/NE^{+} and 6/35 AR^{+}/NE^{-} LuCaP PDXs. FOXA2 was also expressed in the double negative (AR^{-}/NE^{-}) LuCaP. The expression of FOXA2 is associated with NE phenotype. **B & C** Bioinformatics analysis of FOXA2 in publicly available data set**. B.** The expression of FOXA2, AR and NEPCa markers SYP and SOX2 was extracted from NEPCa Beltran dataset that contains RNAseq data derived from 15 NEPCa and 34 adeno-CRPCa samples *(19)*. Increased FOXA2 expression was detected in 13/15 NEPCa and 2/34 adeno-CRPCa. **C.** FOXA2 expression data extracted from microarray GDS1439. FOXA2 expression was detected in 4/6 metastatic CRPCa samples, but not in benign or localized prostatic tissues. The four FOXA2-positive samples express increased levels of SYP, SOX2, CHGA, or ENO2.

Figure 3. FOXA2 knockdown inhibits PC3 cell migration but not proliferation. A: RT-qPCR analyses were conducted to assess the expression levels of FOXA2 in PC3/Control and three PC3/FOXA2-KD cell clones. All three clonal lines demonstrated reduced mRNA levels compared to the PC3/Control cells. **B.** Western blot analysis confirmed loss of FOXA2 expression in PC3/FOXA2-KD cells. β-actin served as the loading control. **C.** Cell proliferation assays were conducted using the IncuCyte zoom method. FOXA2 knockdown did not decrease the proliferation of PC3 cells. **D.** Chemotaxis assays. PC3/Control and PC3/FOXA2-KD cells were seeded on collagen I-coated transwell inserts with 0.5% FBS-containing media in the top chamber and 10% FBS-containing media in the bottom wells. The bottoms of the collagen-coated inserts were imaged every two hours to visualize the cells that had traversed. PC3/Control cells migrated through the collagen I matrix into the bottom wells, whereas PC3/FOXA2-KD cells could not do so. Error bars reported as standard deviation. **E.** PC3/Control and PC3/FOXA2-KD cells (clones II and III) were inoculated subcutaneously into SCID mice. The tumor size was measured one week post injection, 3 times/week. No statistically significant changes were observed in the tumor growth among the three experimental groups. Error bars reported as standard deviation.

Figure 4. FOXA2 knockdown decreases PCa-mediated bone destruction. PC3/Control (n=5) and PC3/FOXA2-KD (n=5) cells were injected into the tibiae of SCID mice. Animals were x-ray imaged weekly for 5 weeks and tumor progression with bone destruction was monitored. PC3/Control cells grew in all the bones injected and generated lytic bone lesions, whereas PC3/FOXA2-KD cells caused less bone destruction in fewer tibiae.

Figure 5. TRAP staining. **A & B** are images of a tibia injected with PC3/Control cells and **C & D** with PC3/FOXA2-KD cells. B and D are higher magnification images of A and C, respectively. Red arrows in panels A & B indicated TRAP-positive osteoclasts and red arrowheads PCa PC3 cells. Green pound signs in panels C & D indicated bone cells. Scale bars represent 50 μm.

Figure 6. FOXA2 regulates the expression of PTHLH. A. RT-qPCR to assess the expression of FOXA2 and PTHLH. FOXA2 knockdown caused a decrease in the mRNA levels of PTHLH. *p < 0.05, t-test. Error bars reported as standard deviation. **B-D.** Chromatin Immunoprecipitation analysis. ChIP assays were conducted by using PC3/Control and PC3/FOXA2-KD cells. FOXA2 bound to both FOXA2 binding sites (A2BS1 & A2BS2) in the regulatory region of the PTHLH gene. Additionally, histone marks of active promoters (H3K4me3 and H3K27Ac3) were enriched in the PTHLH regulatory regions.

Figure 7. Differential gene expression analysis. A & B. GO analysis of RNA-seq data to identify pathways that are altered (A: activated and B: inhibited by FOXA2) following FOXA2 knockdown in PC3 cells. **C.** Volcano plot to show the top genes that are differentially expressed in FOXA2-KD cells.

Table 1. Tissue distribution of PCa metastases and FOXA2 expression in UMTMA22

	FOXA2 negative	FOXA2 positive	Subtotal
Bone	64	11	75
Lymph Node	23	6	29
Lung	2	3	5
Liver	7	7	14
Periaortic Mass	0	1	1
Total			124

Table 2. The expression of FOXA2 and synaptophysin (SYP) in UWTMA22

	Frequency	Percent
$FOXA2^{-}$ SYP^{-}	64	61.54
$FOXA2^{-}$ SYP^{+}	16	15.38
$FOXA2^{+}$ SYP^{-}	10	9.62
$FOXA2^{+}$ SYP^{+}	14	13.46
Total	104	100

Fig. 1

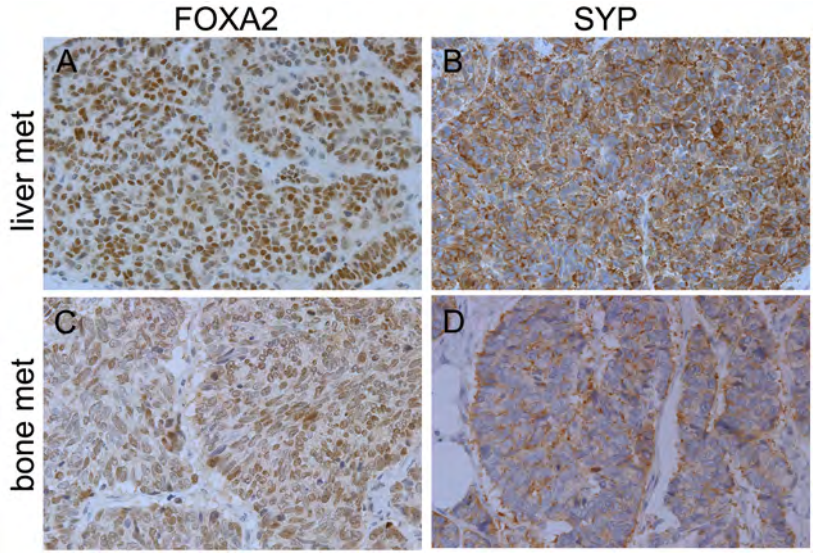


Fig. 2

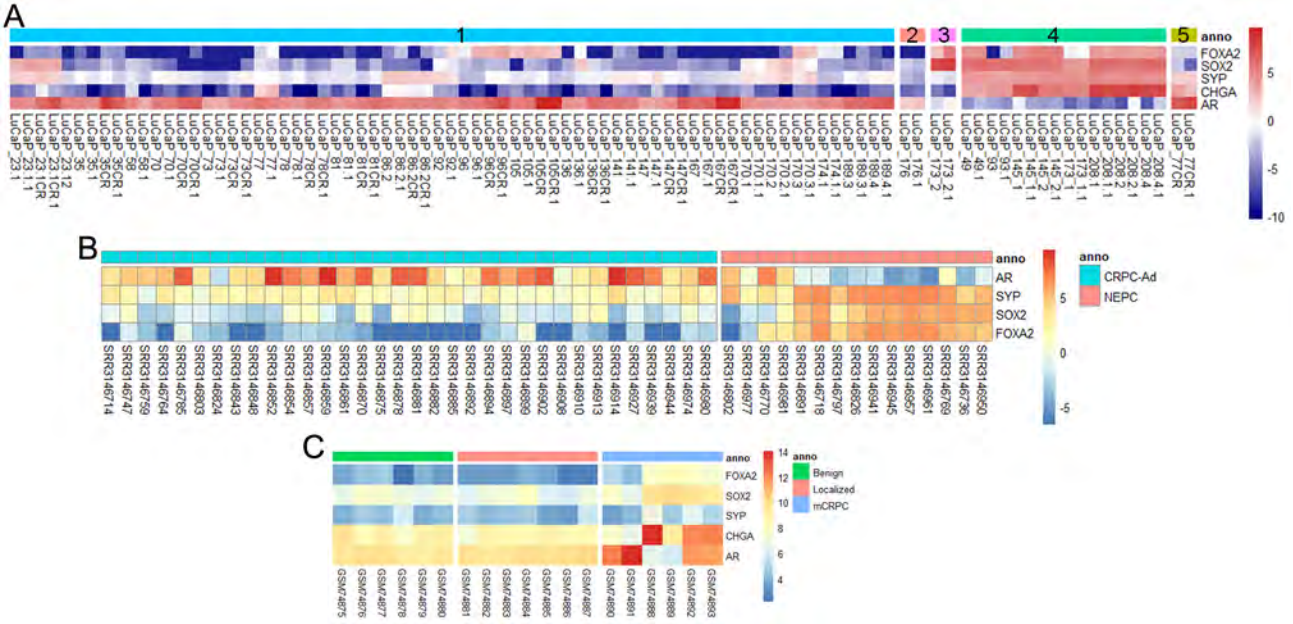

A
1
2
3
4
5
anno
FOXA2
SOX2
SYP
CHGA
AR
5
0
-5
-10
B
anno
AR
SYP
SOX2
FOXA2
anno
CRPC-Ad
NEPC
5
0
-5
C
anno
FOXA2
SOX2
SYP
CHGA
AR
14
12
10
8
6
4
anno
Benign
Localized
mCRPC

Fig. 3

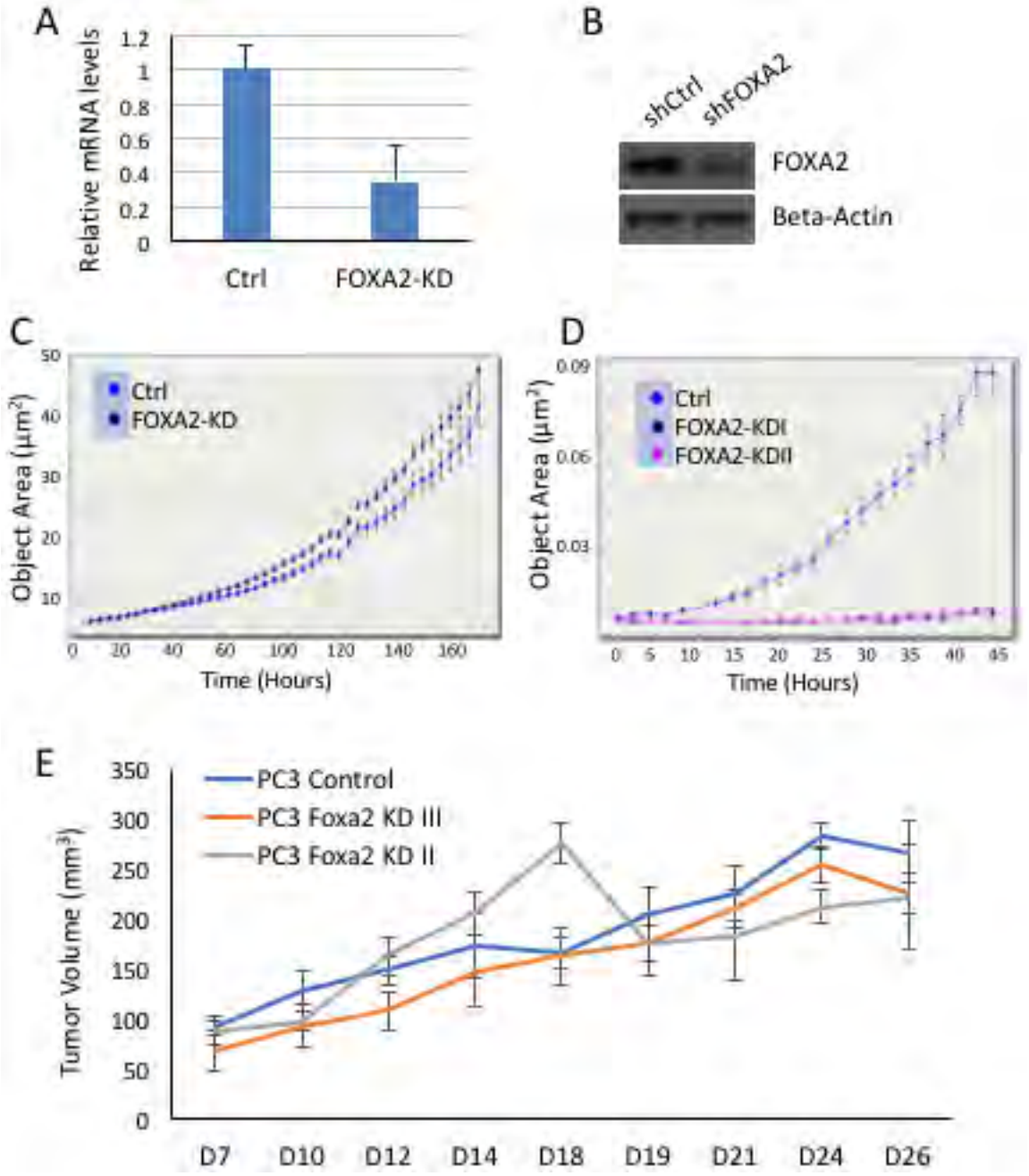

A
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
FOXA2-KD
B
shCtrl
shFOXA2
FOXA2
Beta-Actin
C
Object Area (µm²)
50
40
30
20
10
Ctrl
FOXA2-KD
0
20
40
60
100
120
140
160
Time (Hours)
D
Object Area (µm²)
0.09
0.06
0.03
Ctrl
FOXA2-KDI
FOXA2-KDII
0
5
10
15
20
25
30
35
40
45
Time (Hours)
E
Tumor Volume (mm³)
350
300
250
200
150
100
50
0
PC3 Control
PC3 Foxa2 KD III
PC3 Foxa2 KD II
D7
D10
D12
D14
D18
D19
D21
D24
D26

Fig. 4

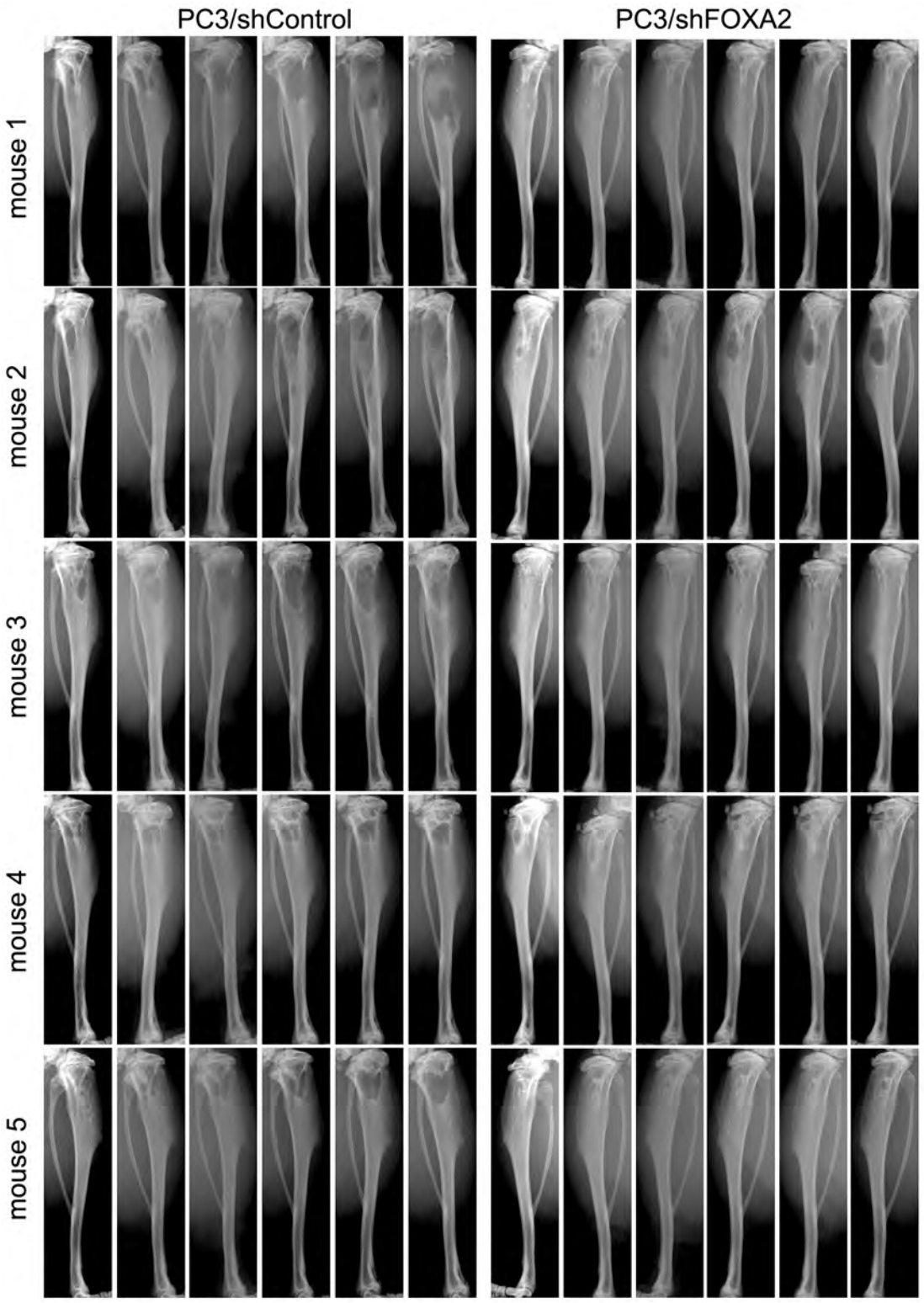

PC3/shControl
PC3/shFOXA2
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5

Fig. 5

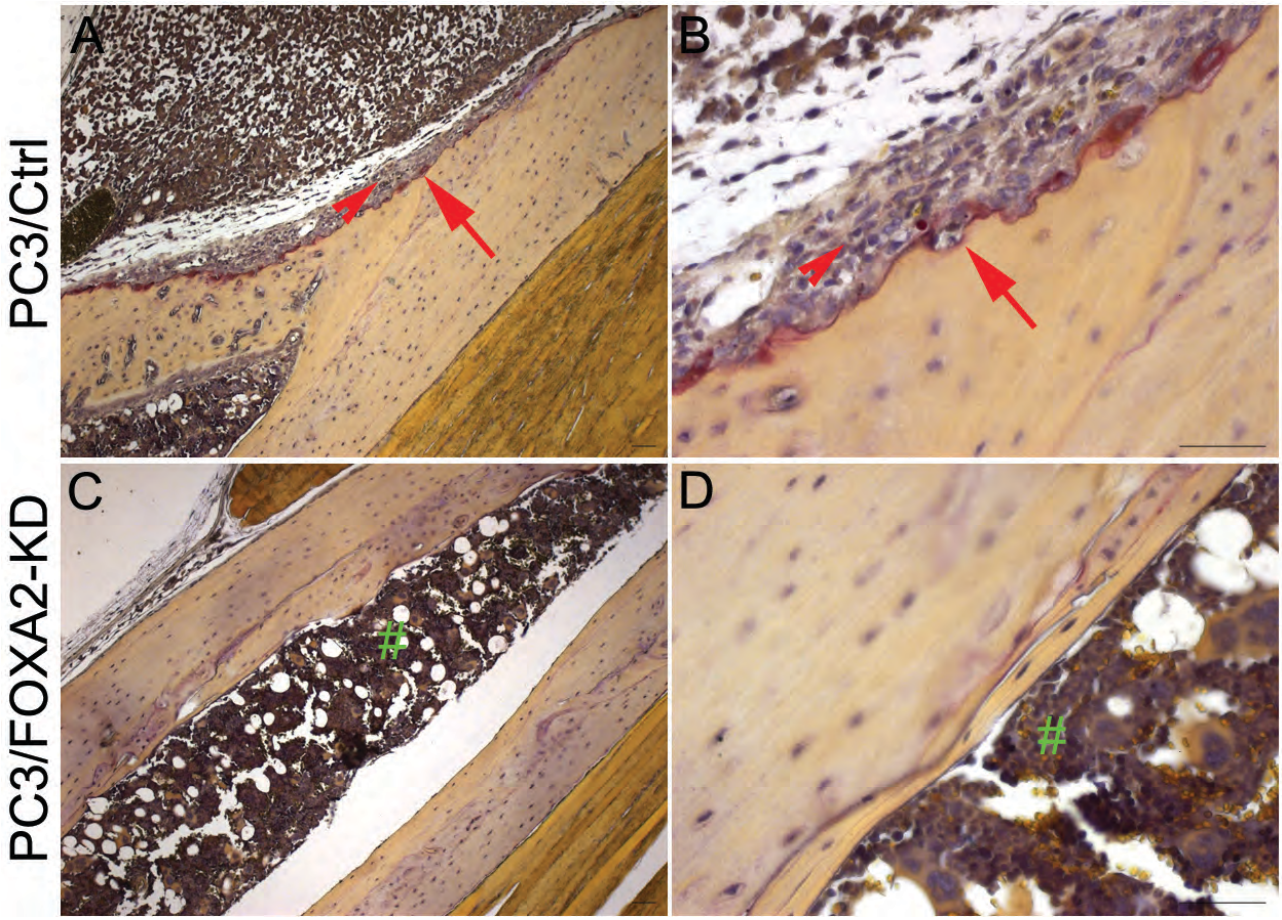


Fig. 6

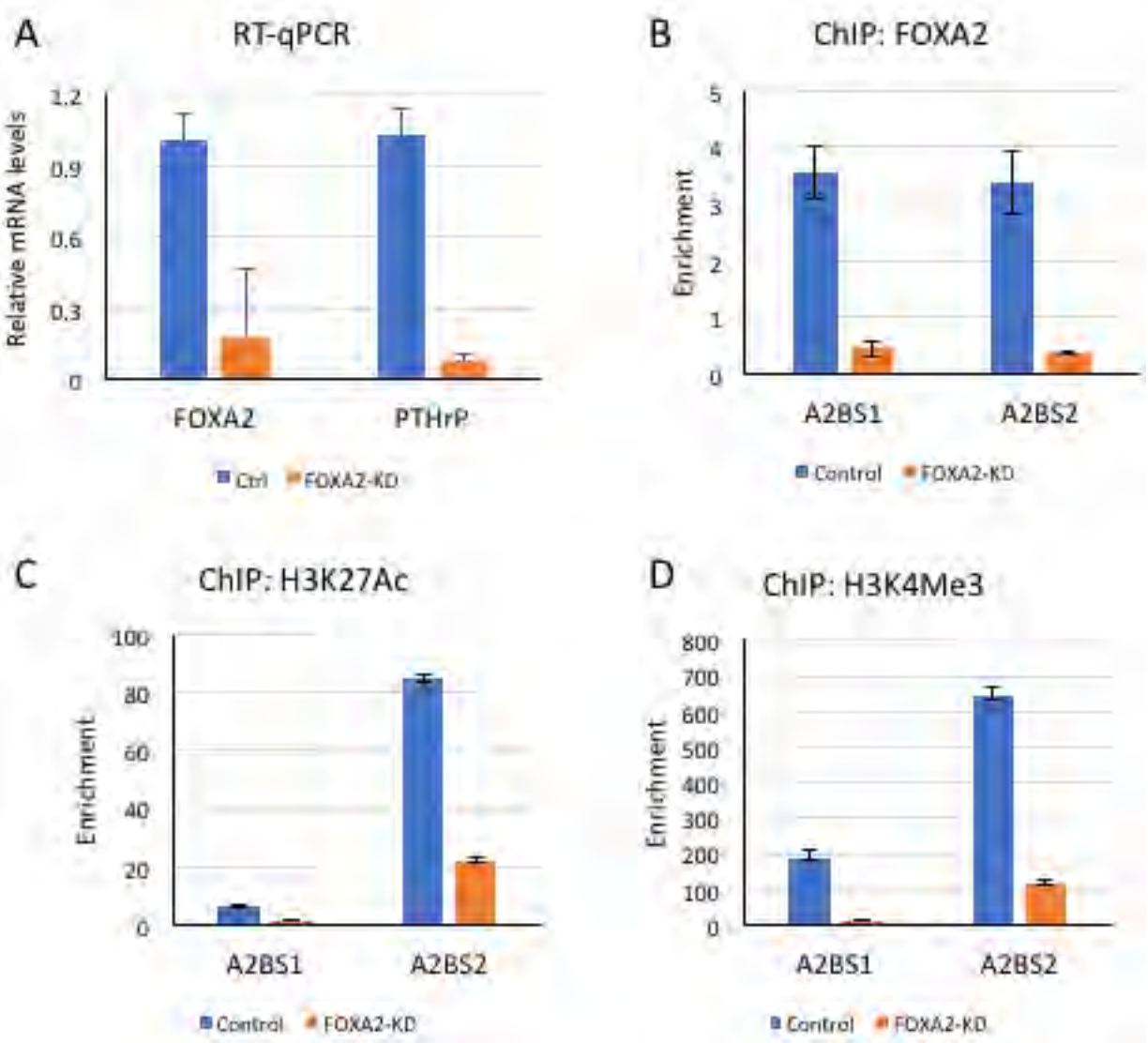
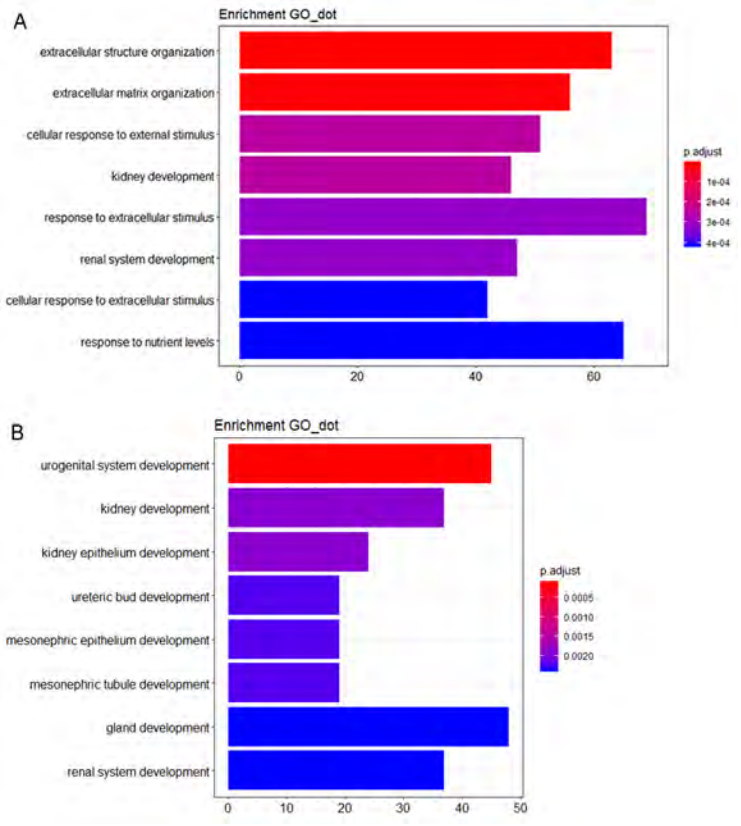
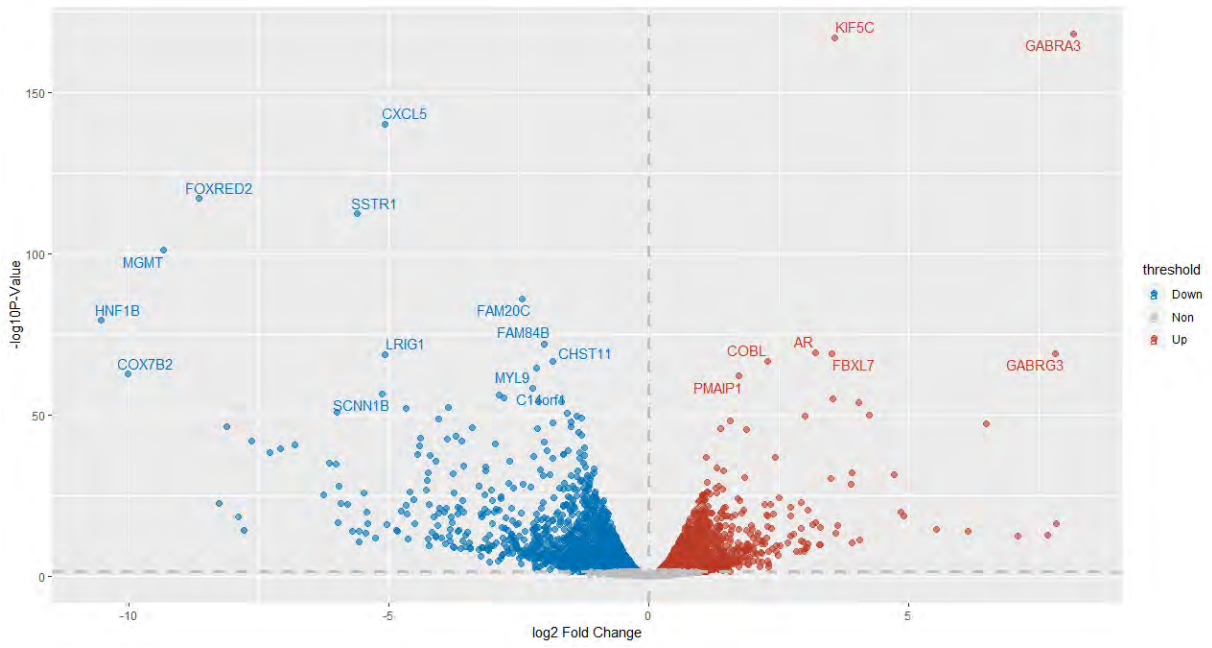

A
RT-qPCR
Relative mRNA levels
1.2
0.9
0.6
0.3
0
FOXA2
PTHrP
Ctrl
FOXA2-KD
B
ChIP: FOXA2
Enrichment
5
4
3
2
1
0
A2BS1
A2BS2
Control
FOXA2-KD
C
ChIP: H3K27Ac
Enrichment
100
80
60
40
20
0
A2BS1
A2BS2
Control
FOXA2-KD
D
ChIP: H3K4Me3
Enrichment
800
700
600
500
400
300
200
100
0
A2BS1
A2BS2
Control
FOXA2-KD

Fig. 7


A
Enrichment GO_dot
extracellular structure organization
extracellular matrix organization
cellular response to external stimulus
kidney development
response to extracellular stimulus
renal system development
cellular response to extracellular stimulus
response to nutrient levels
0
20
40
60
p.adjust
1e-04
2e-04
3e-04
4e-04
B
Enrichment GO_dot
urogenital system development
kidney development
kidney epithelium development
ureteric bud development
mesonephric epithelium development
mesonephric tubule development
gland development
renal system development
0
10
20
30
40
50
p.adjust
0.0005
0.0010
0.0015
0.0020

C


KIF5C
GABRA3
CXCL5
FOXRED2
SSTR1
MGMT
HNF1B
COX7B2
FAM20C
FAM84B
LRIG1
CHST11
MYL9
C14orf4
SCNN1B
COBL
AR
FBXL7
PMAIP1
GABRG3
150
100
50
0
-10
-5
0
5
-log10P-Value
log2 Fold Change
threshold
Down
Non
Up

FOXA2 Regulates Integrin α1 Expression Facilitating Prostate Cancer Bone Colonization

Zachary M. Connelly[1], Shu Yang[1], Siyuan Cheng[1], Nazih Khater[2], Zaki Al Yafeai[3], A. Wayne Orr[4], Colm Morrissey[5], Eva Corey[5], Xiuping Yu[1,2*]

[1]Dept of Biochemistry and Molecular Biology, [2]Dept of Urology, [3]Dept of Molecular and Cellular Physiology [4]Dept of Pathology, LSU Health Sciences Center, Shreveport, LA

[5]Dept of Urology, University of Washington, Seattle, WA

* Correspondence to: xyu@lsuhsc.edu

Running Title: FOXA2/integrin axis in Prostate Cancer Bone Metastasis

Key words: FOXA2, integrin, prostate cancer, bone metastases, collagen I

Abstract

Metastasis to the bone is a major cause of mortality in PCa patients. Understanding the mechanisms by which the PCa cells grow in the bone is critical for the development of new therapeutic approaches for the treatment of advanced PCa. Forkhead box A2 (FOXA2) is expressed in a subset of PCa bone metastases. FOXA2 knockdown (KD) decreases PCa-induced bone lesions. In this study, we explored the mechanisms that mediated FOXA2's pro-metastasis function. We found FOXA2-KD decreased the mRNA and protein levels of the collagen I-binding integrin α1 in PCa cells. Chromatin immunoprecipitation assay demonstrated that FOXA2 binds directly to the regulatory region of the integrin α1 (*ITGA1*) gene, indicating that FOXA2 regulates the expression of ITGA1. Consistent with the decreased levels of integrin α1, FOXA2-KD cells exhibited reduced adhesion, spreading, and integrin signaling on a cell culture surface coated with collagen I. Blocking integrin α1 signaling with a neutralizing antibody reproduced the decreased adhesion phenotype of PC3/FOXA2-KD cells. Inversely, overexpression of integrin α1 rescued adherence properties. Taken together, these results show that FOXA2 upregulates expression of integrin α1 in PCa cells, promoting adherence to extracellular matrix in bone tissues.

Keywords: FOXA2, Integrin α1, Prostate Cancer, Bone

Introduction

Heavily treated PCa acquires neuroendocrine (NE) phenotype. Recent studies indicate that after PCa becomes resistant to the second-generation anti-androgens, NE differentiation is observed in 20-30% of PCa cases (5). NEPCa cells are highly aggressive and metastatic. They lose prostate epithelial differentiation and express no or low levels of AR and AR targets, thus NEPCa cells are resistant to androgen deprivation therapy. A subset of NEPCa patients develop bone metastases (6). Understanding the mechanisms by which PCa cells become established in the bone is important for the identification of new targets to treat PCa bone metastases.

FOXA2 is a forkhead transcription factor that is expressed in both human and mouse NEPCa. We have recently shown that PC3 cells, an aggressive PCa cell line that display NEPCa features, highly express FOXA2(15). Knocking down FOXA2 in PC3 cells resulted in a decreased bone lesions following intra-tibial injection. In this study, we aimed to determine the mechanisms that mediate FOXA2's function in promoting PCa bone metastases.

Tropism of metastatic cells, a key component of cancer site-specific homing, is primarily accredited to expression of specific integrin molecules. Integrins are a family of adhesion proteins that mediate interactions of cells with extracellular matrix (12, 13). Stromal adherence is particularly critical for metastatic cells to survive and proliferate in a new microenvironment. The integrin family of genes consists of 18 ITGA and 8 ITGB genes encoding 18

α and 8 β monomer proteins, which form 24 different αβ heterodimers (12, 13). Extracellular matrix (ECM) proteins, including laminin, collagen, fibronectin, and vitronectin directly interact with integrins (12, 13). Upon binding to ECM proteins, integrins dimerize, resulting in the activation of various signaling cascades involved in the regulation of cell adhesion, survival, migration, wound healing, and cell differentiation (12, 13). Integrins α1β1, α2β1, α10β1 and α11β1 are known to interact with collagen I (14), a major component of bone ECM. Understanding the regulation of integrins and their roles in the communication between cancer cells and bone ECM may provide important information for preventing the establishment of PCa bone metastasis.

In this study, using the PC3/FOXA2-KD cells, we investigated how FOXA2 regulates expression of integrin α1 to promote cellular adhesion and spreading on bone ECM proteins. This study provides the first evidence on the role of FOXA2/integrin α1 axis in NEPCa bone metastases.

Results

FOXA2 regulates the expression of integrin α1

Three monoclonal lines were established to knock down the expression of FOXA2 in PC3 cells. As shown in Figs. 1A & 1B, the expression of FOXA2 was decreased at both the mRNA and protein levels. We have previously shown that FOXA2-KD decreased PCa-mediated bone destruction. We have also conducted RNA-Seq experiments to identify the genes whose expression was altered by FOXA2 inactivation. We found the expression of multiple integrins

decreased in the FOXA2-KD cells (Fig. 1C). RT quantitative (q)-PCR confirmed a significant reduction in mRNA expression of integrins α1, α7, αv, β2, β3, and β4 (Fig. 2A).

Integrin α1 is of special interest because it binds to collagen I, a major component of the extracellular matrix in bone. Western blot results confirmed lower levels of integrin α1 in PC3/FOXA2-KD cells compared to PC3/Control cells (Fig. 2B).

FOXA2 is a pioneer transcription factor; therefore, we evaluated whether FOXA2 is recruited to the regulatory region of *ITGA1*. These results revealed enrichment of FOXA2 at both predicted FOXA2-binding sites on the *ITGA1* regulatory region in PC3/Control cells, but FOXA2 occupancy was significantly decreased in PC3/FOXA2-KD cells compared to PC3/Control cells (Fig. 2C). Concurrent with the reduced FOXA2 binding, binding of H3K27AC3, the histone marker for active enhancers, and H3K4me3, the marker for active promoters, were also decreased (Fig. 2C). Together, these results indicate that FOXA2 directly regulates *ITGA1* expression in PC3 cells.

FOXA2 knockdown reduces integrin signaling in PCa cells, resulting in decreased ability to spread on and adhere to collagen I

Integrin binding to ECM activates multiple intracellular signaling cascades to promote cell survival. Therefore, we next evaluated the effects of FOXA2 knockdown on adhesion to multiple matrix proteins, including collagen I, collagen IV, laminin, vitronectin, and fibronectin and assessed the downstream integrin

signaling. FOXA2 knockdown resulted in a significant decrease in PC3 cells adhesion to collagen I (Supp. Fig. 1), concomitant with decreased expression of integrin α1 (Figs. 2A & 2B). However, adhesion to collagen I was not fully abolished following FOXA2 knockdown. In addition to α1, there are four additional α integrins (α1, α2, α10, and α11) that bind to collagen I and mediate adhesion. While our qPCR results show PC3 cells express little to no integrins α10 and α11, they express high levels of Integrin α2. However, we failed to detect alterations in α2 expression following FOXA2 knockdown, providing plausible mechanism of retained adhesion of PC3/FOXA2-KD cells to collagen I (Fig. 2B).

Additionally, we examined whether FOXA2 knock down alters spreading of PC3 cells. Phalloidin staining was used to visualize the surface area of the cells. FOXA2 knockdown decreased the spreading of PC3 cells (Fig. 3).

An important event during the cell adherence/spreading is formation of focal adhesion complexes that are assembled by integrins and FAKs as well as other signaling proteins at the leading edge of cells. Therefore, we assessed whether FOXA2 knockdown affects the formation of focal adhesions. Phosphorylated FAK (pFAK at Tyr 397), an indicator of active focal adhesion, was detected as early as in 30 min in PC3/Control cells (Fig. 3), whereas the PC3/FOXA2-KD cells did not display any pFAK by 120 min. To determine whether focal adhesions eventually form in PC3/FOXA2-KD cells, we extended the time course to 3, 6, and 12 hr. The results

revealed the loss of pFAK foci in PC3/FOXA2-KD cells even 12 hour post seeding (Fig. 4).

Integrin β1 is the binding partner of integrin α1. Upon ligand binding, integrins α1 and β1 dimerize and initiate the formation of the focal adhesion complex. Therefore, we next examined the co-localization of integrin β1 (12G10 staining) with pFAK. The results in Fig. 4 showed that PC3/Control cells, but not PC3/FOXA2-KD cells, displayed co-staining of integrins β1 at the pFAK foci, further supporting that the loss of FOXA2 prevents the formation of focal adhesion complexes and interferes with the signaling cascades involved in cell spreading.

Next, we examined whether this phenomenon was specific to the collagen I matrix. The results showed when PC3 cells were seeded on fibronectin, neither the PC3/Control nor the PC3/FOXA2-KD cells showed any pFAK foci or spreading on fibronectin (Supp. Fig. 2). Together with the decreased adhesion of PC3 cells on collagen IV, fibronectin, laminin, and vitronectin (Supp. Fig. 6), these data support that FOXA2 promotes the formation of pFAK foci and the adhesion/spreading of PCa cells on collagen I matrix and that FOXA2-modulated PCa/matrix interaction is collagen-specific.

Blocking integrin α1 decreases PC3 cells adhesion to collagen I

To determine whether integrin α1 is involved in facilitating the adhesion of PC3 cells to collagen I, we used an α1 neutralizing antibody to block integrin α1 signaling. As shown in Fig. 5, approximately 75% and 85% PC3/Control cells adhered to the

matrix at 15 and 30 min post-seeding, respectively, compared to only 50% and 60% of FOXA2-KD PC3 cells at the same time points. Incubation of PC3/Control cells with a neutralizing antibody recapitulated the adhesion profile observed with the PC3/FOXA2-KD cells (Fig. 5).

Lastly, we examined whether the matrix-coated surface adhesion results can be repeated in the context of cell-to-cell adhesion. PC3 cells were seeded onto confluent osteoblastic MC3T3 cell monolayers. Similar to what was observed with the adhesion to collagen I, 42 and 57 % of PC3/Control cells attached to MC3T3 cells at 15 and 30 min, respectively. PC3/FOXA2-KD cells adhered at lower rates of 21 and 25% at the same times (Suppl. Fig. 3).

Overexpression of integrin α1 restores adhesion and spreading of PC3/FOXA2-KD cells.

To determine whether integrin α1 is the main mediator of FOXA2-induced cell adherence, we established PC3/FOXA2-KD ITGA1-overexpressing (PC3/FOXA2-KD/ITGA1) cells and examined whether the restoration of integrin α1 expression in PC3/FOXA2-KD cells rescued cellular adhesion properties. Integrin α1 was overexpressed in both PC3/FOXA2-KD clones I and III. GFP-expressing plasmids were used as controls. Western blot and RT-qPCR analysis confirmed that integrin α1 was overexpressed at both the transcript and protein levels (Figs. 6A & 6B). Overexpression of integrin α1 rescued the cells ability to adhere to collagen I (Fig. 6C) and restored focal adhesion (Fig. 6D).

Discussion

Metastasis is the main contributor to mortality in men with advanced prostate cancer (PCa); and bone is the most common site of PCa metastasis (1-4). Following the extravasation of PCa cells, successful establishment of bone metastases requires efficient adhesion of PCa cells to the bone matrix, enabling PCa cells to acquire a foothold in the bone and initiate the vicious cycle of bone destruction and cancer proliferation. We recently found that the expression of FOXA2 is critical for PCa PC3 cells to grow in the bone. In this study, we found knocking down FOXA2 in PC3 cells decreased the expression of multiple integrins, including ITGA1, a collagen 1 binding integrin, resulting in disrupted focal adhesion signaling in PCa cells and decreased ability to attach to and spread on collagen I. These results revealed an important role of FOXA2 and integrin α1 axis in promoting the establishment of PCa in the bone.

Integrins play a pivotal role in the interactions between PCa cells and extracellular matrix, a critical step for the establishment of cancer metastases (12). Our work is the first to show that FOXA2 regulates the expression of integrin α1, which in turn activates integrin signaling, promotes interaction of PCa cells with the bone ECM protein collagen I, and enables PCa cells to grow in bone tissue. However, we did not observe complete loss of adhesion to collagen I in FOX2-KD cells, possibly due to the remaining expression of integrin α1 and the unchanged expression of integrin α2 that is also involved in promoting cancer bone metastasis (18-

20). Additionally, our data indicate that the mRNA levels of multiple integrins, including integrins αv and β3, decreased in PC3/FOXA2-KD cells. Integrin dimer αvβ3 has also been shown to mediate interaction between cancer cells and bone matrix (21). This suggests that FOXA2 could control PCa interaction with bone matrix via multiple mechanisms. Nonetheless, our findings provide direct evidence for the role of FOXA2 in promoting interaction of PCa cells with the bone microenvironment via regulation of integrin α1 expression.

Involvement of integrin α1 in the interaction between PCa and the bone matrix are consistent with a previous study that showed that PC3 cells express high levels of integrin α1 and demonstrate a high binding affinity to a collagen I matrix (22). However, the functional involvement of integrin α1 in PCa bone metastasis had not yet been established. Our study contributes to this line of work and reveals integrin α1 as a key regulator for mediating FOXA2 function in promoting the establishment of PCa in the bone microenvironment.

Our study revealed a mechanism that FOXA2-controled integrin α1 promotes the interaction between NEPCa and bone microenvironment. As for FOXA2-negative PCa bone metastases, different mechanism(s), such as the above-mentioned integrin α2, may mediate interactions between PCa cells and bone matrix. Integrin α2 expression is found on LNCaP and C4-2B cells, which have been shown to successfully grow in the bone matrix as well (20).

Besides bone, FOXA2-regulated integrin expression may play roles in other metastatic sites. For example, collagen I is one of the ECM components and the most abundant collagen in liver (31). We speculate that FOXA2 regulated integrin α1 may also play a role in the establishment of PCa metastases in liver.

In conclusion, in this study, we identified integrin α1 as a mediator of FOXA2's involvement in promoting PCa interaction with bone matrix and PCa establishment in the bone. Understanding how PCa interacts with the bone environment provides important information that can be used to design therapies to prevent PCa bone metastasis.

Material and Methods

Cell culture and establishment of PC3 FOXA2 knockdown cells

The culture and establishment of PC3 FOXA2 knockdown cells were described previously. Because FOXA2 knockdown efficiency gradually decreased, three single clones were generated from PC3/shFOXA2 cells and designated as PC3/FOXA2-KD I, -II, and -III. ITGA1 over-expressing cells were established using lenti-viral ITGA1 plasmid. GFP plasmid was used as negative control.

RNA isolation and real-time PCR

RNA was extracted using Purelink RNA Mini Kit following manufacturer's protocol (Ambion, Life Technologies, Waltham, MA). For reverse transcription, 1μg of total RNA was used for cDNA synthesis. Quantitative (q)-PCR was conducted to assess

the expression levels of FOXA2 and the indicated α and β Integrins (34). Gene expression was normalized by GAPDH.

Western blot analysis

Cells were lysed as described previously. Antibodies used were listed in Table 1.

Cellular adhesion and spreading assays

Cell culture surfaces were coated with ECM proteins, including collagen I (50 μg/mL), collagen IV (20 μg/mL), fibronectin (5 μg/mL), vitronectin (20 μg/mL), or laminin (25 μg/mL) overnight. Cells were trypsinized, washed with media containing 10% FBS followed by two washes with serum-free media. The cells were re-suspended in serum-free media and incubated with CellTracker Green CMFDA (12.5 μg/mL) (Thermo Fisher Scientific, Waltham, MA) at 37 °C for 30 minutes. At completion of incubation, cells were washed to remove excess fluorophore, seeded on a specific matrix for 15 or 30 min. Floating and adherent cells were collected separately and lysed in 100 mM sodium hydroxide. The fluorescence intensity of the cell lysates was read, and percent adhesion was calculated. The adhesion assays were also conducted by seeding PC3 cells onto bone cells. Pre-osteoblastic MC3T3 cells were seeded and grown into a monolayer for 48 hours to allow for 100% confluency and the secretion of bone matrices (35). Cellular adhesion assays were repeated under the same conditions as described above. To study the involvement of integrin α1 in mediating PC3 cells adhesion to collagen, neutralizing antibody to integrin α1 (15 μg/ml,

EDM Millipore, Burlington, MA) was incubated with cells for an additional 30 min after CellTracker Green CMFDA was washed out. IgG was used as a negative control. For spreading assays, cells were seeded onto the collagen I and then stained with Phalloidin to allow visualization of the surface area of the cells. Phosphorylated Focal Adhesion Kinases (pFAKs at Tyr 397) staining and co-localization of integrin β1 (12G10 staining, Martin Humpries, University of Manchester, UK) were used to evaluate the formation of focal adhesion complexes. The time course ranged from 15 min to 12 hr.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation was conducted as described previously using the EDM Millipore Magna ChIP kit (Burlington, MA). Based on the public available ChIP-seq data (36), there are two FOXA2 binding sites in the regulatory region of the integrin α1 gene, designated as A2BS1 and A2BS2. Primers were designed to cover both FOXA2 binding sites. Primer sequences are A2BS1 (Forward: 5'-GATTTGGCCCGGAGAACGAG-3' and Reverse: 5'-CTTCGCGGGTCCGTGTTTAG-3') and A2BS2 (Forward: 5'-GGCCATCGACTTCGACTCTC-3' and Reverse: 5'-CCAGCTCGATGGTGTGGTAA-3'). FOXA2 enrichment was normalized to IgG.

Immunofluorescence staining

PC3/Control and PC3/FOXA2-KD cells were cultured on collagen I or fibronectin for the given amount of time per experiment. Cells

were fixed in 4% formaldehyde followed by incubation with primary antibody overnight at 4°C and secondary antibody (1:1000) for 2 hours at room temperature. DAPI was used to visualize cell nuclei. Slides were mounted with Fluoromount G (SouthernBiotech, Birmingham, AL) and staining was imaged at 60x magnification using a Nikon microscope. Phalloidin staining images were used to quantify cell surface area by using Nikon software, following appropriate mask application. Data are presented as ROI and scaled to μM^2. Antibodies are listed in Table 1.

Statistical analysis

Statistical analyses were conducted using a two-sided Student's t test and a p-value of 0.05 was considered statistically significant.

Acknowledgements

We would like to thank Dr. Robert Matusik for advice on the research and critical reading of the manuscript, Dr. Martin Humphries for the 12G10 antibody clone of active β1 integrin. This research is supported by Department of Defense grant W81XWH-12-1-0212 and Prostate Cancer Biorepository Network (W81XWH-14-2-0183), NIH R03 CA212567, R01 CA226285, U54 GM104940 , HL098435, HL133497, and HL141155, the PNW Prostate Cancer SPORE P50 CA097186 and P01 CA163227, LSUHSC FWCC and Office of Research funding to XY, Carroll Feist Pre-doctoral Fellowship to ZC, and American Heart Association Pre-doctoral Fellowship to Z.A.Y.

Conflict of Interest

We declare no conflicts of interest.

References:

1. Vessella RL, Corey E. Targeting factors involved in bone remodeling as treatment strategies in prostate cancer bone metastasis. Clin Cancer Res. 2006;12(20 Pt 2):6285s-90s.
2. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol. 2000;31(5):578-83.
3. Gandaglia G, Abdollah F, Schiffmann J, Trudeau V, Shariat SF, Kim SP, et al. Distribution of metastatic sites in patients with prostate cancer: A population-based analysis. Prostate. 2014;74(2):210-6.
4. Sottnik JL, Keller ET. Understanding and targeting osteoclastic activity in prostate cancer bone metastases. Curr Mol Med. 2013;13(4):626-39.
5. Epstein JI, Amin MB, Beltran H, Lotan TL, Mosquera JM, Reuter VE, et al. Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am J Surg Pathol. 2014;38(6):756-67.
6. Aprikian AG, Cordon-Cardo C, Fair WR, Zhang ZF, Bazinet M, Hamdy SM, et al. Neuroendocrine differentiation in metastatic prostatic adenocarcinoma. J Urol. 1994;151(4):914-9.
7. Jackson DA, Rowader KE, Stevens K, Jiang C, Milos P, Zaret KS. Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition. Mol Cell Biol. 1993;13(4):2401-10.
8. Mirosevich J, Gao N, Matusik RJ. Expression of Foxa transcription factors in the developing and adult murine prostate. Prostate. 2005;62(4):339-52.
9. Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R, Matusik RJ. Expression and role of Foxa proteins in prostate cancer. Prostate. 2006;66(10):1013-28.
10. Park JW, Lee JK, Witte ON, Huang J. FOXA2 is a sensitive and specific marker for small cell neuroendocrine carcinoma of the prostate. Mod Pathol. 2017.
11. Qi J, Nakayama K, Cardiff RD, Borowsky AD, Kaul K, Williams R, et al. Siah2-dependent concerted activity of HIF and FoxA2 regulates formation of neuroendocrine phenotype and neuroendocrine prostate tumors. Cancer Cell. 2010;18(1):23-38.
12. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. Nat Rev Cancer. 2018;18(9):533-48.

13. Goel HL, Li J, Kogan S, Languino LR. Integrins in prostate cancer progression. Endocr Relat Cancer. 2008;15(3):657-64.
14. Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 2010;339(1):269-80.
15. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, et al. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate. 2011;71(15):1668-79.
16. Corey E, Quinn JE, Bladou F, Brown LG, Roudier MP, Brown JM, et al. Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. Prostate. 2002;52(1):20-33.
17. Bluemn EG, Coleman IM, Lucas JM, Coleman RT, Hernandez-Lopez S, Tharakan R, et al. Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling. Cancer Cell. 2017;32(4):474-89.e6.
18. Sottnik JL, Daignault-Newton S, Zhang X, Morrissey C, Hussain MH, Keller ET, et al. Integrin alpha2beta 1 (alpha2beta1) promotes prostate cancer skeletal metastasis. Clin Exp Metastasis. 2013;30(5):569-78.
19. Kostenuik PJ, Singh G, Orr FW. Transforming growth factor beta upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. Clin Exp Metastasis. 1997;15(1):41-52.
20. Hall CL, Dubyk CW, Riesenberger TA, Shein D, Keller ET, van Golen KL. Type I collagen receptor (alpha2beta1) signaling promotes prostate cancer invasion through RhoC GTPase. Neoplasia. 2008;10(8):797-803.
21. Kwakwa KA, Sterling JA. Integrin alphavbeta3 Signaling in Tumor-Induced Bone Disease. Cancers (Basel). 2017;9(7).
22. Sariisik E, Docheva D, Padula D, Popov C, Opfer J, Schieker M, et al. Probing the interaction forces of prostate cancer cells with collagen I and bone marrow derived stem cells on the single cell level. PLoS One. 2013;8(3):e57706.
23. Perez-Balaguer A, Ortiz-Martinez F, Garcia-Martinez A, Pomares-Navarro C, Lerma E, Peiro G. FOXA2 mRNA expression is associated with relapse in patients with Triple-Negative/Basal-like breast carcinoma. Breast Cancer Res Treat. 2015;153(2):465-74.
24. Vorvis C, Hatziapostolou M, Mahurkar-Joshi S, Koutsioumpa M, Williams J, Donahue TR, et al. Transcriptomic and CRISPR/Cas9 technologies reveal FOXA2 as a tumor suppressor gene in pancreatic cancer. Am J Physiol Gastrointest Liver Physiol. 2016;310(11):G1124-37.
25. Chand V, Pandey A, Kopanja D, Guzman G, Raychaudhuri P. Opposing Roles of the Fork-head box genes FoxM1 and FoxA2 in Liver Cancer. Mol Cancer Res. 2019.

26. Wang J, Zhu CP, Hu PF, Qian H, Ning BF, Zhang Q, et al. FOXA2 suppresses the metastasis of hepatocellular carcinoma partially through matrix metalloproteinase-9 inhibition. Carcinogenesis. 2014;35(11):2576-83.
27. Li CM, Gocheva V, Oudin MJ, Bhutkar A, Wang SY, Date SR, et al. Foxa2 and Cdx2 cooperate with Nkx2-1 to inhibit lung adenocarcinoma metastasis. Genes Dev. 2015;29(17):1850-62.
28. Li C, Lu S, Shi Y. MicroRNA-187 promotes growth and metastasis of gastric cancer by inhibiting FOXA2. Oncol Rep. 2017;37(3):1747-55.
29. Connelly ZM, Yang S, Chen F, Yeh Y, Khater N, Jin R, et al. Foxa2 activates the transcription of androgen receptor target genes in castrate resistant prostatic tumors. Am J Clin Exp Urol. 2018;6(5):172-81.
30. Aggarwal R, Huang J, Alumkal JJ, Zhang L, Feng FY, Thomas GV, et al. Clinical and Genomic Characterization of Treatment-Emergent Small-Cell Neuroendocrine Prostate Cancer: A Multi-institutional Prospective Study. J Clin Oncol. 2018;36(24):2492-503.
31. Aycock RS, Seyer JM. Collagens of normal and cirrhotic human liver. Connect Tissue Res. 1989;23(1):19-31.
32. Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell. 2005;8(5):393-406.
33. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nature medicine. 2016;22(3):298-305.
34. Chen J, Green J, Yurdagul A, Jr., Albert P, McInnis MC, Orr AW. alphavbeta3 Integrins Mediate Flow-Induced NF-kappaB Activation, Proinflammatory Gene Expression, and Early Atherogenic Inflammation. Am J Pathol. 2015;185(9):2575-89.
35. Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res. 1999;14(6):893-903.
36. Freese NH, Norris DC, Loraine AE. Integrated genome browser: visual analytics platform for genomics. Bioinformatics. 2016;32(14):2089-95.

Figure 3. FOXA2 knockdown decreases the expression of multiple integrins in PC3 cells. A: RT-qPCR analyses were conducted to assess the expression levels of FOXA2 in PC3/Control cells and three PC3/FOXA2-KD cell clones. All three clonal lines demonstrated reduced mRNA levels compared to the PC3/Control cells. **B.** Western blot analysis confirmed loss of FOXA2 expression in PC3/FOXA2-KD cells. β-actin served as the loading control. **C**. The expression of integrins in PC3/Control and PC3/FOXA2-KD cells. Log2 expression levels were extracted from RNA-seq data.

Figure 2. FOXA2 regulates the expression of ITGA1. A. RT-qPCR to assess the expression of integrins. FOXA2 knockdown caused a decrease in the mRNA levels of integrins α1, α7, αv, β2, β3, and β4. *$p < 0.05$, **$p < 0.01$, t-test. Error bars reported as standard deviation. **B.** Western blot to assess the expression of integrins α1 and α2 in PC3/Control cells and three independent FOXA2 knockdown clones (PC3/FOXA2 KD I, II, III). The level of integrin α1 was lower in PC3/FOXA2-KD cells than PC3/Control cells. However, FOXA2 knockdown did not change the expression level of integrin α2. **C.** Chromatin Immunoprecipitation analysis. ChIP assays were conducted by using PC3/Control and PC3/FOXA2-KD cells. FOXA2 bound to both FOXA2 binding sites (A2BS1 & A2BS2) in the regulatory region of the ITGA1 gene. Additionally, H3K4me3 (histone mark of active promoters) and

H3K27Ac3 (histone mark of active enhancers) were enriched in the ITGA1 regulatory regions.

Figure 3. FOXA2 knockdown prevents cellular spreading and the activation of focal adhesion kinase. PC3/Control and PC3/FOXA2-KD cells were seeded on collagen-I for 15, 30, 60, or 120 minutes. Immunofluorescence staining was conducted to visualize the cellular cytoskeleton (Phallodin, green) or phosphorylated FAK (pFAK at Tyr 397, red). DAPI (blue) was used to visualize the nuclei. As early as 30 minutes, PC3 Control cells started to spread out and displayed the formation of focal adhesions. PC3/FOXA2-KD cells failed to spread out on the collagen-I surface and pFAK foci were absent at the cell membrane.

Figure 4. Integrin α1 is involved in PC3 cells' adhesion and integrin activation on collagen I. Immunofluorescence staining was conducted to examine the co-localization of phosphorylated-FAK (pFAK, green) and integrin β1 (12G10, Red) in PC3/Control and PC3/FOXA2-KD cells that were seeded on Collagen I. PC3 control cells displayed co-localization of pFAK and integrin β1 starting at 3 hours, whereas PC3/FOXA2-KD cells did not show any co-localization of pFAK and integrin β1 at the time points examined.

Figure 5. Blocking integrin α1 decreases PC3 cells' adherence to collagen I. PC3/Control and PC3/FOXA2-KD cell were seeded on collagen-I coated surfaces for 15 or 30 minutes. Compared to control cells, PC3/FOXA2-KD cells displayed decreased cell

adherence. Furthermore, incubating PC3 control cells with neutralizing antibodies to integrin α1 recapitulated the adherence profile observed in the FOXA2-KD cells. ns: not significant, * $p < 0.05$, t-test. Error bars reported as standard deviation.

Figure 6. Integrin α1 overexpression in PC3/FOXA2-KD cells restores their adhesion properties. **A & B**. RT-qPCR (A) and Western blot (B) to assess the expression levels of ITGA1 in PC3 cells. **C.** Cellular adherence assays. The cells were seeded on collagen I for 15 or 30 minutes. Integrin α1 overexpression restored cellular adherence. *$p<0.05$, t-test. Error bars reported as standard deviation. **D.** Integrin α1 overexpression restored focal adhesions. The cells were seeded on collagen I for 30 minutes. Activated focal adhesion was visualized by the co-localization of phosphorylated-FAK (pFAK, green) and integrin β1 (12G10, Red). PC3/Control cells displayed co-localization of active integrin β1 with substantial pFAK. In the PC3/FOXA2-KD cells, both pFAK and active integrin β1 foci were lost. However, co-localization of pFAK/integrin β1 was restored in the PC3/FOXA2-KD/ITGA1 overexpressing cells.

Supplemental Figure 1. Cell adherence of PC3 cells on various ECM proteins. PC3/Control and PC3/FOXA2-KD cells were seeded on different extracellular matrices for 30 minutes. The percentage of cells adhered to each matrix was assessed. PC3/Control and PC3/FOXA2-KD cells did not display statistically significant difference in their adherence to the cell culture surface coated with

559 Collagen IV, Fibronectin, Laminin, or Vitronectin. Error bars
560 reported as standard deviation.
561
562 **Supplemental Figure 2**. Cell spreading analysis. **A & B.**
563 PC3/Control and PC3/FOXA2 KD cells were seeded on fibronectin
564 for 15, 30, and 60 minutes. Cells were fixed and stained for
565 Phalloidin (F-actin) and p-FAK Y397. Neither types of cells could
566 spread or activate integrin signaling on fibronectin. Quantitation of
567 cell surface area from Phalloidin stain is shown below for each time
568 point. **C & D.** cell spreading assays on collagen I. More PC3/Control
569 cells were able to spread and activate integrin signaling on Collagen
570 I compared to PC3/FOXA2-KD cells. Quantitation of cell surface
571 area from Phalloidin stain is shown below for each time point.
572
573 **Supplemental Figure 3.** FOXA2 promotes cellular adhesion to
574 bone cells. MC3T3 cells were seeded in a monolayer and allowed
575 to grow into 100% confluency. PC3/Control and PC3/FOXA2-KD
576 cells were seeded on top of the MC3T3 cells for 15 or 30 minutes.
577 Significantly more PC3/Control cells (56%) adhered to bone cells
578 compared to PC3/FOXA2-KD cells (27%) at 30 minutes. *$p<0.05$,
579 t-test.
580 **Tables**
581 Table 1. List of antibodies

Catolog Number	Antigen	Dilution	Application
SC 271034	Integrin α1	1: 500	Western Blot
SA 05-246	Integrin α1	1µg/ml	Neutralizing

SC 9089	Integrin α2	1: 1000	Western Blot
CS 8556	pFAK	1:400	IF
Martin Humphries	12G10	1:400	IF
A12379	Phallodin	1:1000 (secondary)	IF

582
583

Fig. 1

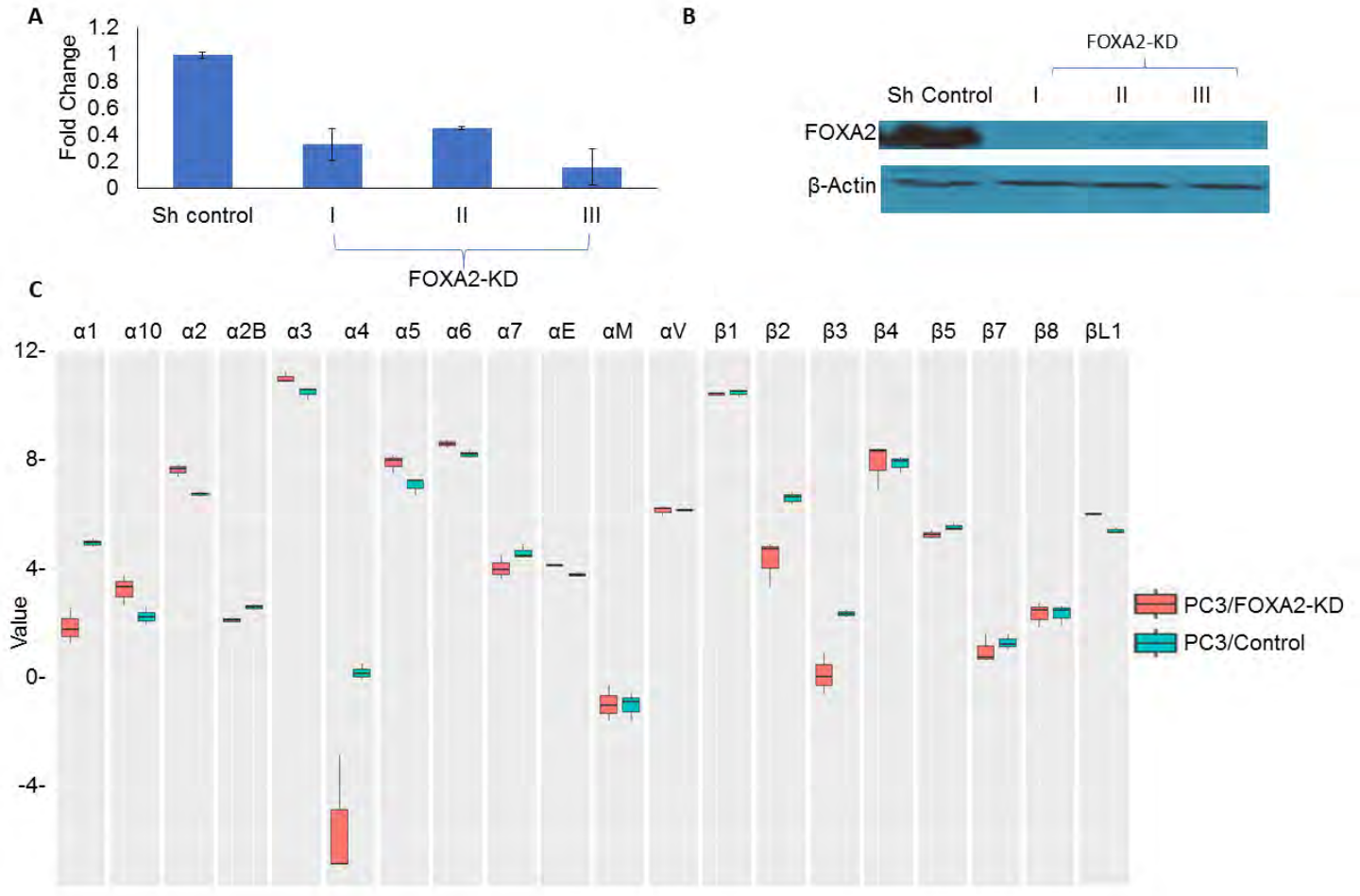

A
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Sh control
I
II
III
FOXA2-KD
B
FOXA2-KD
Sh Control
I
II
III
FOXA2
β-Actin
C
α1
α10
α2
α2B
α3
α4
α5
α6
α7
αE
αM
αV
β1
β2
β3
β4
β5
β7
β8
βL1
12-
8-
4-
0-
-4-
Value
PC3/FOXA2-KD
PC3/Control

Fig. 2

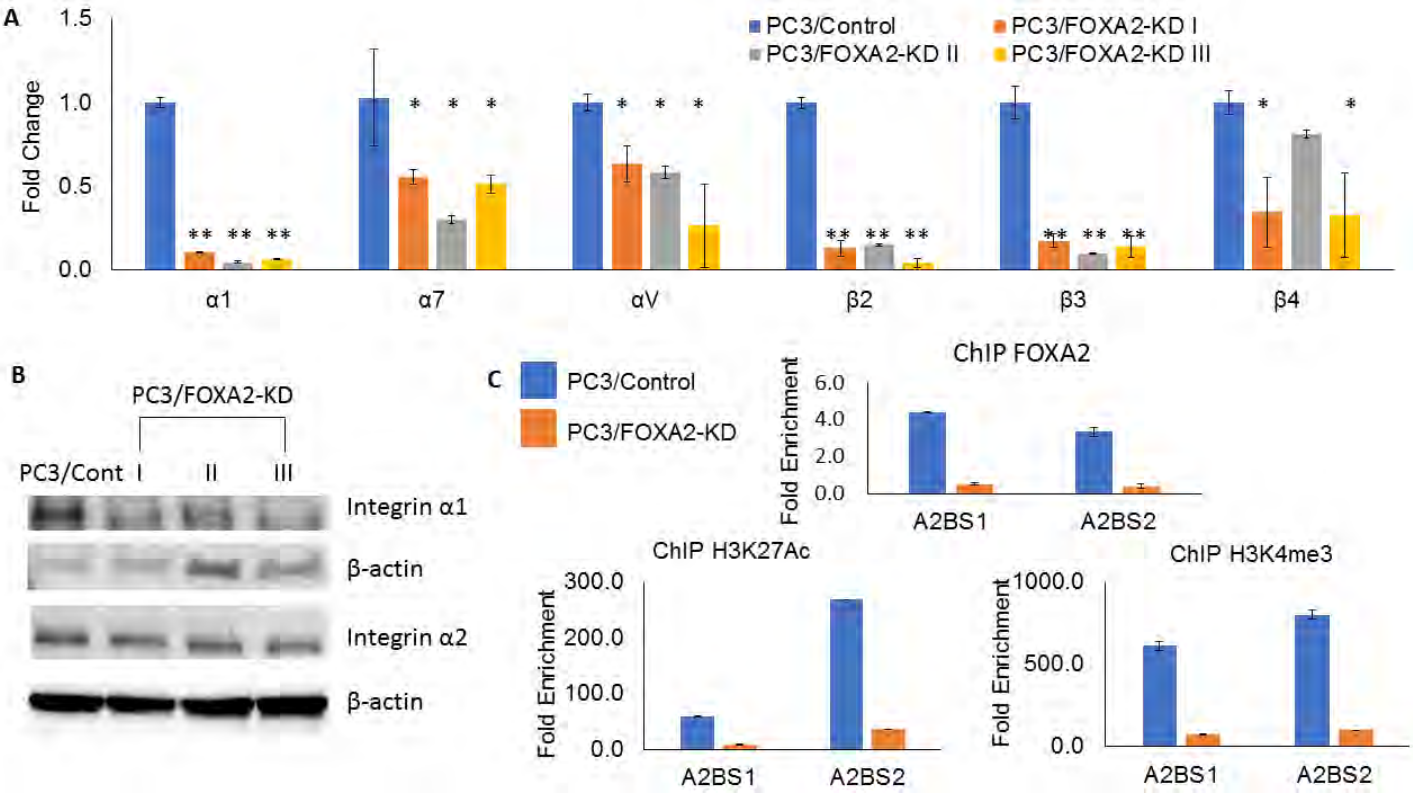

A
PC3/Control
PC3/FOXA2-KD I
PC3/FOXA2-KD II
PC3/FOXA2-KD III
Fold Change
1.5
1.0
0.5
0.0
α1
α7
αV
β2
β3
β4
B
PC3/FOXA2-KD
PC3/Cont
I
II
III
Integrin α1
β-actin
Integrin α2
β-actin
C
PC3/Control
PC3/FOXA2-KD
ChIP FOXA2
Fold Enrichment
6.0
4.0
2.0
0.0
A2BS1
A2BS2
ChIP H3K27Ac
Fold Enrichment
300.0
200.0
100.0
0.0
A2BS1
A2BS2
ChIP H3K4me3
Fold Enrichment
1000.0
500.0
0.0
A2BS1
A2BS2

Fig. 3

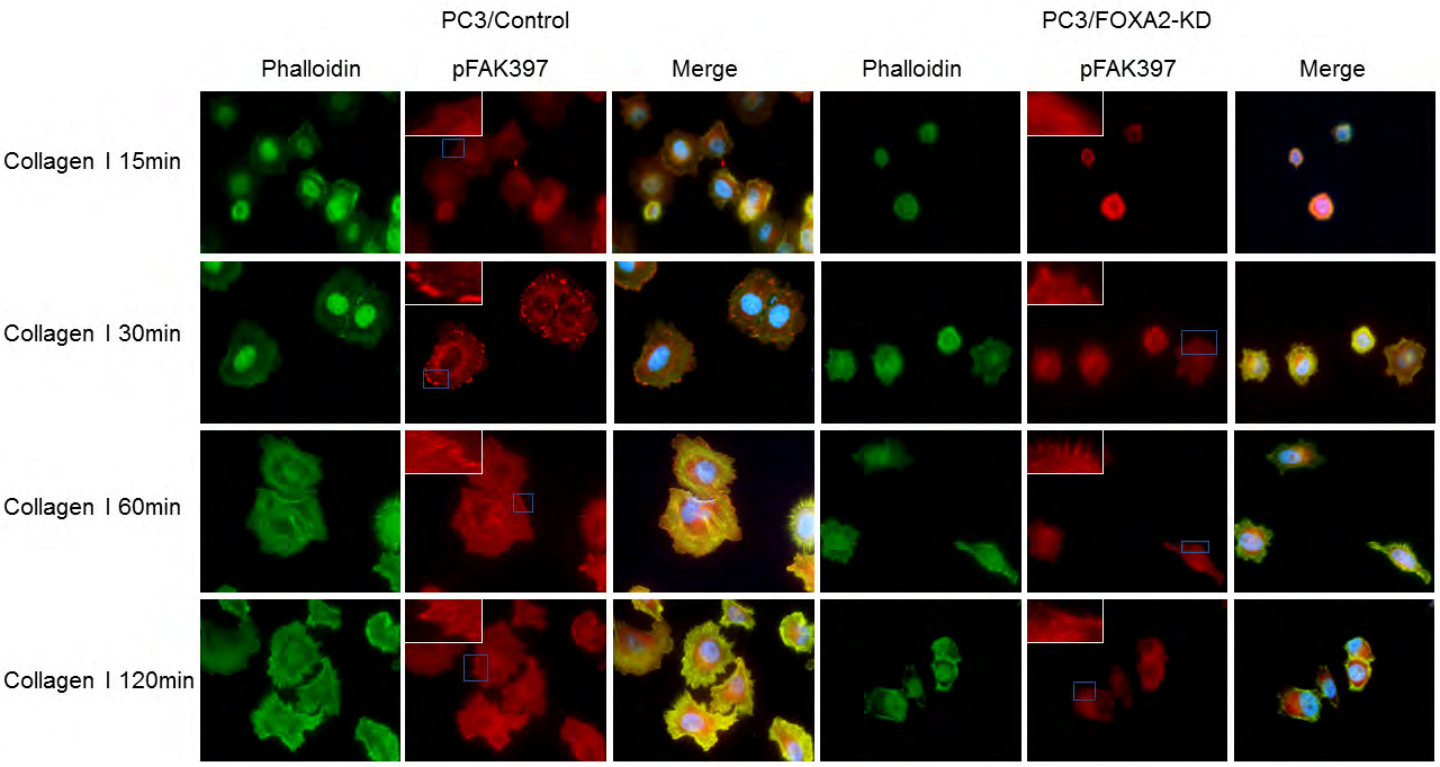

PC3/Control
PC3/FOXA2-KD
Phalloidin
pFAK397
Merge
Phalloidin
pFAK397
Merge
Collagen I 15min
Collagen I 30min
Collagen I 60min
Collagen I 120min

Fig. 4

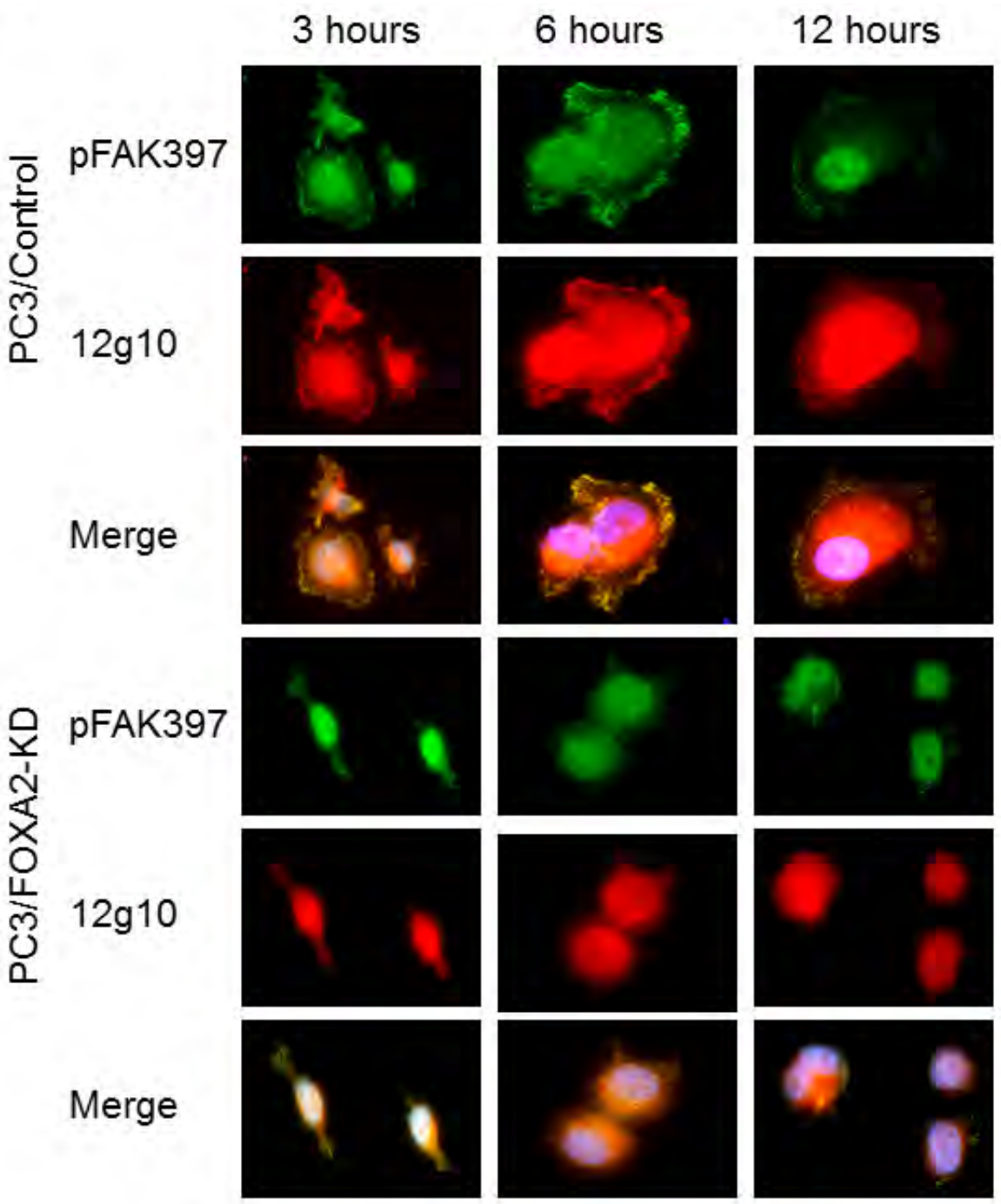

3 hours
6 hours
12 hours
PC3/Control
pFAK397
12g10
Merge
PC3/FOXA2-KD
pFAK397
12g10
Merge

Fig. 5

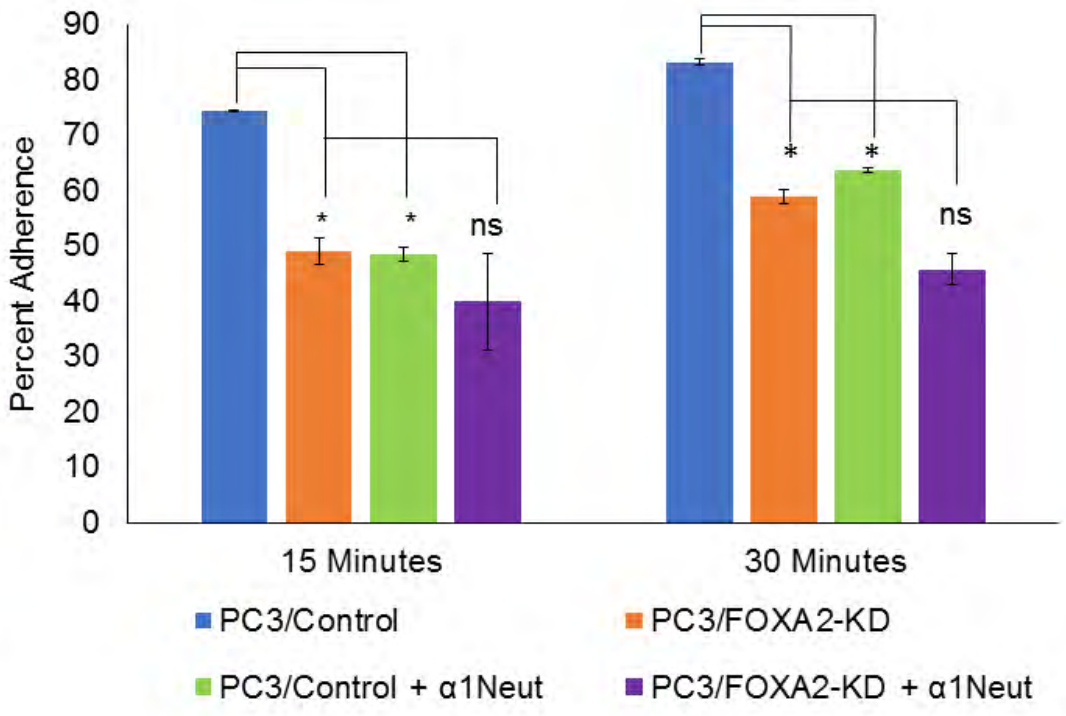

Percent Adherence
90
80
70
60
50
40
30
20
10
0
*
*
ns
*
*
ns
15 Minutes
30 Minutes
PC3/Control
PC3/FOXA2-KD
PC3/Control + α1Neut
PC3/FOXA2-KD + α1Neut

Fig. 6

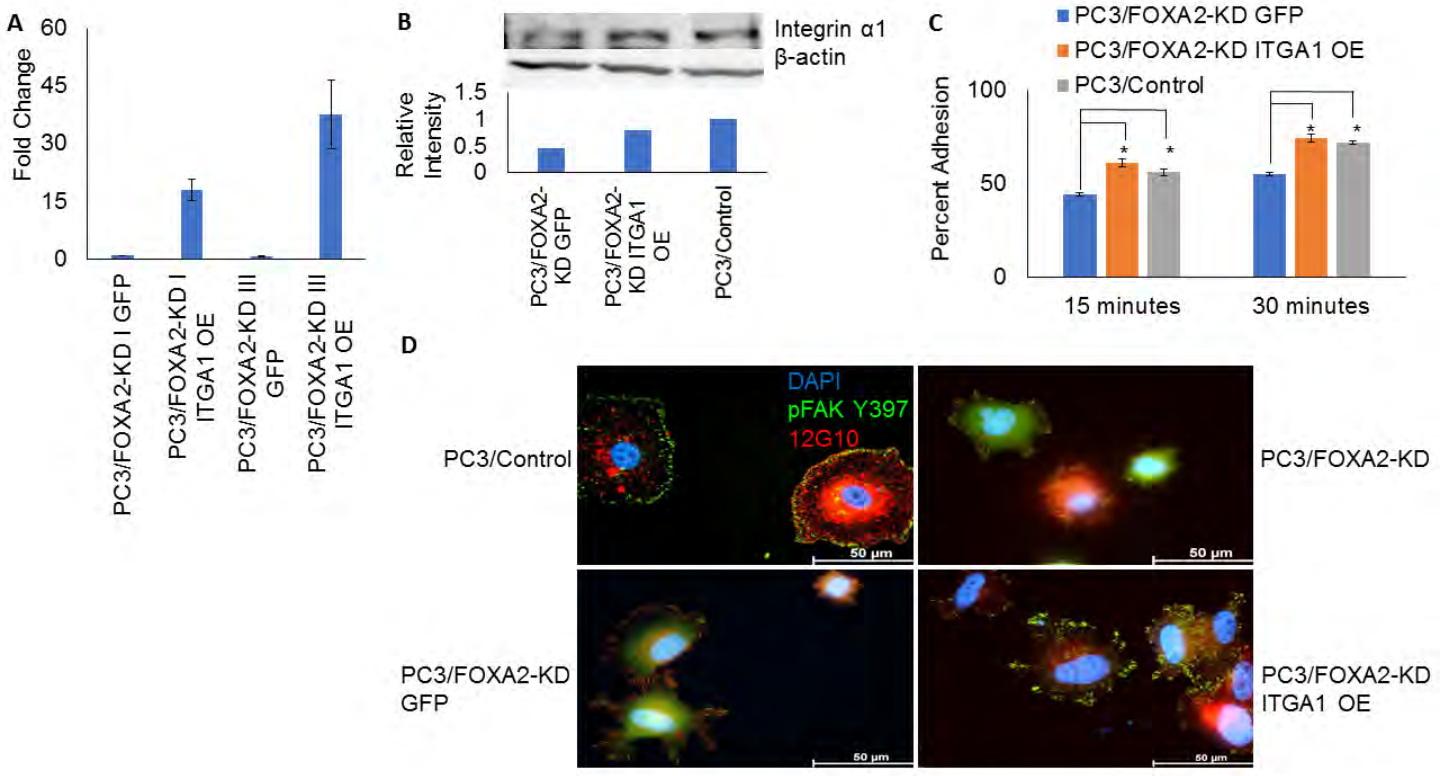

A
Fold Change
60
45
30
15
0
PC3/FOXA2-KD I GFP
PC3/FOXA2-KD I ITGA1 OE
PC3/FOXA2-KD III GFP
PC3/FOXA2-KD III ITGA1 OE
B
Integrin α1
β-actin
Relative Intensity
1.5
1
0.5
0
PC3/FOXA2-KD GFP
PC3/FOXA2-KD ITGA1 OE
PC3/Control
C
PC3/FOXA2-KD GFP
PC3/FOXA2-KD ITGA1 OE
PC3/Control
Percent Adhesion
100
50
0
15 minutes
30 minutes
D
DAPI
pFAK Y397
12G10
PC3/Control
PC3/FOXA2-KD
PC3/FOXA2-KD GFP
PC3/FOXA2-KD ITGA1 OE
50 μm

Fig. S1

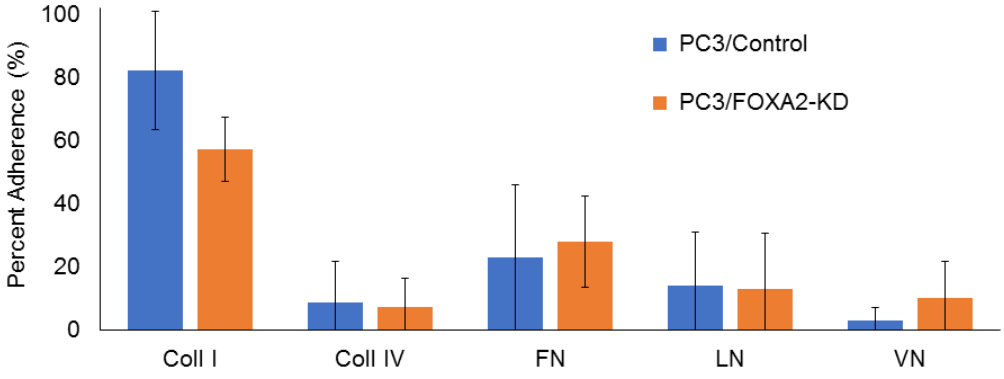

Percent Adherence (%)
100
80
60
40
20
0
PC3/Control
PC3/FOXA2-KD
Coll I
Coll IV
FN
LN
VN

Fig. S2

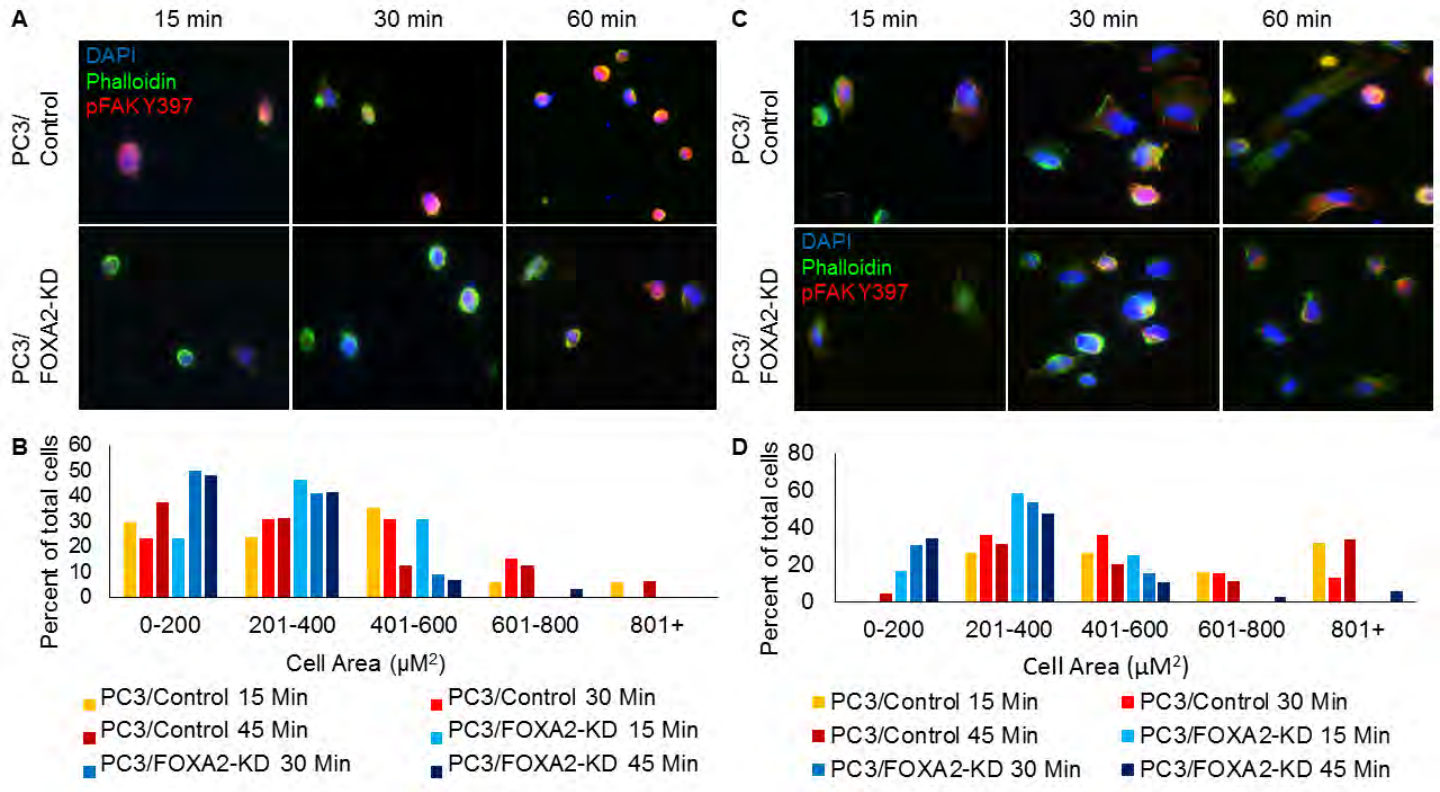
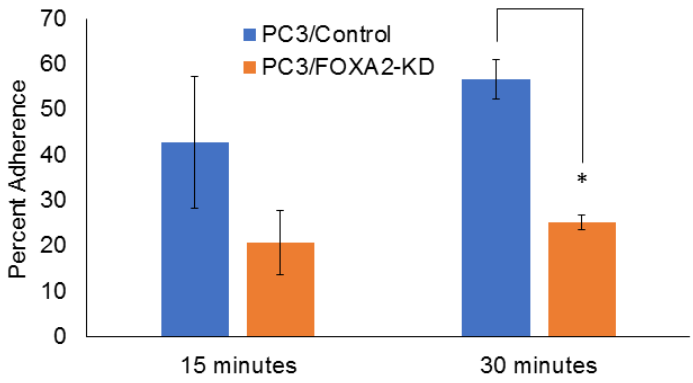

A
15 min
30 min
60 min
PC3/ Control
PC3/ FOXA2-KD
DAPI
Phalloidin
pFAKY397
C
15 min
30 min
60 min
PC3/ Control
PC3/ FOXA2-KD
DAPI
Phalloidin
pFAKY397
B
Percent of total cells
0
10
20
30
40
50
60
0-200
201-400
401-600
601-800
801+
Cell Area (μM2)
PC3/Control 15 Min
PC3/Control 30 Min
PC3/Control 45 Min
PC3/FOXA2-KD 15 Min
PC3/FOXA2-KD 30 Min
PC3/FOXA2-KD 45 Min
D
Percent of total cells
0
20
40
60
80
0-200
201-400
401-600
601-800
801+
Cell Area (μM2)
PC3/Control 15 Min
PC3/Control 30 Min
PC3/Control 45 Min
PC3/FOXA2-KD 15 Min
PC3/FOXA2-KD 30 Min
PC3/FOXA2-KD 45 Min

Fig. S3


70
60
50
40
30
20
10
0
Percent Adherence
PC3/Control
PC3/FOXA2-KD
*
15 minutes
30 minutes

Am J Clin Exp Urol 2018;6(5):172-181
www.ajceu.us /ISSN:2330-1910/AJCEU0084720

Original Article

Foxa2 activates the transcription of androgen receptor target genes in castrate resistant prostatic tumors

Zachary M Connelly[1], Shu Yang[1], Fenghua Chen[1], Yunshin Yeh[3], Nazih Khater[2], Renjie Jin[4], Robert Matusik[4], Xiuping Yu[1]

Departments of [1]Biochemistry and Molecular Biology, [2]Urology, LSU Health Sciences Center, Shreveport, Louisiana 71103, USA; [3]Department of Pathology, Overton Brooks VA Medical Center, Shreveport, LA 71101, USA; [4]Department of Urologic Surgery, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Received August 28, 2018; Accepted September 11, 2018; Epub October 20, 2018; Published October 30, 2018

Abstract: Prostate cancer (PCa) is the leading cancer among men. Androgen Deprivation Therapy (ADT) is a common treatment for advanced PCa. However, ADT eventually fails and PCa relapses, developing into castration-resistant prostate cancer (CRPCa). Although alternative pathways such as cancer stem-cell pathway and neuroendocrine differentiation bypass androgen receptor (AR) signaling, AR remains the central player in mediating CRPCa. In this study, we identified a mechanism that retains AR signaling after androgen deprivation. The TRAMP SV40 T antigen transgenic mouse is a model for PCa. The expression of SV40 T-antigen is driven by the androgen-responsive, prostate specific, Probasin promoter. It has been recognized that in this model, T-antigen is still expressed even after androgen ablation. It is unclear how the androgen-responsive Probasin promoter remains active and drives the expression of T-antigen in these tumors. In our study, we found that the expression of Foxa2, a forkhead transcription factor that is expressed in embryonic prostate and advanced stage prostate cancer, is co-expressed in T-antigen positive cells. To test if Foxa2 activates AR-responsive promoters and promotes the expression of T-antigen, we established the prostate epithelial cells that stably express Foxa2, NeoTag1/Foxa2 cells. Neotag1 cells were derived from the Probasin promoter driven SV40 T-antigen transgenic mouse. We found ectopic expression of Foxa2 drives the T-antigen expression regardless of the presence of androgens. Using this model system, we further explored the mechanism that activates AR-responsive promoters in the absence of androgens. Chromatin immunoprecipitation revealed the occupancy of both H3K27Ac, an epigenetic mark of an active transcription, and Foxa2 at the known AR target promoters, Probasin and FKBP5, in the absence of androgen stimulation. In conclusion, we have identified a mechanism that enables PCa to retain the AR signaling pathway after androgen ablation.

Keywords: Prostate cancer, castrate resistant, Foxa2, AR signaling, T-antigen, LADY mice, TRAMP mice

Introduction

Prostate cancer (PCa) is the most common non-skin cancer and second leading cause of cancer-related death in American men. Androgen deprivation therapy is the gold standard treatment for advanced stage PCa. However, prostatic tumors eventually become resistant to androgen deprivation and progress into castrate-resistant PCa (CRPCa). Identifying mechanisms that drive the development of CRPCa has been a major focus of the field. While loss/reduction of AR signaling and the emergence of neuroendocrine features were observed in a subset of CRPCa (less than 30%) [1], reactivation of AR signaling more commonly occurs in CRPCa [2]. For example, the rising of PSA, a well-established AR-regulated gene, accompanies disease progression and recurrence of PCa, indicating that AR signaling is active in CRPCa. In the effort to identify mechanisms that drive the development of CRPCa, previous studies have demonstrated that AR is still the central player in sustaining PCa growth after androgen deprivation. These studies have identified several mechanisms that activate the AR signaling in CRPCa, including AR amplification/mutations, AR activation by growth factors/crosstalk with other signaling pathways, and AR variants that confer ligand-independent activation of AR signaling [3]. These studies have greatly improved our understanding of CRPCa

progression and have resulted in the development of second-generation androgen-deprivation drugs [4, 5].

Mouse models are useful tools for studying disease progression in PCa. Two of the commonly used transgenic mouse models for PCa research are the LADY and TRAMP mice, both of which express SV40 T-antigen [6, 7]. In these mice, the expression of T-antigen is driven by the AR-responsive Probasin promoter, allowing for T-antigen expression confined to prostatic tissues. Although driven by androgen-responsive promoters, it was noticed that T-antigen is still expressed in the prostatic tumors after castration when they progress to CRPCa. The re-expression of T-antigen in mouse CRPCa mirrors human PCa progression, where the levels of PSA rise again after PCa fails androgen deprivation therapy. In this study, we investigated the mechanisms that activate AR-responsive promoters and drive the expression of AR target genes after androgen deprivation.

Foxa2 is a member of the forkhead (Foxa) family of transcription factors. Foxa proteins act as pioneer transcription factors [8]. Their binding precedes the binding of other transcription factors to the regulatory elements of target genes. The forkhead domain of Foxa protein can displace linker histones and relax chromatin structure. In developing prostates, Foxa2 is expressed in embryonic prostates (in both human and mouse) when prostates undergo budding morphogenesis [9]. At adult prostates, Foxa2 is expressed in rare neuroendocrine cells [10]. In PCa, Foxa2 expression was detected in advanced stage cancer tissues; and the expression of Foxa2 is positively associated with neuroendocrine phenotype [11]. Previous studies indicate that active Wnt/β-Catenin signaling induces the expression of Foxa2 in PCa [12]. In this study, we found the expression of Foxa2 activates AR-responsive promoters and drives the expression of T-antigen after androgen deprivation.

Materials and methods

Animal maintenance

TRAMP mice were maintained at LSU Health Sciences Center-Shreveport animal facility. Mice were castrated at 18-22 week of age and sacrificed 2 days to 4 weeks post-castration. All the animal experiments were approved by the Institutional Animal Care and Use Committee. Archived tissues derived from 12T-10 LADY mice were used for this study.

Cell culture

NeoTag1 cells were previously established and were cultured in Dulbecco's Modified Eagles Media (Hyclone) with the following additives: 10% Heat-Inactivated Fetal Bovine Serum (Atlanta Biologicals, Flowery Branch, GA), 1% Penicillin-streptomycin (Gibco), 4 μg/ml Bovine Pituitary Extract (Atlanta Biologicals), 10 ng/ml Epithelial Growth Factor (Sigma-Aldrich, St. Louis, MO), 1% Insulin-transferrin-selenium X (Gibco). For androgen treatment experiments, cells were washed and cultured overnight in DMEM with 5% charcoal-stripped Serum and 1% Penicillin-Streptomycin. The next day media was changed and ethanol (vehicle control) or 1 nM synthetic androgens (R1881) was supplemented.

RNA isolation and real-time PCR

After completion of treatment, cells were harvested and RNA extraction was performed following manufactures protocol (Purelink RNA Mini Kit, Ambion, Life Technologies). Total RNA was quantitated on a Nanodrop spectrophotometer and 1 μg RNA was used to synthesize cDNA according to manufactures protocol. cDNA was used for transcript analysis for the following genes: AGR2, FKBP5, AR, Synaptophysin, Chromogranin A, and NSE (Neuronal Specific Enolase). Ct values were all normalized to GAPDH and then to the Neotag1 empty vector ethanol control and reported as relative mRNA levels. See Primer Sequence **Table 2** for details.

Protein analysis

Following cell treatments, cells were lysed using passive lysis buffer (Promega), sonicated 10 seconds at 30% power, and centrifuged at 12,000 rcf for 30 minutes. Supernatants were collected and subject to SDS-PAGE at 100 V for 120 minutes. Proteins were transferred to nitrocellulose membrane at 100 V for 90 minutes. Membranes were blocked in 5% non-fat milk for one hour and then incubated overnight

Table 1. Antibodies

Sample	Application	Dilution	Catalog Number
Androgen Receptor	Western Blot/IF	1:1000/	SC-816 (N-20), Santa Cruz
Androgen Receptor	ChIP	4 ug/ml	Ab74272, Abcam
Foxa2	Western Blot/IF	1:1000	SC-9187 (P-19), Santa Cruz
Foxa2	ChIP	4 ul	17-10258, Millipore
T-antigen	Western Blot/IF	1:1000/	SC-25326 (H-1), Santa Cruz
β-actin	Western Blot	1:1000	SC-47778 (C4), Santa Cruz
H3K27AC3	ChIP	1:100	8173 (D5E4), Cell Signaling
IgG	ChIP	4 ul	17-10258, Millipore

Table 2. Primer sequence

Sample	Application	Sequence Forward	Sequence Reverse
Androgen Receptor	qRT-PCR	TGTGGAGATGAAGCTTCTGGCTGT	TGGTACAATCGTTTCTGCTGGCAC
Foxa2	qRT-PCR	ATCCGACTGGAGCAGCTACTACGC	CGCGGACATGCTCATGTATGTGTT
FKBP5	qRT-PCR	GGCGACAGGTCTTCTACTTAC	GATATCTTCACCCTGCTCAGTC
AGR2	qRT-PCR	GTTCTCCTCAACCTGGTCTATG	GTCAGGGATGGGTCTACAAAC
GAPDH	qRT-PCR	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC
Synaptophysin	qRT-PCR	TACCGAGAGAACAACAAAGGGCCA	CGGATGAGCTAACTAGCCACATGA
Chromogranin A	qRT-PCR	GGACACCAAGGTGATGAAGT	GATTCTGGTGTCGCAGGATAG
Neural Specific Enolase	qRT-PCR	ACCACATCAACAGCAGGATTGCAC	TCCCATCCAGTTCCAACATCAGGT
Probasin	ChIP	ACATCTACCATTCCAGTTAAGA	TTCTTGGAGTACTTTACTAGGC
FKBP5	ChIP	ACCCCCATTTTAATCGGAGAAC	TTTTGAAGAGCACAGAACACCCT

with primary antibody of interest. All antibodies were used at a 1:1000 dilution. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (a 1:5000, GE Healthcare, Pittsburgh, PA) for 1 hour at room temp. Lastly, enhanced chemiluminescence detection reagents (PerkinElmer, Waltham, MA) was incubated on membranes for 5 minutes and then exposed on x-ray film or processed in a chemidoc imaging system (Bio-rad). Normalization to β-actin is done for all protein bands. See antibody **Table 1** for details.

Chromatin immunoprecipitation

Cells were treated with R1881 or vehicle control (ethanol) for two hours and then fixed with 1% formaldehyde. EDM Millipore Magna ChIP kit was used and their protocol was followed. DNA fragmentation was optimized from Bioruptor (Bioruptor) sonication for 40 cycles on high producing approximately 200 bp length fragments. Following de-crosslink, proteinase K and RNase treatment, DNA was purified using Qiagen quick DNA isolation kit and was subjected to SYBR Green Real-Time PCR. All Relative DNA levels were normalized to IgG immunoprecipitation control and enrichment is shown. Antibodies used were ChIP-grade and can be found in the antibody **Table 1**.

Histology, immunohistochemistry, and cell imaging

Immunohistochemistry protocol was conducted as described previously [12, 13]. Tissue was harvested from mice and fixed in 10% buffered formalin overnight, processed, and paraffin embedded. Sections of tissue were cut to 5 microns and heated to remove residual paraffin. Tissue sections were de-deparaffinized, rehydrated, and microwaved at 30% power for 20 minutes in boiling antigen unmasking solution (Vector Laboratories, Burlingame, CA) for antigen retrieval. To block for endogenous peroxidases, slides were incubated with 15% hydrogen peroxide. The antigen-antibody interaction was visualized using the Vectastain Elite ABC Peroxidase kit (Vector Laboratory) following the manufacturer's protocol with additional DAKO DAB-chromogen System (DAKO, Carpinteria, CA). Slides were then counter-

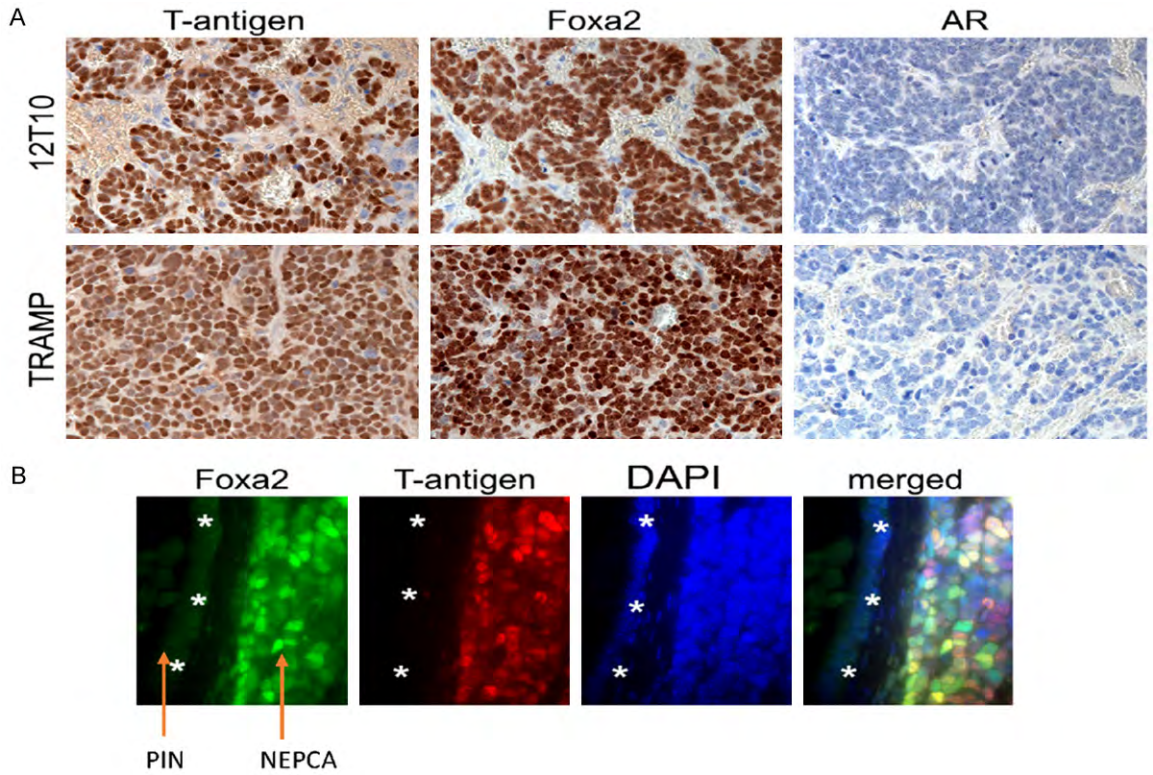


Figure 1. T-antigen and Foxa2 are co-expressed in prostatic tumors of transgenic mice. A. Immunohistochemistry staining of T-antigen, Foxa2, and AR conducted on serial sections derived from prostatic tumors of 12T-10 LADY and TRAMP mice. While nuclear AR is not expressed in the cancer cells, the expression of T-antigen is concomitant with Foxa2 in these cells. *Indicate areas that is negative for nuclear AR but positive for T-antigen and Foxa2. B. Immunofluorescence staining of T-antigen and Foxa2 conducted on a section of TRAMP tumor. *Indicate T-antigen and Foxa2 negative area.

stained with hematoxylin, dehydrated, coverslipped and then imaged using a Zeiss microscope.

Cell proliferation

To quantify the differences in cell proliferation rates, two different assays were used. For Incucyte ZOOM method, 500 cells were seeded in a 96 well plate and once attached, regular cell culture media were replaced with DMEM media containing 5% charcoal-stripped serum without other additives. Cells were then placed in the Incucyte ZOOM Live Imager scanner and images were taken every four hours. After 24 hours of cell culture in androgen-depleted media, ethanol or androgens was added back to the media and cell proliferation continued for five days with a media change every two days. All images were analyzed on the Incucyte ZOOM Software with appropriate mask applied. Total area for each time point was quantitated and averaged across each group and shown with the standard deviation. Under the same cell culture conditions, WST1 cell proliferation was also conducted following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). WST1 was added to cell culture media, incubated with the cells for 30 minutes and then the absorbance was read. Absorbance at 440 nm (630 nm reference wavelength) was measured and readings were averaged for each experimental group and the standard deviation was shown.

Immunofluorescence

Following the same cell culture conditions previously described for Chromatin Immunoprecipitation, cells were then fixed in 4% formaldehyde for 20 minutes at 4 degrees C. After fixation, cells were washed, blocked with 10% horse serum and 1% BSA in 0.2% TBS-T for 1 hour and then incubated with antibody of interest overnight at 4 degrees C. The next day, cells were washed, and secondary antibody was

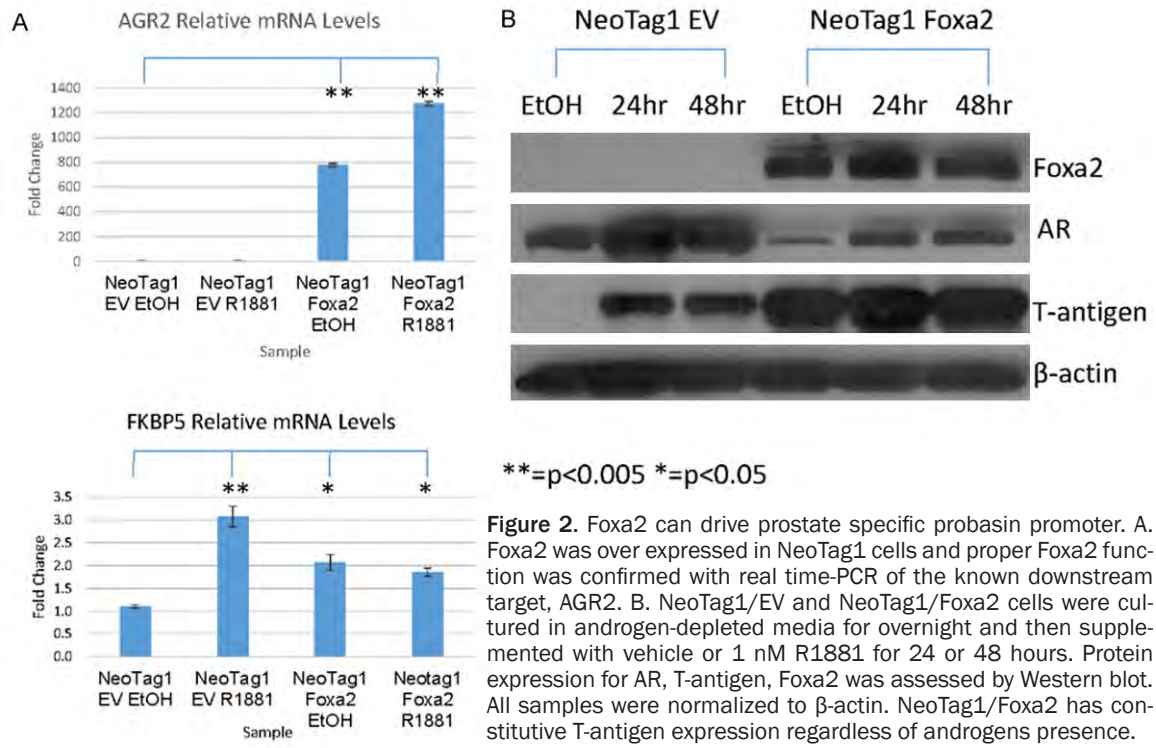


Figure 2. Foxa2 can drive prostate specific probasin promoter. A. Foxa2 was over expressed in NeoTag1 cells and proper Foxa2 function was confirmed with real time-PCR of the known downstream target, AGR2. B. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and then supplemented with vehicle or 1 nM R1881 for 24 or 48 hours. Protein expression for AR, T-antigen, Foxa2 was assessed by Western blot. All samples were normalized to β-actin. NeoTag1/Foxa2 has constitutive T-antigen expression regardless of androgens presence.

applied at a dilution of 1:1000 for two hours. Cells were washed and then incubated with DAPI. Slides were mounted with Fluoromount G and staining was imaged at 60x magnification using Nikon microscope. Images were processed using Nikon Software. Antibody information can be found in the antibody **Table 1**.

Results

Foxa2 and T-antigen are co-expressed in castrate-resistant PCa

Using prostatic tumors derived from castrated TRAMP mice, we conducted immunostaining of T-antigen and AR. While the expression of AR was not detectable in these tumors, T-antigen was found to be present in the relapsed tumors (**Figure 1A**). 12T-10 LADY mice develop NEPCa at about 1 year old. These NEPCa tumors express little to no AR (**Figure 1A**), but T-antigen remains expressed in the NEPCa tumors (**Figure 1A**). To test if Foxa2 is co-expressed in T-antigen positive tumors, we performed immunostaining of Foxa2 on serial sections that were used for T-antigen and AR staining. We found that Foxa2 was positive in the tissues where T-antigen was reactivated (**Figure 1A**). The co-expression of T-antigen with Foxa2 was also confirmed by dual immunofluorescence staining of Foxa2 and T-antigen. As shown in **Figure 1B**, T-antigen was only expressed in the regions where Foxa2 was expressed. In summary, we see a concomitant expression of Foxa2 and T-antigen in castrate-resistant PCa developed in LADY and TRAMP transgenic mice.

Ectopic expression of Foxa2 enables androgen-independent expression of T-antigen in NeoTag1 cells

To test the hypothesis that Foxa2, which is co-expressed with T-antigen in prostatic tumors, drives the expression of T-antigen in the absence of nuclear AR, we ectopically expressed Foxa2 in NeoTag1 cells. NeoTag1 is a prostate epithelial cell line derived from 12T-7 LADY mouse. NeoTag1 cells express endogenous AR but not Foxa2, and their cell proliferation is androgen-dependent. In this study, the Foxa2 gene was stably integrated into NeoTag1 cells. The resulting NeoTag1/Foxa2 and empty vector (EV) control (NeoTag1/EV) cells were cultured with or without androgen (1 nM R1881) for 24 and 48 hours. Quantitative (q) RT-PCR was conducted to assess the expression levels of Agr2 (Foxa2 target gene) and FKBP5 (AR target gene) in NeoTag1 cells. As shown in **Figure 2**, Western

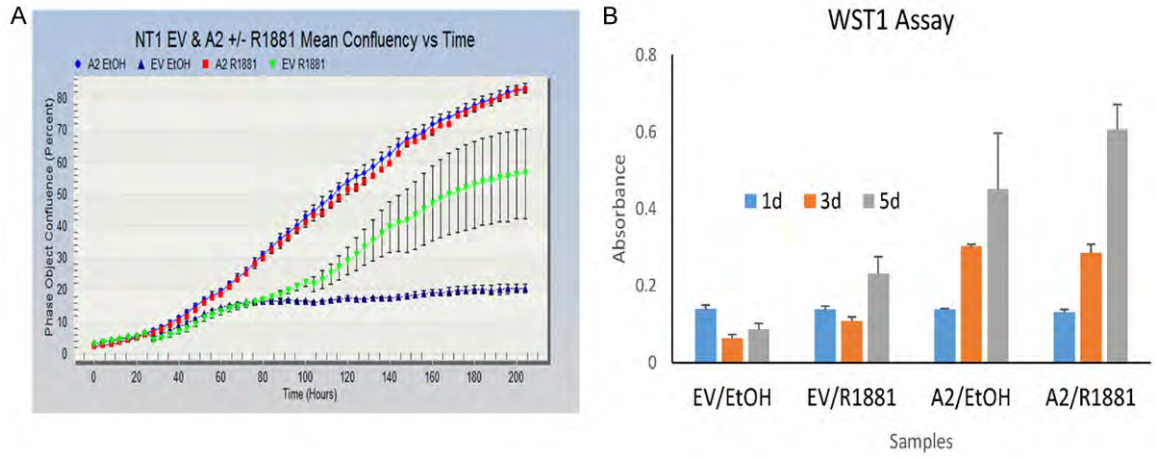


Figure 3. Cell proliferation assays. A. The proliferation of NeoTag1/EV and NeoTag1/Foxa2 was assessed using IncuCyte Zoom method. One thousand cells were seeded in each well of 96-well plate (time 0 hour) in cell culture media containing 10% fetal bovine serum. 24 hours later, the cells were cultured in androgen-depleted media for overnight and then supplemented with ethanol or 1 nM R1881. Pictures were taken every 4 hours. NeoTag1/EV cells display an androgen-dependent cell proliferation pattern, whereas NeoTag1/Foxa2 cells grew faster than empty vector control cells, regardless of the presence of androgens. B. WST1 cell proliferation assays. Cells were seeded and treated similarly as described above. Cells were seeded in media containing 10% fetal bovine serum, switched to media containing 5% charcoal stripped serum on day 1, and then supplemented with ethanol or 1 nM R1881 starting day 2.

blot results indicated that Foxa2 transgene was expressed in NeoTag1/Foxa2 but not in NeoTag1/EV cells. Ectopic Foxa2 induced the expression of Agr2 in NeoTag1 cells (**Figure 2A**), confirming that the Foxa2 transgene is functional. Also, qRT-PCR results indicated a constitutive expression of AR androgen-responsive gene, FKBP5, in NeoTag1/Foxa2 cells. As expected, the expression of FKBP5 was induced by androgen treatment in NeoTag1/EV cells, but the level of FKBP5 mRNA was higher in Neotag1/Foxa2 than NeoTag1/EV cells, even in the absence of androgens (**Figure 2A**). Western blot results indicated a similar constitutive expression of T-antigen in NeoTag1/Foxa2 cells. Androgen treatment activated the expression of T-antigen in Neotag1/EV cells, but T-antigen was constitutively expressed in Neotag1/Foxa2 cells, regardless of the presence of androgens (**Figure 2B**). Surprisingly, the total AR protein level was lower in NeoTag1 cells that ectopically express Foxa2 compared with NeoTag1/EV cells.

Ectopic expression of Foxa2 confers androgen-independent cell proliferation of NeoTag1 cells

We also examined if NeoTag1/Foxa2 cells proliferate constitutively. Two types of methods were used to assess cell proliferation, Incucyte zoom method, and WST1 cell proliferation assays. The Incucyte zoom uses live cell imaging and generates a cell confluency map. After the cells were seeded for one day, NeoTag1/Foxa2 and control NeoTag1/EV cells were cultured in androgen-depleted media and cell confluency reading began. All experiment groups started at the same confluency, however, the Foxa2 cells grew at a much faster rate than empty vector control cells over the course of the experiment, regardless of the presence of androgens (**Figure 3A**). Consistent with the previous report, androgen treatment stimulated cell proliferation of Neotag1/EV cells. But NeoTag1/EV cells grew at a rate slower than Foxa2-expressing cells, regardless of androgens' presence. NeoTag1/Foxa2 cells displayed almost identical cell proliferation pattern in the presence or absence of androgens, indicating that they do not dependent on androgens for cell proliferation anymore. Also, we conducted WST-1 assays (**Figure 3B**). Similar to what is seen with the Incucyte experiments, cell proliferation remains faster when Foxa2 was overexpressed.

Nuclear AR level is low in NeoTag1/Foxa2 cells

Numerous studies have shown that AR is still the central player in activating androgen

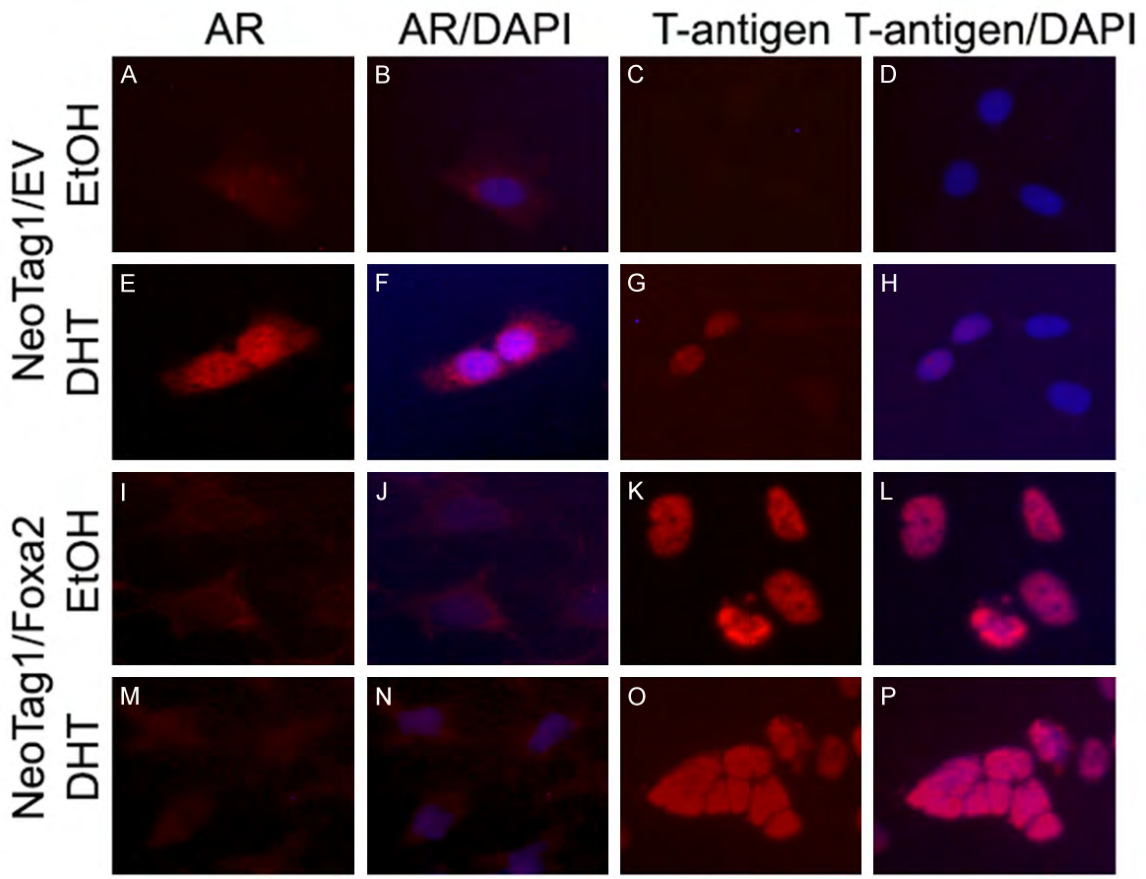


Figure 4. Decreased nuclear androgen receptor in NeoTag1/Foxa2 cells. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and then supplemented with ethanol or 1 nM R1881 for 2 hours. In the NeoTag1/EV cells, 2-hour androgen treatment provoked androgen receptor nuclear localization and T-antigen expression in a subset of cells. In the NeoTag1/Foxa2 expression, very low levels of androgen receptor protein were present, however T-antigen expression remained high regardless of androgen stimulation.

signaling in castrate-resistant PCa. However, our data indicated that AR protein level was decreased in NeoTag1/Foxa2 cells (**Figure 2A**). Also, qRT-PCR results revealed that the mRNA levels of AR decreased (**Figure 4A**). Furthermore, we conducted immunofluorescence staining and found that 2-hour androgen treatment induced AR nuclear translocation in Neotag1/EV cells, but AR level was lower in Neotag1/Foxa2 cells, and no nuclear translocation of AR protein was detected in Neotag1/Foxa2 cells. Concomitant with androgen-induced AR's nuclear translocation in NeoTag1/EV cells, T-antigen expression was detected in a subset of Neotag1/EV cells after 2-hour androgen treatment (full induction of T-antigen expression was only detected in NeoTag1/EV cells after 6-hour androgen treatment, data not shown). However, T-antigen was found to be constitutively expressed in the nuclei of NeoTag1/Foxa2 cells, regardless of androgen's presence. Together, these data support that AR is not the mechanism to sustain the constitutive expression of T-antigen in Neotag1/Foxa2 cells.

AR-responsive promoters are active in NeoTag1/Foxa2 cells after androgen deprivation

The constitutive expression of T-antigen, which can account for the androgen-independent proliferation of Neotag1/Foxa2 cells, suggests that the promoter that drives the expression of T-antigen transgene is active in these cells. To determine Foxa2's involvement in the direct regulation of androgen-responsive promoters and if Foxa2 alters AR's occupancy on these promoters, chromatin immunoprecipitation (ChIP) was conducted using Neotag1/Foxa2

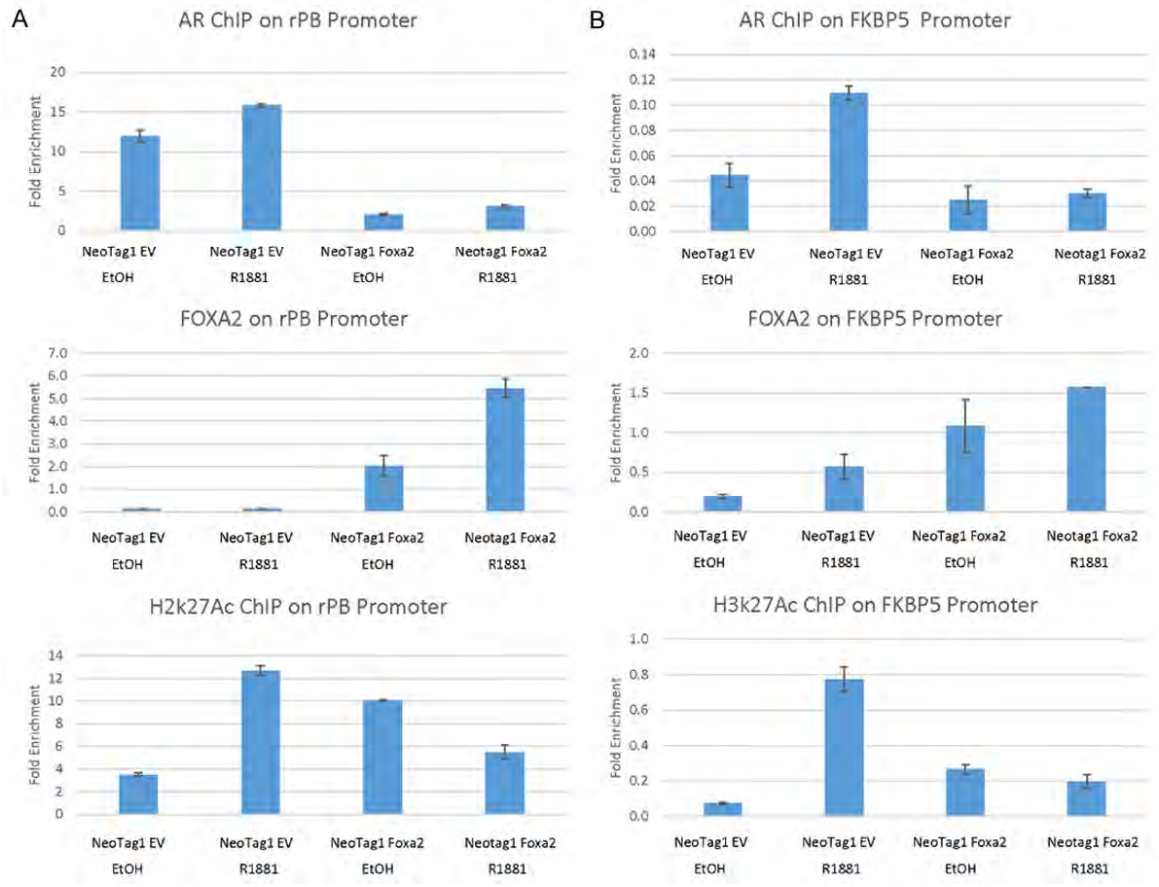


Figure 5. Foxa2 retains active chromatin state in the absence of androgens. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and then supplemented with ethanol or 1 nM R1881 for two hours. Cells were then fixed and Chromatin Immunopreciptation was conducted. A. Probsasin promoter analysis. B. FKBP5 promoter analysis. In NeoTag1/Foxa2 cells, AR's occupancy on both promoters diminished, but Foxa2 and H3k27Ac were recruited to the promoters.

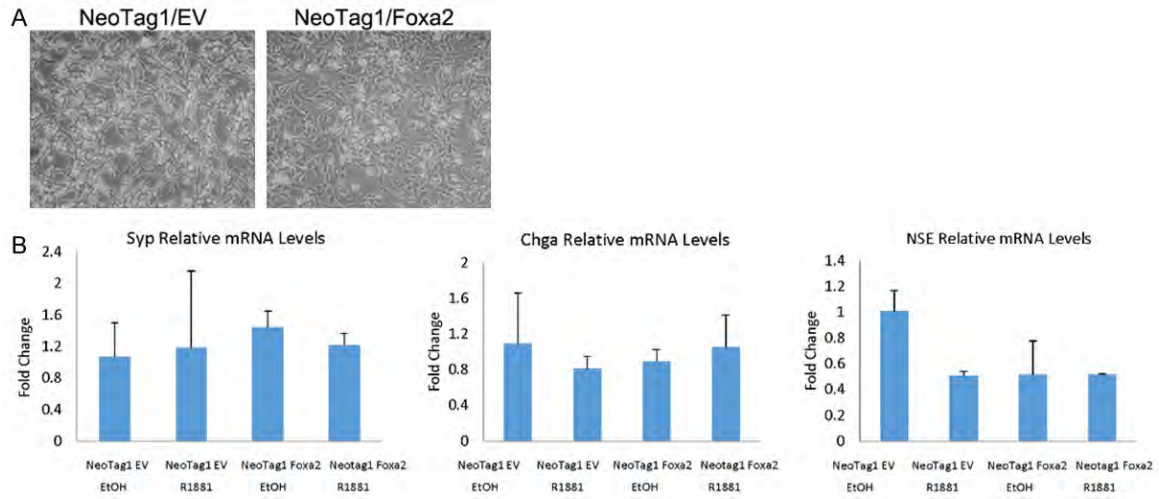


Figure 6. Ectopic expression of Foxa2 failed to induce NE differentiation. A. cell morphology of Neotag1/EV and NeoTag1/Foxa2 cells. B. qRT-PCR. The mRNA levels of NEPCa markers including synaptophysin (Syp), chromogranin A (Chga), and NSE were assessed by qRT-PCR. Over-expression of Foxa2 did not cause NE differentiation in Neotag1 cells.

and empty vector control cells. As shown in **Figure 5**, histone 3 lysine 27 triacetylation (H3K27Ac3, a mark of active transcription) was enriched on androgen-responsive promoters, including transgenic rat Probasin promoter and endogenous FKBP5 promoter in Neotag1/EV cells treated with androgens as well as in Neotag1/Foxa2 cells with or without androgen treatment. It is interesting to note that when Foxa2 was overexpressed and no androgens were present, H3K27Ac3's enrichment at AR-responsive promoters remained elevated. AR was recruited to transgenic Probasin promoter and FKBP5 promoter in Neotag1/EV cells when they were treated with androgens. However, AR's occupancy on these promoters was lower in Neotag1/Foxa2 cells, no matter these cells were treated with androgens or not. Consistent with the differential protein levels, Foxa2's occupancy on Probasin and FKBP5 promoters was detected in NeoTag1/Foxa2 cells but not in Neotag1/EV cells. Foxa2's enrichment to androgen-responsive promoters in NeoTag1/Foxa2 cells suggests that Foxa2 directly regulates these promoters.

Ectopic expression of Foxa2 fails to induce NE differentiation of NeoTag1 cells

Neuroendocrine PCa cells express little to no AR and reduced AR signaling promotes NE differentiation [14]. Given the decreased transcript levels and protein expression of AR in NeoTag1/Foxa2 cells, and the association of Foxa2 with neuroendocrine phenotype [10], we hypothesized NeoTag1/Foxa2 cells acquire some NE phenotype and examined if ectopic expression of Foxa2 induced NE differentiation of Neotag1 cells. As shown in **Figure 6**, we did not observe any morphological changes supporting that NE phenotype is emerging. We also assessed the mRNA levels of known NEPCa markers, Synaptophysin, Chromogranin A, and NSE (neuron specific enolase), but did not observe any increases in the expression of these genes. Taken together, these data indicate that Foxa2, although its expression is associated with the NE phenotype of PCa, is not sufficient to drive the development and/or promote the NE differentiation by itself.

Discussion

In LADY and TRAMP mice, the expression of T-antigen is driven by the androgen-responsive Probasin promoter [6, 7]. Using prostatic tissues derived from these transgenic mice, we found that the re-expression T-antigen is concomitant with the expression of Foxa2. Using NeoTag1 cells, which are derived from prostate of probasin promoter driven SV40 T-antigen transgenic mouse, we found that Foxa2 confers androgen-independent expression of T-antigen. These data indicate that the expression of Foxa2 provides a mechanism to activate AR-responsive promoters and induce the re-expression of T-antigen in castrate-resistant prostatic tumors in mice.

While Foxa2 activates the transcription/expression of a subset of AR target genes in mouse tumors, whether Foxa2 exerts a similar effect in human PCa remains unclear. In human PCa, the expression of Foxa2 is associated with NEPCa [10]. A recent study has shown that Foxa2 is present in approximately 75% of NEPCa patients [15]. While in pure NEPCa (also called small-cell carcinomas, occurring in less than 5% PCa patients), AR level is low to none, NEPCa often presents as mixed prostatic adenocarcinomas with NE features, where AR expression in the various stages of PCa is yet clearly determined. Foxa2's modulation on AR signaling may vary at different stages during NEPCa progression. Our data indicate that Foxa2 expression downregulates AR protein (**Figure 2B**), suggesting that the emergence of Foxa2 can be a mechanism for the loss of AR expression. However, in the Foxa2-expressing cells, AR signaling still retains, even though AR protein level is low. This may reflect a transition stage in PCa when AR expression gets gradually lost and PCa cells transit from adenocarcinoma to neuroendocrine cancer.

AR-responsive promoters remain active in the Foxa2-expressing cells (**Figure 5**); however, AR is not the likely driver that activates this response since AR level is low, and AR doesn't seem to be recruited to the AR-responsive Probasin or FKBP5 promoters in the Foxa2 expressing cells. This supports the argument that Foxa2 recruitment to the promoter regions is in parallel with the active transcription of the promoters. It remains to be determined if Foxa2 is the sole driver to activate transcription of these AR-responsive promoters.

Foxa2-expressing NeoTag1 cells continue proliferating, even when they are cultured in androgen-depleted media. However, this does not necessarily mean Foxa2 drives the rapid proliferation of these cells in the absence of androgens. The Foxa2-mediated T-antigen expres-

sion could be the cause of such proliferation rates observed. Future studies on mice where Foxa2 is present and T-antigen is absent and in human cells where Foxa2 is present are required to conclude if Foxa2 has any substantial role in sustaining the cell proliferation in CRPCa.

Similar to the undefined role of Foxa2 in controlling cell proliferation, it remains unclear whether Foxa2 exerts comparable transactivation function in regulating the expression of AR target genes in human PCa tissues. It is possible that Foxa2's effect on AR signaling is model-specific. Additional research using human PCa cells will be needed to delineate whether Foxa2's effect on AR target genes is a common phenomenon or it applies only to cells that carry T-antigen transgene. Regardless, here, we present the first study using a PCa mouse model to reveal Foxa2's regulation of androgen-responsive promoters in the absence of androgen, ultimately retaining cell survival.

Acknowledgements

We thank Dr. Jennifer Carroll for the help with the IncuCyte cell proliferation assays, Dr. Wayne Orr for the use of his Nikon microscope. This research is supported by DOD grant (W81XWH-12-1-0212), NIH grants (R03 CA-212567 and R01 CA226285), LSUHSC start-ups (FWCC and Office of Research) to XY, and Carroll Feist Pre-doctoral Fellowship to ZC.

Disclosure of conflict of interest

None.

Address correspondence to: Xiuping Yu, Department of Biochemistry and Molecular Biology, LSU Health Sciences Center, Shreveport, Louisiana 71103, USA. E-mail: xyu@lsuhsc.edu

References

[1] Beltran H, Tomlins S, Aparicio A, Arora V, Rickman D, Ayala G, Huang J, True L, Gleave ME, Soule H, Logothetis C, Rubin MA. Aggressive variants of castration-resistant prostate cancer. Clin Cancer Res 2014; 20: 2846-50.

[2] Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. N Engl J Med 2004; 351: 1488-90.

[3] Dehm SM, Tindall DJ. Alternatively spliced androgen receptor variants. Endocr Relat Cancer 2011; 18: R183-96.

[4] Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 2009; 324: 787-90.

[5] Reid AH, Attard G, Barrie E, de Bono JS. CYP17 inhibition as a hormonal strategy for prostate cancer. Nat Clin Pract Urol 2008; 5: 610-20.

[6] Masumori N, Thomas TZ, Chaurand P, Case T, Paul M, Kasper S, Caprioli RM, Tsukamoto T, Shappell SB, Matusik RJ. A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential. Cancer Res 2001; 61: 2239-49.

[7] Greenberg NM, DeMayo FJ, Sheppard PC, Barrios R, Lebovitz R, Finegold M, Angelopoulou R, Dodd JG, Duckworth ML, Rosen JM, et al. The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Mol Endocrinol 1994; 8: 230-9.

[8] Zaret KS, Carroll JS. Pioneer transcription factors: establishing competence for gene expression. Genes Dev 2011; 25: 2227-41.

[9] Mirosevich J, Gao N, Matusik RJ. Expression of Foxa transcription factors in the developing and adult murine prostate. Prostate 2005; 62: 339-52.

[10] Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R, Matusik RJ. Expression and role of Foxa proteins in prostate cancer. Prostate 2006; 66: 1013-28.

[11] Yu X, Gupta A, Wang Y, Suzuki K, Mirosevich J, Orgebin-Crist MC, Matusik RJ. Foxa1 and Foxa2 interact with the androgen receptor to regulate prostate and epididymal genes differentially. Ann N Y Acad Sci 2005; 1061: 77-93.

[12] Yu X, Wang Y, Jiang M, Bierie B, Roy-Burman P, Shen MM, Taketo MM, Wills M, Matusik RJ. Activation of beta-catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. Prostate 2009; 69: 249-62.

[13] Yu X, Cates JM, Morrissey C, You C, Grabowska MM, Zhang J, DeGraff DJ, Strand DW, Franco OE, Lin-Tsai O, Hayward SW, Matusik RJ. SOX2 expression in the developing, adult, as well as, diseased prostate. Prostate Cancer Prostatic Dis 2015; 17: 301-9.

[14] Wright ME, Tsai MJ, Aebersold R. Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Mol Endocrinol 2003; 17: 1726-37.

[15] Park JW, Lee JK, Witte ON, Huang J. FOXA2 is a sensitive and specific marker for small cell neuroendocrine carcinoma of the prostate. Mod Pathol 2017; 30: 1262-1272.

Prostate Cancer and Prostatic Disease (2014), 1–9

www.nature.com/pcan

ORIGINAL ARTICLE

SOX2 expression in the developing, adult, as well as, diseased prostate

X Yu[1], JM Cates[2], C Morrissey[3], C You[1], MM Grabowska[1], J Zhang[1], DJ DeGraff[1], DW Strand[1], OE Franco[1], O Lin-Tsai[1], SW Hayward[1] and RJ Matusik[1]

BACKGROUND: SOX2 is a member of SOX (SRY-related high mobility group box) family of transcription factors.
METHODS: In this study, we examined the expression of SOX2 in murine and human prostatic specimens by immunohistochemistry.
RESULTS: We found that SOX2 was expressed in murine prostates during budding morphogenesis and in neuroendocrine (NE) prostate cancer (PCa) murine models. Expression of SOX2 was also examined in human prostatic tissue. We found that SOX2 was expressed in 26 of the 30 BPH specimens. In these BPH samples, expression of SOX2 was limited to basal epithelial cells. In contrast, 24 of the 25 primary PCa specimens were negative for SOX2. The only positive primary PCa was the prostatic NE tumor, which also showed co-expression of synaptophysin. Additionally, the expression of SOX2 was detected in all prostatic NE tumor xenograft lines. Furthermore, we have examined the expression of SOX2 on a set of tissue microarrays consisting of metastatic PCa tissues. Expression of SOX2 was detected in at least one metastatic site in 15 of the 24 patients with metastatic castration-resistant PCa; and the expression of SOX2 was correlated with synaptophysin.
CONCLUSIONS: SOX2 was expressed in developing prostates, basal cells of BPH, as well as prostatic NE tumors.

Prostate Cancer and Prostatic Disease advance online publication, 5 August 2014; doi:10.1038/pcan.2014.29

INTRODUCTION

The SRY-related high mobility group box (SOX) family of transcription factors is instrumental for diverse developmental processes and in determining cell fate.[1,2] In particular, SOX2 seems to be essential for sustaining pluripotency and neural induction.[2] In combination with Oct4, KLF4 and c-Myc, SOX2 can successfully reprogram somatic cells to become induced pluripotent stem cells,[3] suggesting an important role for SOX2 in controlling the pluripotency of stem/progenitor cells. SOX2 has also been implicated in branching morphogenesis and epithelial cell differentiation during early lung development, as aberrant expression of SOX2 disrupts normal lung branching morphogenesis, resulting in reduced number of airways.[4,5] Mice overexpressing SOX2 exhibit an increased number of basal cells (marked by the expression of p63) and neuroendocrine (NE) cells in the respiratory mucosa,[4] and lung carcinomas are developed in about half of these mice, indicating that SOX2 overexpression is oncogenic.[6]

Expression of SOX2 has been reported in carcinomas arising in several organs, including prostate.[7–10] A recent study shows that SOX2 is expressed in both benign and malignant prostate tissue, but only in a small subpopulation of cells ($< 10\%$).[7] SOX2 is also detected in castration-resistant prostate cancer (PCa) (CRPC) metastasis samples.[10] Several studies have highlighted the functional implication of SOX2 in tumor progression, as a reduction of SOX2 levels has been shown to decrease proliferation and invasion while concomitantly increasing differentiation of cancer cells.[11–15] In PCa, silencing SOX2 by short hairpin RNA decreases both cell proliferation and the invasive capacity of PCa cells,[8,16] and ectopic expression of SOX2 promotes PCa cell growth.[9,10,16]

SOX2 expression is associated with NE tumors. In the skin, SOX2 is expressed in cutaneous NE carcinoma (Merkel cell carcinoma) in addition to a subset of melanomas.[17] SOX2 is also detected in NE carcinomas of the lung,[18] consistent with the observation that SOX2 overexpressing mice have an expansion of NE cells in the respiratory mucosa and develop lung carcinomas. However, the expression of SOX2 in NE PCa is yet to be examined.

NE PCa, or small cell carcinoma of the prostate, is a rare and highly aggressive subtype of PCa. Because it is androgen receptor (AR) negative, NE PCa is naturally androgen independent. Although the vast majority of primary human PCa are adenocarcinomas, immunohistochemical evidence of NE differentiation (NED) can be often found in human PCa, and PCa exhibiting NED is associated with poor prognosis.[19,20] Rapid autopsy of hormone-refractory metastatic PCa has shown NED in most cases,[21] and patients who fail androgen-deprivation therapy can develop NE cancer and adenocarcinoma with NED.[22] However, although NE phenotype is associated with advanced stage PCa, NE and NED in prostate are understudied owing to the scarcity of NE PCa specimens as advanced PCa is usually not biopsied.

A major interest of our laboratory has been NE tumors, largely due to the fact that numerous mouse PCa models readily develop or progress to NE tumors.[23] Our focus has been identifying potential transcription factors that promote the NE phenotype. Because SOX2 can drive NE cell hyperplasia and carcinoma in the murine lung, we postulated that SOX2 may also have a similar role in the prostate. In order to begin to elucidate the relationship between SOX2 and the NE phenotype, we undertook a careful analysis of murine and human prostate to determine the expression pattern of SOX2 and whether SOX2 is co-expressed with NE markers. Specifically, we examined the expression of SOX2 during murine prostate development, in diseased prostate (BPH,

[1]Department of Urologic Surgery, Vanderbilt University Medical Center, Nashville, TN, USA; [2]Department of Pathology, Vanderbilt University Medical Center, Nashville, TN, USA and [3]Department of Urology, University of Washington, Seattle, WA, USA. Correspondence: Dr X Yu or Dr RJ Matusik, Department of Urologic Surgery, Vanderbilt University Medical Center, Nashville, TN 37232, USA.
E-mail: xiuping.yu@vanderbilt.edu or robert.matusik@vanderbilt.edu
Received 2 April 2014; revised 30 May 2014; accepted 27 June 2014

primary prostate adenocarcinoma and NE PCa) and in metastatic PCa. We also studied the expression of SOX2 in prostate NE tumor murine models and analyzed the association of SOX2 expression with the NE phenotype in metastatic human PCa. We found that SOX2 was expressed in embryonic prostates, BPH and NE PCa.

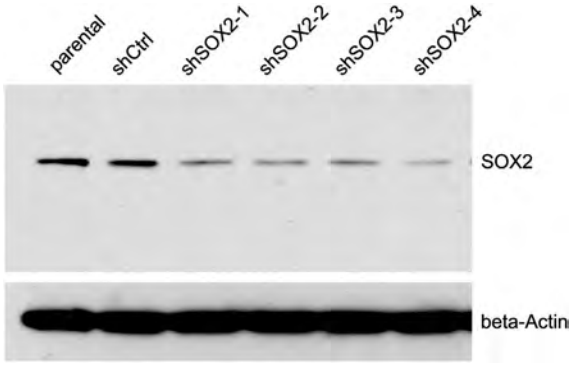


Figure 1. Western blotting analysis. The level of SOX2 was knocked down in PC3 cells and protein lysate was prepared from parental PC3 cells and PC3 cells that have stably integrated with control short hairpin RNA or with variant shSOX2 construct. Beta-actin was used as a loading control.

MATERIALS AND METHODS

Mouse lines

All mice used in this study were housed and handled in accordance with the standard protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Seven-to-eight-week-old CD-1 mice (Harlan, Indiana, IN, USA) were used for timed mating. The day a vaginal copulation plug was observed was designated embryonic day 1. The 12T7,[24] 12T10,[25] and TRAMP[26] are transgenic mouse lines that develop prostatic intraepithelial neoplasia (PIN) or NE PCa. NE10[27] is a prostatic NE tumor xenograft line.

Human samples

Primary prostate specimens were obtained from the Department of Pathology, Vanderbilt University Medical Center, Nashville, TN, USA with approval from the Institutional Review Board. The UWTMA22 and UWTMA46 are tissue microarrays constructed at the University of Washington, Seattle, WA, USA. UWTMA22 consists of metastatic CRPC tissues from 24 patients; UWTMA46 consists of 24 LuCaP xenograft tumors derived from 19 patients, 4 of which are prostatic NE tumors derived from 3 patients.

Validation of SOX2 antibody

The specificity of SOX2 antibody was validated by western blotting using cell lysate collected from control PC3 cells and PC3/shSOX2 cells (Figure 1). The shSOX2 constructs were purchased from Origene (Rockville, MD, USA). For retroviral infection, the shSOX2 or control retroviral vector plasmids were transfected into Phoenix packaging cells. The cell culture media were

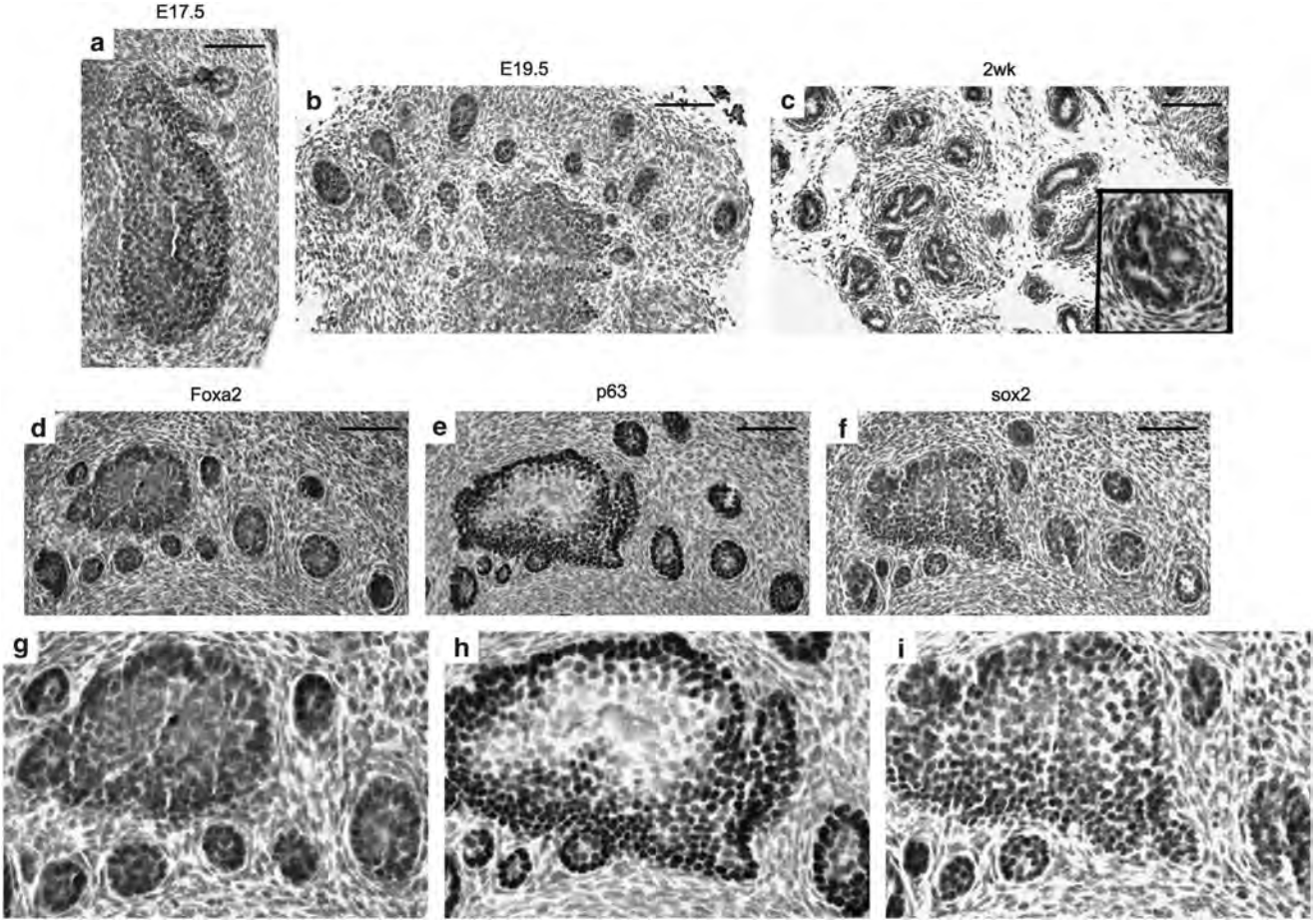


Figure 2. SOX2 expression during murine embryonic prostate development. Nuclear SOX2 staining was detected in the prostatic buds at E17.5 and E19.5 (**a**, **b**). At 2 weeks after birth, the nuclear staining of SOX2 was hardly detected in prostate (**c**, inset is a higher magnification picture). The prostatic buds co-expressed Foxa2 (**d**), p63 (**e**) and SOX2 (**f**). Panels **g**, **h** and **i** are higher magnification picture of panels **d**, **e** and **f**, respectively. Scale bars represent 25 μm.

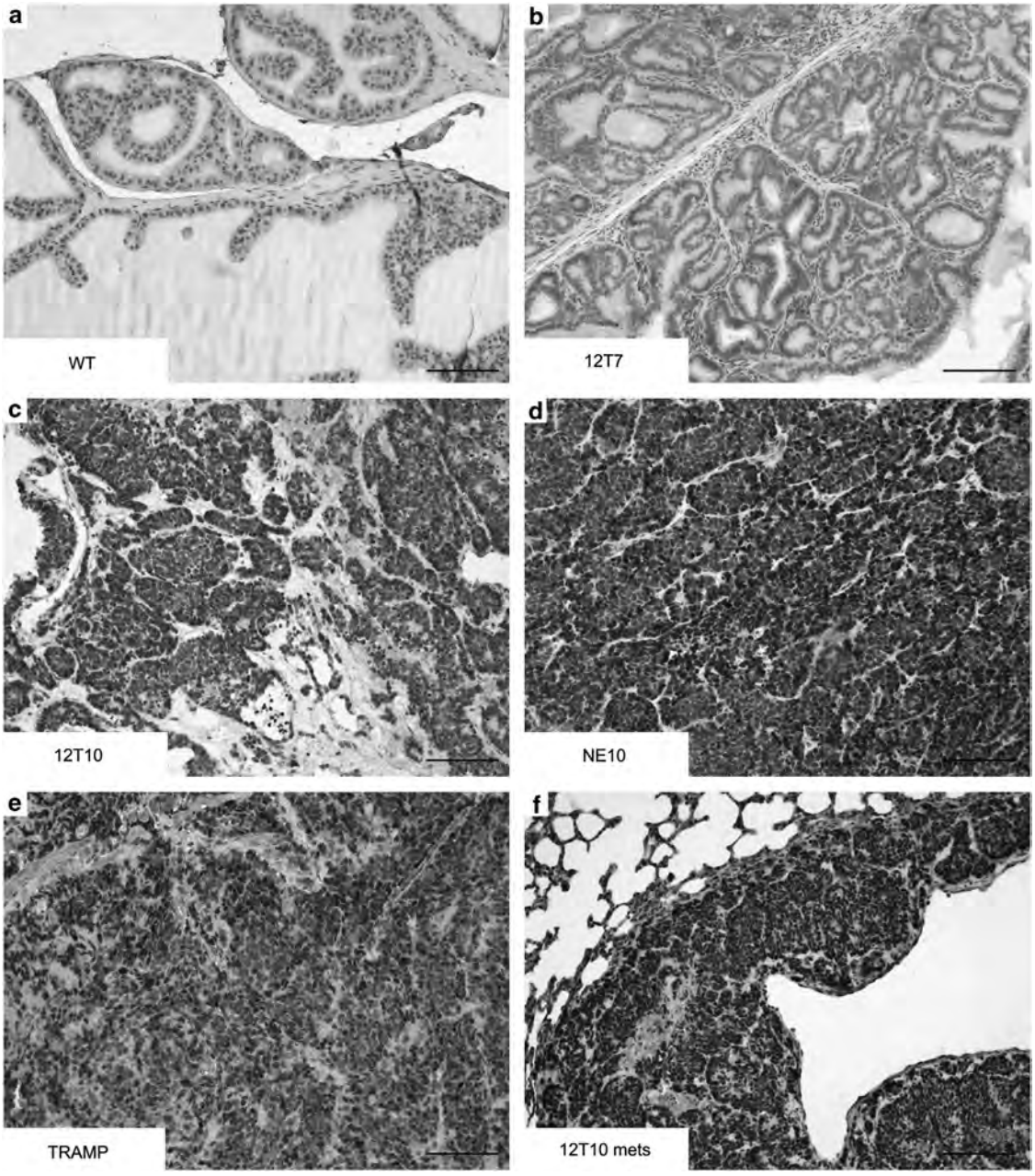


Figure 3. SOX2 expression in neuroendocrine (NE) prostate cancer (PCa) murine models. Immunohistochemical staining was performed on prostate sections derived from wild type (WT, **a**), 12T7 (**b**), 12T10 (**c**), NE10 (**d**) and TRAMP (**e**) mice or on NE PCa lung metastasis in 12T10 mouse (**f**). Strong nuclear SOX2 staining was detected in prostate NE tumors (12T10, NE10, TRAMP and NE PCa metastasis) but not in WT or 12T7 mouse prostates. Scale bars represent 50 μm.

collected 24 h later and used for infecting PC3 cells. The infection procedure was repeated twice, and 10 μg ml^{-1} puromycin was used for selection of PC3 cells that have control or shSOX2 construct stably integrated.

Immunohistochemistry (IHC) and immunofluorescence staining

IHC was conducted as described previously.[28] Prostates were fixed in 10% buffered formalin overnight and processed to paraffin. IHC stains were preformed following routine deparaffinization and rehydration of 5-μm sections. Antigen retrieval was performed by microwaving slides for 20 min in boiling antigen-unmasking solution (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase activity was blocked with DAKO Peroxidase Blocking Reagent (DAKO, Carpinteria, CA, USA) for 15 min. Sections were incubated with primary antibodies overnight at 4 °C in a humidified chamber. Antibodies used were: Foxa2 and p63 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), synaptophysin from BD Transduction Laboratories (San Jose, CA, USA), and SOX2 from Abcam (Cambridge, MA, USA) or Cell Signaling (Danvers, MA, USA). Specific antibody binding was detected using the Vectastain Elite ABC peroxidase kit (Vector Laboratories) according to the manufacturer's protocol with the DAKO DAB-Chromogen System (DAKO). Sections were counterstained with hematoxylin, dehydrated and cover-slipped. For immunofluorescence staining, tissue sections were blocked with phosphate-buffered saline containing 5% normal donkey serum (Vector Laboratories) for 1 h and incubated overnight at 4 °C with primary antibodies. After washing in phosphate-buffered saline, sections were incubated with fluorescence-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for

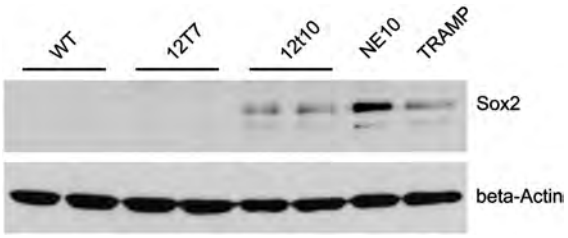


Figure 4. Western blotting analysis. Protein samples were prepared from prostates derived from wild type (WT), 12T7, 12T10, NE10 and TRAMP mice. The expression of SOX2 was detected in 12T10, NE10 and TRAMP prostate neuroendocrine (NE) tumors but not in prostatic intraepithelial neoplasia (12T7) or WT prostates. NE10 tumor exhibits the highest expression level of SOX2.

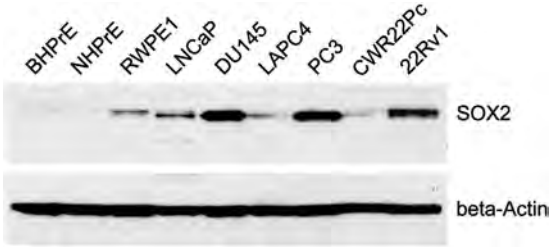


Figure 5. SOX2 expression in established prostate cell lines. Protein samples were prepared from various prostatic cell lines. DU145 and PC3 exhibited the strongest expression of SOX2. Moderate level of SOX2 was observed in 22Rv1 cells. RWPE1, LNCaP, LAPC4 or CWR22Pc cells exhibited low SOX2 expression. The expression of SOX2 was hardly detected in NHPrE and BHPrE cells.

1 h (diluted 1:200 in blocking buffer). Sections were washed in phosphate-buffered saline and cover-slipped using mounting solution with DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories).

Western blotting

Protein lysates were prepared from prostate specimens as described previously.[29] Twenty micrograms of total protein was loaded for electrophoresis. After transfer, membranes were blocked in 5% milk for 1 h, incubated overnight at 4 °C with primary antibodies and incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA, USA) for 1 h. ECL-Plus detection system (PerkinElmer, Waltham, MA, USA) was used to visualize the reaction. Rabbit anti-SOX2 antibodies were from Abcam or Cell Signaling, and β-actin was from Sigma (St Louis, MO, USA).

Statistical analysis of IHC data

SAS 9.3 (SAS Institute, Cary, NC, USA) was used for statistical analysis. The percentage of specific cell types in primary BPH and PCa specimens showing nuclear staining for SOX2 was recorded. Expression of SOX2 and synaptophysin in metastatic CRPC specimens were evaluated for both the percentage of cells stained and intensity of nuclear (SOX2) or cytoplasmic (synaptophysin) staining. Intensity values were categorized as 0 negative, 1+ weak, 2+ moderate, and 3+ strong. The total score was calculated from the intensity value and percentage of staining (intensity × percentage). Associations between SOX2 and synaptophysin expression was examined using Spearman's rank test. All tests with a *P*-value of < 0.05 were considered statistically significant.

RESULTS

SOX2 is expressed during murine embryonic prostate development

Prostate budding morphogenesis occurs around E17 to E18 (time varies among different mouse strains) when solid epithelial cords grow into the surrounding urogenital sinus mesenchyme to form the rudimentary prostate buds. SOX2 expression was detected during prostate budding morphogenesis (Figure 2a and b). Nuclear SOX2 staining was observed in the urogenital sinus epithelial cells, with stronger nuclear staining in cells at the epithelial–mesenchymal interface. Some stromal cells show weak staining for SOX2. The expression pattern of SOX2 in the budding prostate is similar to that of Foxa2 and p63 (Figure 2d–i).[30] At 2 weeks after birth (Figure 2c) and in adults (Figure 3a), nuclear SOX2 staining is essentially undetectable in luminal prostatic epithelial cells. Because of background cytoplasmic staining (Figure 2c and 3a), it is not clear whether SOX2 is expressed in mouse prostate basal epithelial cells at these stages.

Table 1. Human prostate specimens used in this study

Cohort	*Histology*	*Patients*	*SOX2+*	*SOX2 –*
BPH	BPH	30	26	4
Primary PCa	Adenocarcinoma	24	2[a]	22
	Neuroendocrine	1	1	0
UWTMA22	Synaptophysin+	10	8	2
(metastasis)	Synaptophysin –	14	7	7
UWTMA46 (LuCaP)	Adenocarcinoma	16	2	14
	Neuroendocrine	3	3	0

Abbreviation: PCa, prostate cancer. [a]SOX2 was only detected in benign areas.

SOX2 is expressed in NE PCa murine models

In the adult, wild-type prostate luminal epithelial cells were negative for SOX2 (Figure 3a). Similarly, nuclear SOX2 staining was not observed in the 12T7 prostate PIN model, although some luminal epithelia cells exhibit weak cytoplasmic staining (Figure 3b), which was considered to be background. In contrast, strong nuclear staining for SOX2 was observed in the 12T10, NE10 and TRAMP NE tumors (Figure 3c–e). Expression of SOX2 was also detected in prostatic NE tumor metastasis (Figure 3f).

Expression of SOX2 in murine NE PCa was confirmed by western blotting. SOX2 expression was detected in 12T10, NE10 and TRAMP tumor lysates but not in prostates derived from wild-type or 12T7 mice (Figure 4). The lack of SOX2 immunoreactivity on western blots of normal or PIN mouse prostates supports the conclusion that the cytoplasmic IHC staining seen in some sections was indeed non-specific background staining.

Expression of SOX2 in human PCa cell lines

SOX2 expression was examined in established benign or cancerous prostatic cell lines by western blotting (Figure 5). SOX2 was strongly expressed in DU145 and PC3 cells, with lower amounts in CWR22Rv1 cells. DU145 and PC3 are AR-negative cell lines and their growth is independent of the presence of androgen, whereas CWR22Rv1 is AR positive but grows as a castrate-resistant cancer cell line.[31] Low SOX2 expression was detected in RWPE1, LNCaP, LAPC4 and CWR22Pc cell lines. Compared with the three cell lines that have high/moderate level of SOX2, these cell lines are generally less aggressive *in vivo* and are androgen responsive.[32] SOX2 was essentially undetectable in NHPrE and BHPrE cells, two newly established human prostate epithelial cell lines derived from normal and benign prostate tissue, respectively.[33] Each cell line will form normal prostate glandular structure when recombined with inductive embryonic urogenital sinus mesenchyme and grafted under the renal capsule.[33]

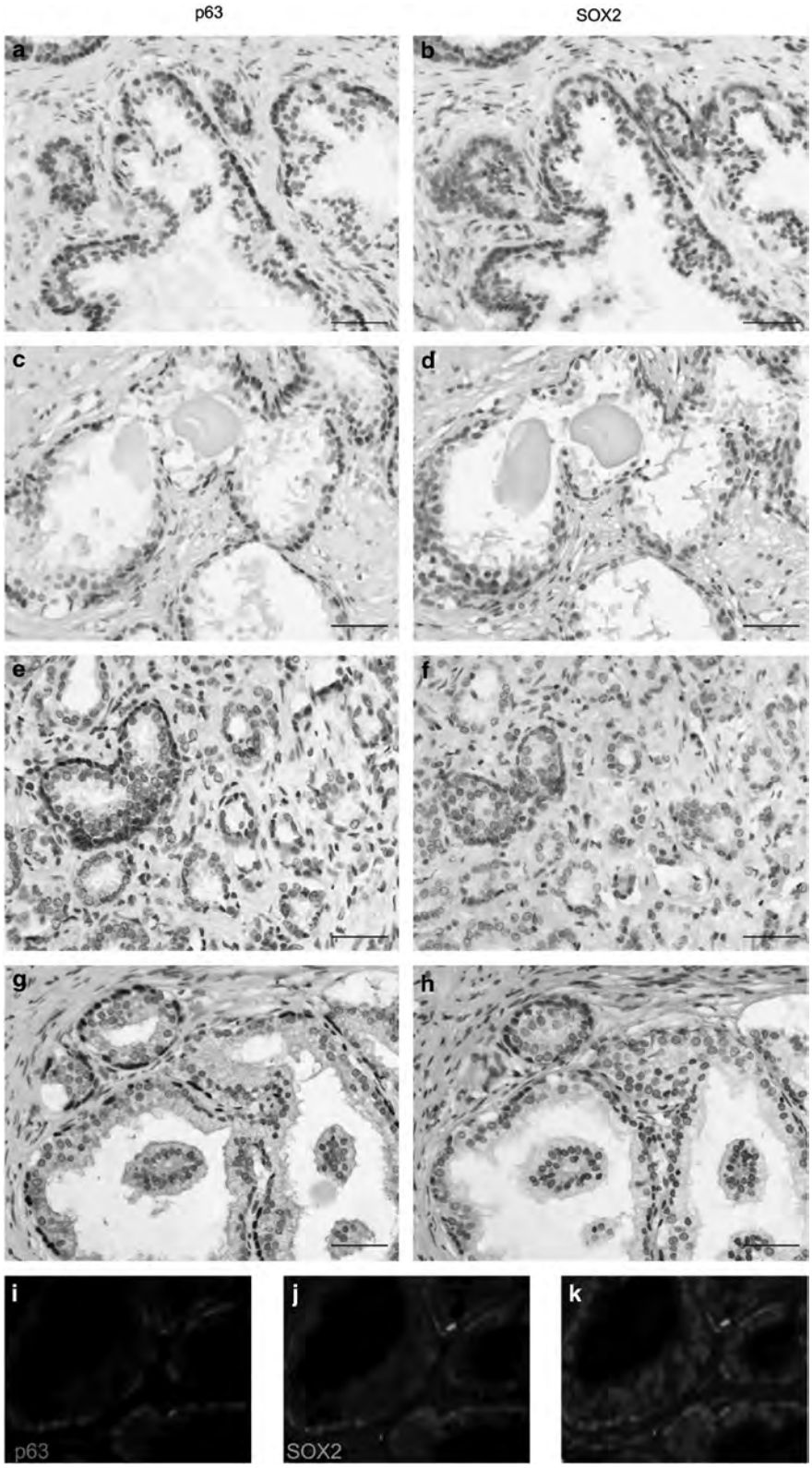


Figure 6. Expression of SOX2 in human BPH and primary prostate adenocarcinoma specimens. Immunohistochemical staining of SOX2 or p63 was performed on serial sections derived from BPH (**a**–**d**) or prostate cancer (PCa) (**e**–**h**) tissues. Panels **a** and **b** represented the 26 of the 30 BPH cases that displayed positive SOX2 staining in basal cell layer; panels **c** and **d** represented the 4 of the 30 BPH cases that showed little or no SOX2 staining but were still positive for p63. Panels **e** and **f** represented the 22 of the 24 primary PCa cases that lost SOX2 expression. Some benign areas in these PCa specimens were positive for p63 but negative for SOX2 staining. Panels **g** and **h** represented cancer-adjacent normal areas in the 2 of the 24 PCa cases where both p63 and SOX2 were expressed. Panels **i**–**k** are images from dual immunofluorescence staining performed on sections derived from BPH specimens. SOX2 (in green) was co-expressed with basal cell marker-p63 (in red). DAPI was used for counterstaining. Scale bars represent 25 μm. A full colour version of this figure is available at the *Prostate Cancer and Prostatic Disease* journal online.

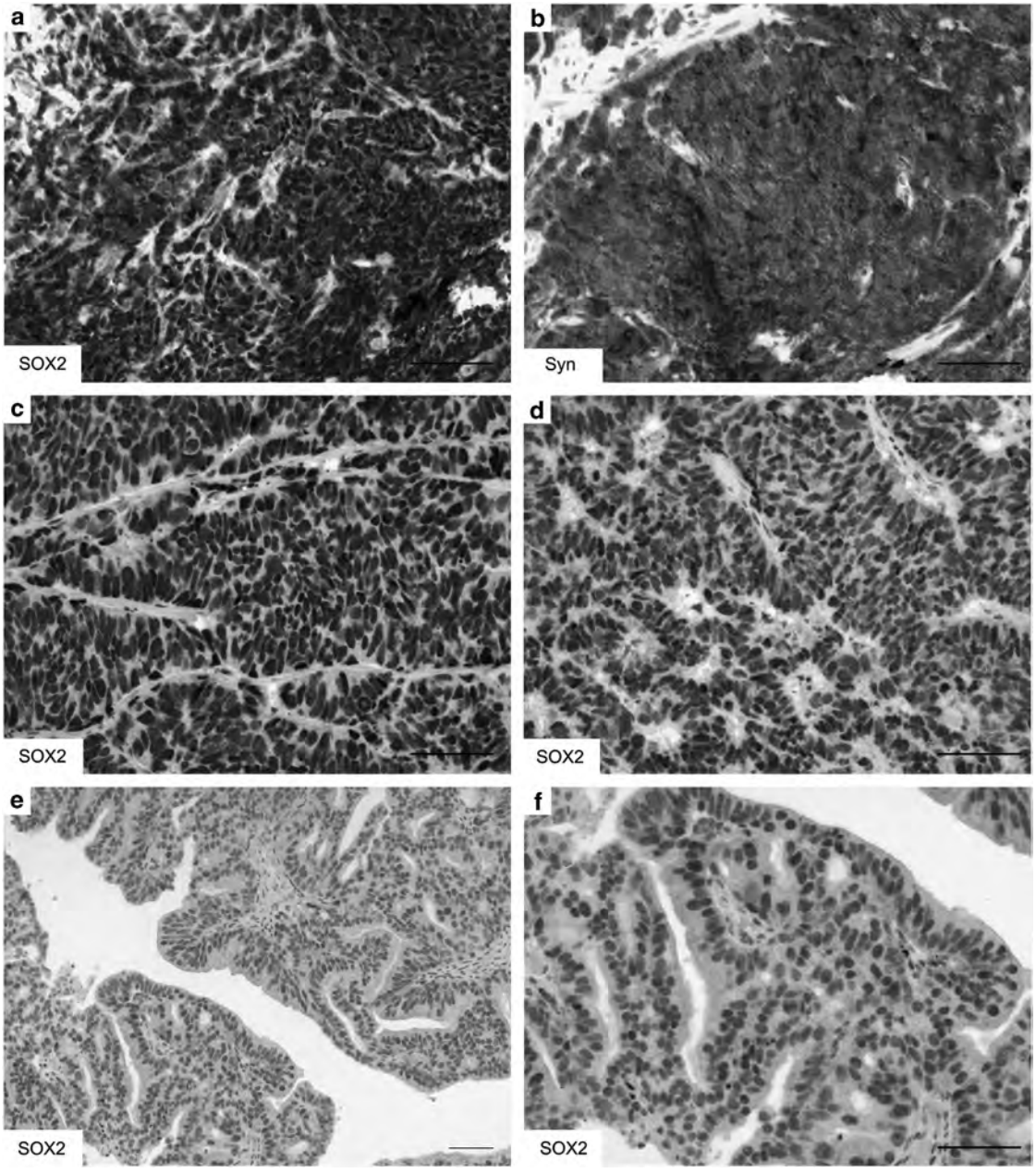


Figure 7. SOX2 expression was detected in human neuroendocrine (NE) prostate cancer (PCa) specimens. (**a**, **b**) Immunohistochemistry staining of SOX2 and NE markers (synaptophysin) was performed on serial sections derived from NE PCa needle biopsies. Both SOX2 and synaptophysin were highly expressed on these sections. The expression of SOX2 was also examined on a set of tissue microarrays consisting of LuCaP xenografts derived from 19 patients. Panels **c** and **d** represented positive SOX2 staining on all the NE PCa cases. Panels **e** and **f** represented the one case that was adenocarcinoma but showed positive SOX2 staining. Panel **f** is a higher magnification picture of panel **e**. Scale bars represent 25 μm.

Expression of SOX2 in benign and malignant human PCa specimens

The expression of SOX2 in benign and malignant human prostatic tissues has been assessed in a recent study where focal expression of SOX2 was detected in 90% of benign tissues, 65% of HGPIN samples and 52% of PCa.[7] Here we examined the expression of SOX2 in BPH, primary prostate adenocarcinoma, prostate NE cancer (small cell carcinoma) and castration-resistant metastatic tumors. All the human specimens examined in this study were listed in Table 1. Most (26 of 30; 87%) BPH specimens showed positive SOX2 staining in prostatic basal cells (Figure 5b), which co-express the basal cell marker p63 (Figure 6a i–k). The other four BPH cases showed little or no SOX2 expression in the basal cell layer despite the presence of p63-positive basal cells (Figure 6c and d). Most (22 of 24; 92%) primary prostate adenocarcinoma specimens were negative for SOX2 staining (Figure 6f). Although some benign prostatic acini or ducts within the tumors still expressed p63 (Figure 6e), they did not exhibit SOX2 immunoreactivity (Figure 6f), suggesting that loss of SOX2 expression is not simply a reflection of an absent basal cell layer. In the two SOX2-positive prostate adenocarcinoma cases, SOX2 expression was only detected in adjacent benign areas but not in cancer areas (Figure 6g and h).

As SOX2 was associated with NE tumor phenotype in the skin[17] and lung[18] and that SOX2 was expressed in murine models of

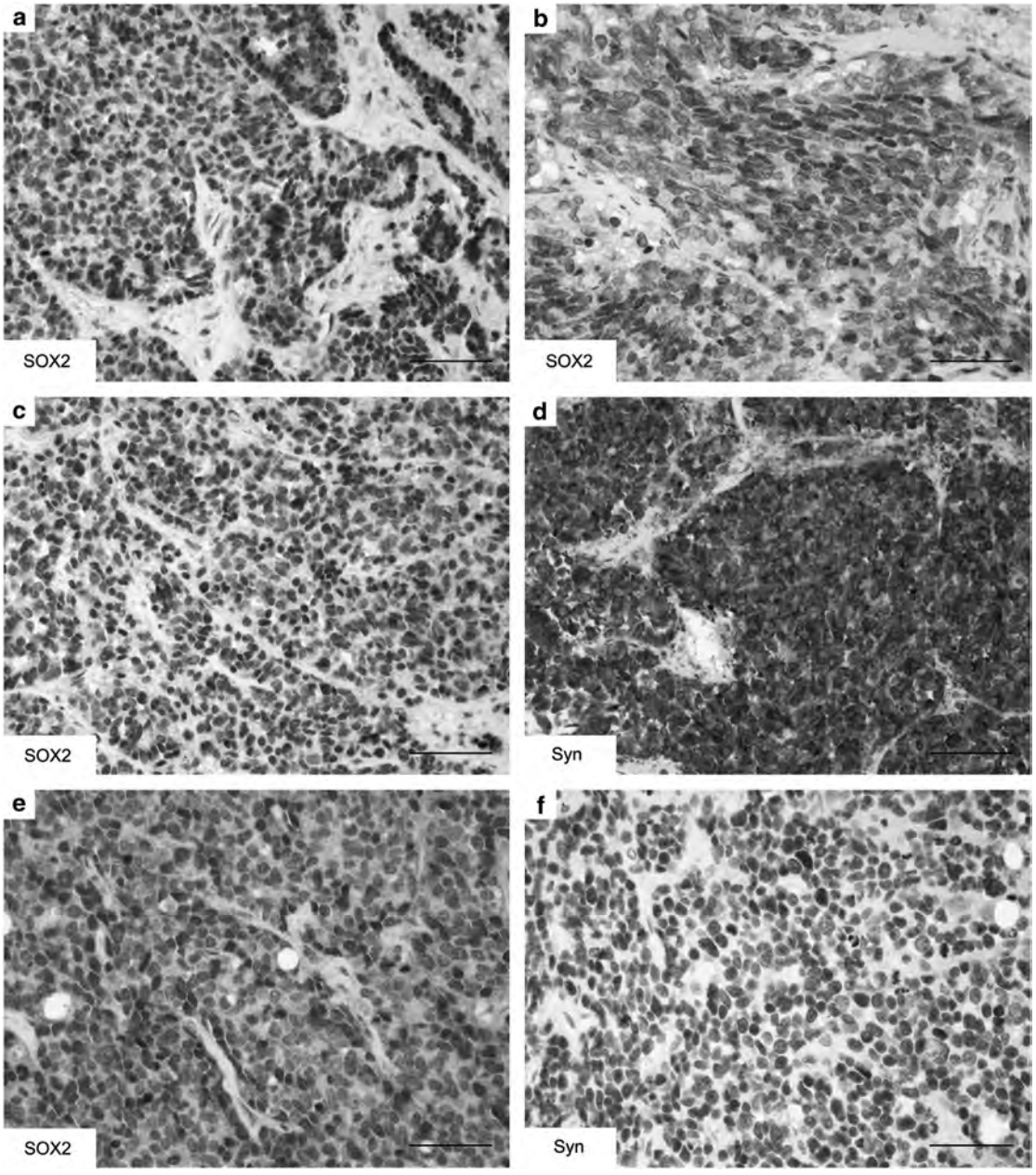


Figure 8. SOX2 expression in metastatic prostate cancer (PCa). The expression of SOX2 was examined on a set of tissue microarrays (UWTMA22) that consist of metastatic castration-resistant PCa tissues. The expression of SOX2 was detected in both soft tissue (**a**, liver metastasis) and bone metastasis (**b**), in both neuroendocrine (NE) (**c**) and non-NE (**e**) PCa metastasis. On serial sections **c** and **d**, both SOX2 and synaptophysin were expressed; however, on serial sections **e** and **f**, SOX2 but not synaptophysin was expressed. Scale bars represent 25 μm.

prostatic NE tumors, we examined the expression of SOX2 in human prostatic NE tumors. A NE tumor biopsy specimen examined exhibited strong nuclear SOX2 expression (Figure 7a), along with co-expression of synaptophysin (Figure 7b). We also examined the expression of SOX2 on a set of prostate tissue microarrays UWTMA46, a collection of LuCaP xenografts. All of the NE tumor specimens derived from three patients were positive for SOX2 (Figures 7c and d). In the non-NE xenografts derived from two patients that were positive for SOX2, one case showed nuclear SOX2 staining in almost half of the epithelial cells (Figures 7e and f, median value 47.5%), whereas the other case showed positive SOX2 staining in only 5% of the epithelial cells (data not shown). The remaining non-NE PCa xenografts derived from other 14 patients were negative for SOX2.

We have also examined the expression of SOX2 on UWTMA22, a set of prostate tissue microarrays that consists of CRPC bone and soft tissue metastases. Of the 24 patients, 15 (63%) were positive for SOX2 in at least one metastatic site (Table 1 and Figure 8), despite the observation that primary adenocarcinomas of the prostate exhibit loss of SOX2 expression. Serial sections from the UWTMA22 were also stained for synaptophysin by IHC and compared with SOX2 expression (Figure 8). The expression of SOX2 was correlated with synaptophysin-positive staining (Spearman's test, $P = 0.037$; Rho = 0.143; Table 2).

Table 2. Correlation of expression of SOX2 and synaptophysin

	Synaptophysin					
	Score 0	*Score 1*	*Score 2*	*Score 3*	P-*value*	*Rho value*
SOX2						
Score 0	121 (76.1)	20 (76.9)	7 (50.0)	9 (56.2)	0.037	0.143
Score 1	10 (6.3)	3 (11.5)	3 (21.4)	2 (12.5)		
Score 2	15 (9.4)	3 (11.5)	3 (21.4)	5 (31.3)		
Score 3	13 (8.2)	0 (0.0)	1 (7.1)	0 (0.0)		

DISCUSSION

In this study, we characterized the expression of SOX2 in murine and human prostatic tissue. Our data demonstrated that SOX2 was expressed during embryonic prostate budding morphogenesis. In the adult prostate, SOX2 expression was primarily detected in prostatic basal epithelial and NE cells. Therefore in the prostate SOX2 is largely an embryonic and NE marker, which is turned off in the luminal compartment following birth.

In the adult human prostate, SOX2 expression was limited to the basal compartment. The expression of SOX2 was lost in 24 primary prostate adenocarcinomas, which agrees with the notion that prostates lose basal cells during cancer progression. Interestingly, in these primary PCa samples, we noticed some p63-positive but SOX2-negative areas, suggesting that loss of SOX2 expression in basal cells occurs before p63 is lost in PCa. Our finding that SOX2 is lost in prostate adenocarcinoma differs from a previous report that showed SOX2 is detected in 51% prostate carcinoma cases. One possible reason for the differences in SOX2 expression in these two cohorts could be because of different treatment regimens used. For example, androgen has been shown to inhibit the expression of SOX2,[10] thus androgen-deprivation therapy may induce the expression of SOX2 in patients who have undertaken antiandrogens. The loss of SOX2 in adenocarcinomas, however, may be transient as SOX2 was detected in 15 of the 24 metastatic hormone refractory PCa patients. The observation that SOX2 is expressed in PCa metastases is consistent with a recent study where SOX2 was detected in majority of castration-resistant metastatic PCa specimens,[10] as well as our characterization of SOX2 expression in established prostate cell lines that suggests an association with castrate resistance as well.

We also showed that the expression of SOX2 was detected in NE tumors of prostate. SOX2 was expressed in human NE and NE tumor murine models examined. In human pathology specimens, SOX2 was expressed in primary NE PCa needle biopsy specimens and in all the NE tumor xenografts. In the metastatic PCa specimens, the expression of SOX2 was correlated with NE marker, synaptophysin. Because NE PCa are AR negative, they do not respond to hormonal therapy. Currently, there is no effective therapy for treating NE PCa, and most patients with NE PCa survive < 1 year.[34] Identifying genes and pathways that are active in NE PCa can improve our understanding on prostatic NE tumor biology and provide potential therapeutic targets. Studies have shown that signaling pathways active in stem cells and in embryonic prostate development are often activated in prostate NE tumors.[30,35–38] Our finding that SOX2, a 'stemness' gene, is expressed in embryonic prostates and in prostate NE tumors provides another piece of evidence supporting the connection of NE phenotype with stem cell features. Moreover, characterizing the expression pattern of SOX2 in well-characterized NE mouse models allows us to further interrogate the molecular underpinnings of NE PCa.

The observation that the majority of adenocarcinomas lose SOX2 expression, whereas increased SOX2 expression is associated with castrate-resistant metastatic PCa, suggests that those patients who express SOX2 may be more likely to progress to castrate-resistant disease. Moreover, the expression of SOX2 in NE tumor and tumors that have undergone NED support the notion that SOX2 may support progression to castrate resistance via a NED pathway. Indeed, a recent study found that ectopic expression of SOX2 drives CRPC growth, indicating that SOX2 is not only expressed in CRPC but also an important driver in promoting PCa progression.[10] Coupled with this observation, our findings support exploring the predictive value of SOX2 for PCa with a larger patient cohort.

In conclusion, SOX2 was expressed during murine prostate development. In human prostate specimens, SOX2 expression was detected in basal cells. The expression of SOX2 was lost in primary prostate adenocarcinoma but was detected in castration-resistant metastatic PCa and in prostatic NE tumors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the patients and their families who were willing to participate in the Prostate Cancer Donor Program and the physicians and the rapid autopsy team at the University of Washington. This research was supported by: NIH to RM (5R01 DK055748-14 and 4R01 CA076142-14); DOD to RM (PC074022); DOD to XY (PC111074); NIH to SWH (5R01 DK067049); Pacific Northwest Prostate Cancer SPORE (P50CA97186); PO1 NIH grant (PO1CA085859); and Richard M. Lucas Foundation.

REFERENCES

1 Bowles J, Schepers G, Koopman P. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* 2000; **227**: 239–255.
2 Schepers GE, Teasdale RD, Koopman P. Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. *Dev Cell* 2002; **3**: 167–170.
3 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–676.
4 Gontan C, de Munck A, Vermeij M, Grosveld F, Tibboel D, Rottier R. Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. *Dev Biol* 2008; **317**: 296–309.
5 Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN *et al.* Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. *PLoS One* 2009; **4**: e8248.
6 Lu Y, Futtner C, Rock JR, Xu X, Whitworth W, Hogan BL *et al.* Evidence that SOX2 overexpression is oncogenic in the lung. *PLoS One* 2010; **5**: e11022.
7 Ugolkov AV, Eisengart LJ, Luan C, Yang XJ. Expression analysis of putative stem cell markers in human benign and malignant prostate. *Prostate* 2011; **71**: 18–25.
8 Bae KM, Su Z, Frye C, McClellan S, Allan RW, Andrejewski JT *et al.* Expression of pluripotent stem cell reprogramming factors by prostate tumor initiating cells. *J Urol* 2010; **183**: 2045–2053.
9 Jia X, Li X, Xu Y, Zhang S, Mou W, Liu Y *et al.* SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell. *J Mol Cell Biol* 2011; **3**: 230–238.
10 Kregel S, Kiriluk KJ, Rosen AM, Cai Y, Reyes EE, Otto KB *et al.* Sox2 is an androgen receptor-repressed gene that promotes castration-resistant prostate cancer. *PLoS One* 2013; **8**: e53701.
11 Chen S, Xu Y, Chen Y, Li X, Mou W, Wang L *et al.* SOX2 gene regulates the transcriptional network of oncogenes and affects tumorigenesis of human lung cancer cells. *PLoS One* 2012; **7**: e36326.
12 Stolzenburg S, Rots MG, Beltran AS, Rivenbark AG, Yuan X, Qian H *et al.* Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer. *Nucleic Acids Res* 2012; **40**: 6725–6740.
13 Basu-Roy U, Seo E, Ramanathapuram L, Rapp TB, Perry JA, Orkin SH *et al.* Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. *Oncogene* 2012; **31**: 2270–2282.
14 Xiang R, Liao D, Cheng T, Zhou H, Shi Q, Chuang TS *et al.* Downregulation of transcription factor SOX2 in cancer stem cells suppresses growth and metastasis of lung cancer. *Br J Cancer* 2011; **104**: 1410–1417.
15 Girouard SD, Laga AC, Mihm MC, Scolyer RA, Thompson JF, Zhan Q *et al.* SOX2 contributes to melanoma cell invasion. *Lab Invest* 2012; **92**: 362–370.

16 Lin F, Lin P, Zhao D, Chen Y, Xiao L, Qin W *et al.* Sox2 targets cyclinE, p27 and survivin to regulate androgen-independent human prostate cancer cell proliferation and apoptosis. *Cell Prolif* 2012; **45**: 207–216.
17 Laga AC, Lai CY, Zhan Q, Huang SJ, Velazquez EF, Yang Q *et al.* Expression of the embryonic stem cell transcription factor SOX2 in human skin: relevance to melanocyte and merkel cell biology. *Am J Pathol* 2010; **176**: 903–913.
18 Sholl LM, Long KB, Hornick JL. Sox2 expression in pulmonary non-small cell and neuroendocrine carcinomas. *Appl Immunohistochem Mol Morphol* 2010; **18**: 55–61.
19 Wang W, Epstein JI. Small cell carcinoma of the prostate. A morphologic and immunohistochemical study of 95 cases. *Am J Surg Pathol* 2008; **32**: 65–71.
20 Yao JL, Madeb R, Bourne P, Lei J, Yang X, Tickoo S *et al.* Small cell carcinoma of the prostate: an immunohistochemical study. *Am J Surg Pathol* 2006; **30**: 705–712.
21 Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M *et al.* Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* 2004; **64**: 9209–9216.
22 Mosquera JM, Beltran H, Park K, MacDonald TY, Robinson BD, Tagawa ST *et al.* Concurrent AURKA and MYCN gene amplifications are harbingers of lethal treatment-related neuroendocrine prostate cancer. *Neoplasia* 2013; **15**: 1–10.
23 Grabowska MM, Degraff DJ, Yu X, Jin RJ, Chen Z, Borowsky AD *et al.* Mouse models of prostate cancer: picking the best model for the question. *Cancer Metastasis Rev* 2014 (in press).
24 Kasper S, Sheppard PC, Yan Y, Pettigrew N, Borowsky AD, Prins GS *et al.* Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. *Lab Invest* 1998; **78**: 319–334.
25 Masumori N, Thomas TZ, Case T, Paul M, Kasper S, Chaurand P *et al.* A probasin-large T antigen transgenic mouse line develops prostate adeno and neuroendocrine carcinoma with metastatic potential. *Cancer Res* 2001; **61**: 2239–2249.
26 Chiaverotti T, Couto SS, Donjacour A, Mao JH, Nagase H, Cardiff RD *et al.* Dissociation of epithelial and neuroendocrine carcinoma lineages in the transgenic adenocarcinoma of mouse prostate model of prostate cancer. *Am J Pathol* 2008; **172**: 236–246.
27 Masumori N, Tsuchiya K, Tu WH, Lee C, Kasper S, Tsukamoto T *et al.* An allograft model of androgen independent prostatic neuroendocrine carcinoma derived from a large probasin promoter-T antigen transgenic mouse line. *J Urol* 2004; **171**: 439–442.
28 Yu X, Wang YQ, Jiang M, Bierie BB, Hayward SW, Shen MM *et al.* Activated beta-catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. *Prostate* 2009; **69**: 249–262.
29 Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ. Wnt/beta-Catenin activation promotes prostate tumor progression in a mouse model. *Oncogene* 2011; **30**: 1868–1879.
30 Mirosevich J, Gao N, Matusik RJ. Expression of Foxa transcription factors in the developing and adult murine prostate. *Prostate* 2005; **62**: 339–352.
31 Sramkoski RM, Pretlow TG 2nd, Giaconia JM, Pretlow TP, Schwartz S, Sy MS *et al.* A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cell Dev Biol Anim* 1999; **35**: 403–409.
32 Dagvadorj A, Tan SH, Liao Z, Cavalli LR, Haddad BR, Nevalainen MT. Androgen-regulated and highly tumorigenic human prostate cancer cell line established from a transplantable primary CWR22 tumor. *Clin Cancer Res* 2008; **14**: 6062–6072.
33 Jiang M, Strand DW, Fernandez S, He Y, Yi Y, Birbach A *et al.* Functional remodeling of benign human prostatic tissues in vivo by spontaneously immortalized progenitor and intermediate cells. *Stem Cells* 2010; **28**: 344–356.
34 Beltran H, Tagawa ST, Park K, MacDonald T, Milowsky MI, Mosquera JM *et al.* Challenges in recognizing treatment-related neuroendocrine prostate cancer. *J Clin Oncol* 2012; **30**: e386–e389.
35 Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R, Matusik RJ. Expression and role of Foxa proteins in prostate cancer. *Prostate* 2006; **66**: 1013–1029.
36 Simon RA, di Sant'Agnese PA, Huang LS, Xu H, Yao JL, Yang Q *et al.* CD44 expression is a feature of prostatic small cell carcinoma and distinguishes it from its mimickers. *Hum Pathol* 2009; **40**: 252–258.
37 Palapattu GS, Wu C, Silvers CR, Martin HB, Williams K, Salamone L *et al.* Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. *Prostate* 2009; **69**: 787–798.
38 Sotomayor P, Godoy A, Smith GJ, Huss WJ. Oct4A is expressed by a subpopulation of prostate neuroendocrine cells. *Prostate* 2009; **69**: 401–410.

Research Paper

Androgen receptor differentially regulates the proliferation of prostatic epithelial cells *in vitro* and *in vivo*

Shu Yang[1,*], Ming Jiang[2,3,*], Magdalena M. Grabowska[4], Jiahe Li[1], Zachary M. Connelly[1], Jianghong Zhang[4], Simon W. Hayward[5], Justin M. Cates[6], Guichun Han[7], Xiuping Yu[1]

[1]Department of Biochemistry and Molecular Biology, LSU Health Sciences Center, Shreveport, LA, USA

[2]Laboratory of Nuclear Receptors and Cancer Research, Center for Basic Medical Research, Nantong University School of Medicine, Nantong, Jiangsu, China

[3]Institute of Medicine and Public Health, Division of Epidemiology, Department of Medicine, Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, TN, USA

[4]Department of Urologic Surgery, Vanderbilt University Medical Center, Nashville, TN, USA

[5]Department of Surgery, NorthShore University HealthSystem Research Institute, Evanston, IL, USA

[6]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

[7]Women's Health Division, Michael E. DeBakey Institute, and Department of Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA

[*]These authors have contributed equally to this work

Correspondence to: *Xiuping Yu,* ***email:*** *xyu@lsuhsc.edu*

Keywords: *prostate, androgen receptor*

Received: *April 23, 2016* ***Accepted:*** *August 24, 2016* ***Published:*** *September 7, 2016*

ABSTRACT

Androgens regulate the proliferation and differentiation of prostatic epithelial cells, including prostate cancer (PCa) cells in a context-dependent manner. Androgens and androgen receptor (AR) do not invariably promote cell proliferation; in the normal adult, endogenous stromal and epithelial AR activation maintains differentiation and inhibits organ growth. In the current study, we report that activation of AR differentially regulates the proliferation of human prostate epithelial progenitor cells, NHPrE1, *in vitro* and *in vivo*. Inducing AR signaling in NHPrE1 cells suppressed cell proliferation *in vitro*, concomitant with a reduction in MYC expression. However, ectopic expression of AR *in vivo* stimulated cell proliferation and induced development of invasive PCa in tissue recombinants consisting of NHPrE1/AR cells and rat urogenital mesenchymal (UGM) cells, engrafted under renal capsule of adult male athymic mice. Expression of MYC increased in the NHPrE1/AR recombinant tissues, in contrast to the reduction seen *in vitro*. The inhibitory effect of AR signaling on cell proliferation *in vitro* were reduced by co-culturing NHPrE1/AR epithelial cells with prostatic stromal cells. In conclusion, these studies revealed that AR signaling differentially regulates proliferation of human prostatic epithelia cells *in vitro* and *in vivo* through mechanisms involving stromal/epithelial interactions.

INTRODUCTION

Androgen deprivation therapy is the gold standard treatment for advanced stage PCa [1]. Initially, PCa responds to the treatment, resulting in tumor regression. However, these tumors almost invariably progress to castration-resistant PCa (CRPCa) in which androgen ablation can no longer suppress disease progression [2]. Studies have several mechanisms that contribute to the development of CRPCa, including androgen receptor (AR) AR mutations, AR activation by growth factors, constitutively active AR variants, and increased intra-prostatic androgen synthesis [2]. Although neuroendocrine differentiation and cancer stem cell pathways may bypass AR, alterations in the androgen signaling pathway are still considered a predominant factor in mediating the emergence of resistance to androgen deprivation therapy in PCa.

Although blocking AR signaling causes prostate tumors to shrink in PCa patients and animal models, it has long been recognized that androgen deprivation therapy fails to produce complete responses. One explanation for the incomplete regression may be the presence of distinct populations of prostatic cells that respond to androgenic stimulation anomalously. The prostate gland has an epithelial parenchyma surrounded by a stroma. The epithelial tissue is composed of basal cells and tall columnar secretory luminal cells with occasional neuroendocrine cells [3]. AR is expressed in both the stromal and epithelial tissues although the distribution between cell types varies among species. In humans, AR is expressed in virtually all luminal, many basal epithelial cells, and many cells of the stroma. Castration results in a reduction in the total volume of the prostate. In rats, the initial target of androgen loss is the microvasculature immediately adjacent to the epithelial cells with loss of epithelium occurring subsequent to the loss of vasculature [4, 5]. Experimental models have demonstrated that epithelial apoptosis following castration is due to a failure of androgen to occupy stromal, but not epithelial, AR [6, 7]. Androgen ablation leads to a preferential loss of the luminal phenotype. It does not however lead to a complete regression of the gland, and mechanisms such as Wnt/ß-catenin signaling seem to play a protective role, maintaining the viability of some portion of the tissue [8]. This maintenance of tissue is important in seasonally breeding animals [9], but is problematic in the context of cancer therapy in humans, where it allows the preservation of cancer cells from androgen deprivation therapy.

Studies using *in vitro* cell culture methods have shown that AR signaling exerts mixed effects on the growth of cultured prostatic cells [10–12]. Some AR-expressing PCa cells (such as LNCaP [10]) depend on androgens for proliferation/survival. However, other PCa cell lines are insensitive to androgens or show growth inhibition responses upon androgen exposure. For example, proliferation of PC3 cells, an AR-negative PCa cell line, is inhibited by ectopic-expression of AR [13, 14]. Similarly, proliferation of ARCaP cells that express low levels of AR is inhibited by androgen treatment both *in vitro* and *in vivo* [11]. LNCaP 104-R2, a sub-line cells derived from LNCaP after long-term androgen deprivation [12], expresses increased levels of AR. Unlike their parental cell line, LNCaP, androgen treatment induces cell cycle arrest and suppresses the cell proliferation of LNCaP 104-R2 [12]. Additionally, several recent studies have characterized the role of AR by ectopically expressing AR in normal prostatic epithelial cells [15–17]. These studies have revealed that AR signaling induces luminal epithelial differentiation and suppresses proliferation of these cells. Although these studies have established the roles of AR in *in vitro* cultured prostatic cells, it is not yet clear whether inducing AR signaling produces similar proliferation-regulation *in vivo*.

In this study, we ectopically expressed AR in human prostatic epithelial progenitor NHPrE1 cells and used a unique tissue recombination technique to investigate the roles of AR signaling in modulating prostatic cell proliferation *in vitro* and *in vivo*. NHPrE1 is a cell line derived from normal human prostate epithelial cells; NHPrE1 cells have some progenitor features [18]. When recombined with inductive rat urogenital sinus mesenchyme (UGM), NHPrE1 cells are able to generate benign secretory ductal-acinar architecture *in viv*o [18]. Thus, the benign nature of NHPrE1 cells makes them a suitable model system for investigating the molecular mechanisms of human prostatic carcinogenesis.

RESULTS

Ectopic expression of AR confers a functional AR-mediated signaling in NHPrE1 cells

NHPrE1 is an epithelial cell line derived from a normal human prostate that has some stem/progenitor features but does not express AR in 2D culture *in vitro*. When recombined with rat UGM and grafted *in vivo*, NHPrE1 cells form organized, functional prostatic glandular structures and therefore can be considered to represent untransformed prostate epithelium [18]. In order to study the role of AR in prostatic cells, we stably integrated full-length AR cDNA into NHPrE1 cells (NHPrE1/AR); NHPrE1 cells stably transduced with empty vector (EV) served as control cells. Ectopic expression of AR in NHPrE1/AR cells under the CMV promoter was by Western blot (Figure 1A) and quantitative (q)RT-PCR (Figure 1B). We also determined whether ectopic expression of AR enabled functional androgen signaling in NHPrE1 cells by examining the expression of two well-established androgen-regulated genes (PSA and FKBP5) by qRT-PCR. Results (Figures 1C and 1D) demonstrated that androgen treatment induced expression of both PSA and FKBP5 in NHPrE1/AR cells. staining for AR was conducted to examine cellular localization in response to androgens in NHPrE1/AR cells. Upon androgen treatment, ectopically expressed AR translocated from the cytoplasm to the cell nucleus (Figure 1E). These results that ectopic expression of AR confers functional AR signaling in NHPrE1 cells.

Inducing AR signaling inhibits the proliferation of NHPrE1 cells *in vitro*

To investigate whether ectopically expressed AR plays a functional role in modulating proliferation of NHPrE1 cells, we cultured NHPrE1/EV or NHPrE1/AR cells in medium supplemented with 5% charcoal-stripped serum with or without the addition of androgens (10 nM DHT or 1 nM R1881). As shown in Figure 2A (WST-

1 assay) and Figure 2B (IncuCyte cell proliferation assay), androgen treatment did not affect proliferation of NHPrE1/EV cells but markedly inhibited proliferation of NHPrE1/AR cells. It was also noticed that 70% NHPrE1/AR cells died after they were cultured in the presence of androgens for 4 days. Additionally, we treated NHPrE1/AR cells with an AR inhibitor (bicalutamide, 10 μM) [19]. As shown in Figure 2C, bicalutamide did not alter the proliferation of NHPrE1/AR cells in the absence of androgen, but partially restored proliferation of NHPrE1/AR cells in the presence of androgen ($p<0.05$). It has been well documented that inducing

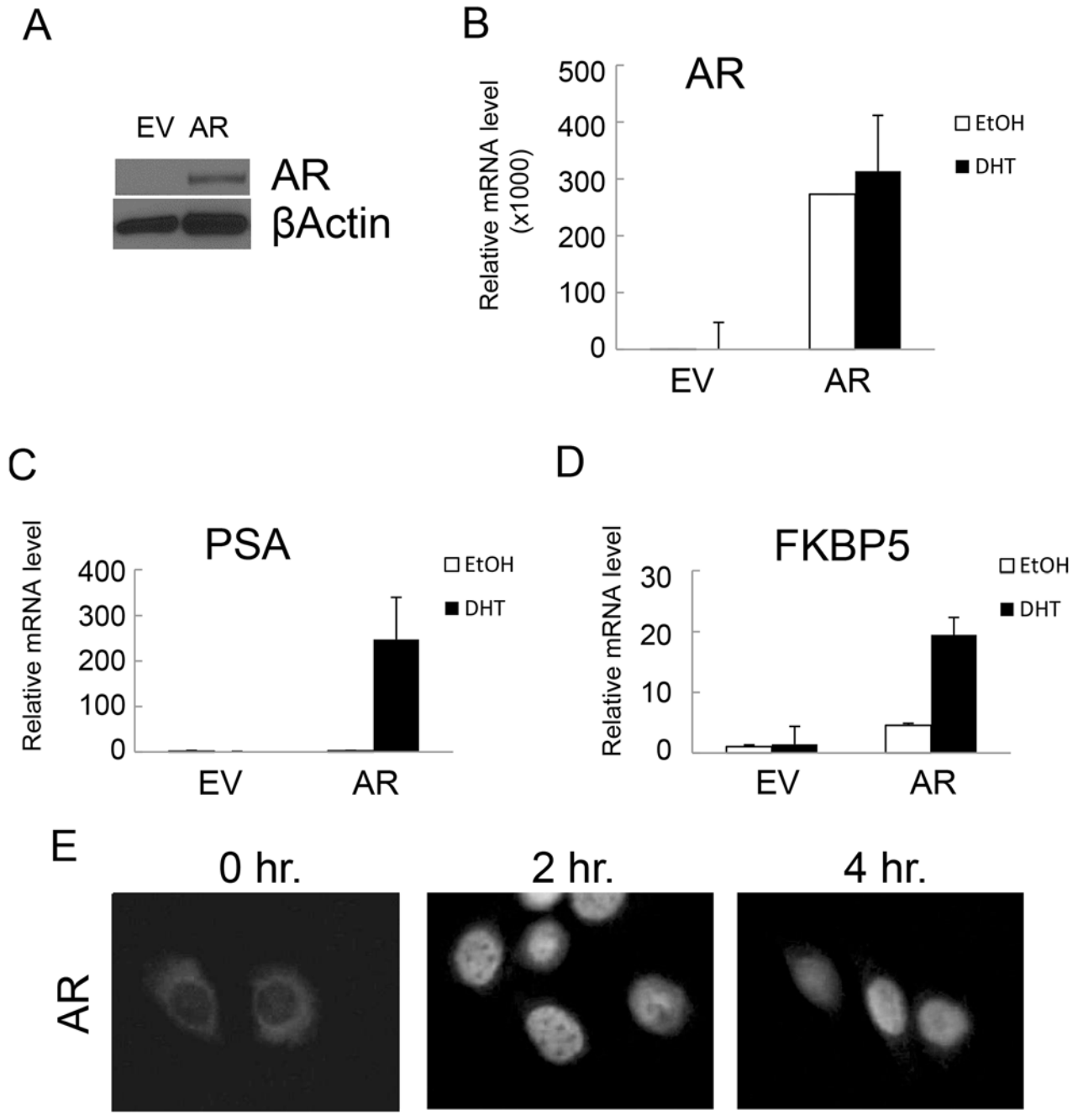


Figure 1: Ectopic expression of AR conferred functional AR-mediated androgen signaling in NHPrE1 cells. Retroviral vector pLNCX or pLNCX-AR was used to generate NHPrE1 cells with empty vector (EV) control or AR transgene. **A.** Western blot to analyze the expression of AR in NHPrE1/EV (EV) or NHPrE1/AR (AR) cells. Beta-actin served as a loading control. **B-D.** quantitative (q)RT-PCR to assess the levels of AR (B) and androgen responsive genes PSA (C) and FKBP5 (D). Androgen treatment (DHT, 10 nM) induced the expression of PSA and FKBP5. The expression of GAPDH was used to normalize the qPCRs. **E.** staining of AR. NHPrE1/AR cells were cultured in androgen-depleted medium for 24 hours and then treated with R1881 (1nM) for 2 or 4 hours. AR was conducted to examine the nuclear translocation of AR upon androgen treatment.

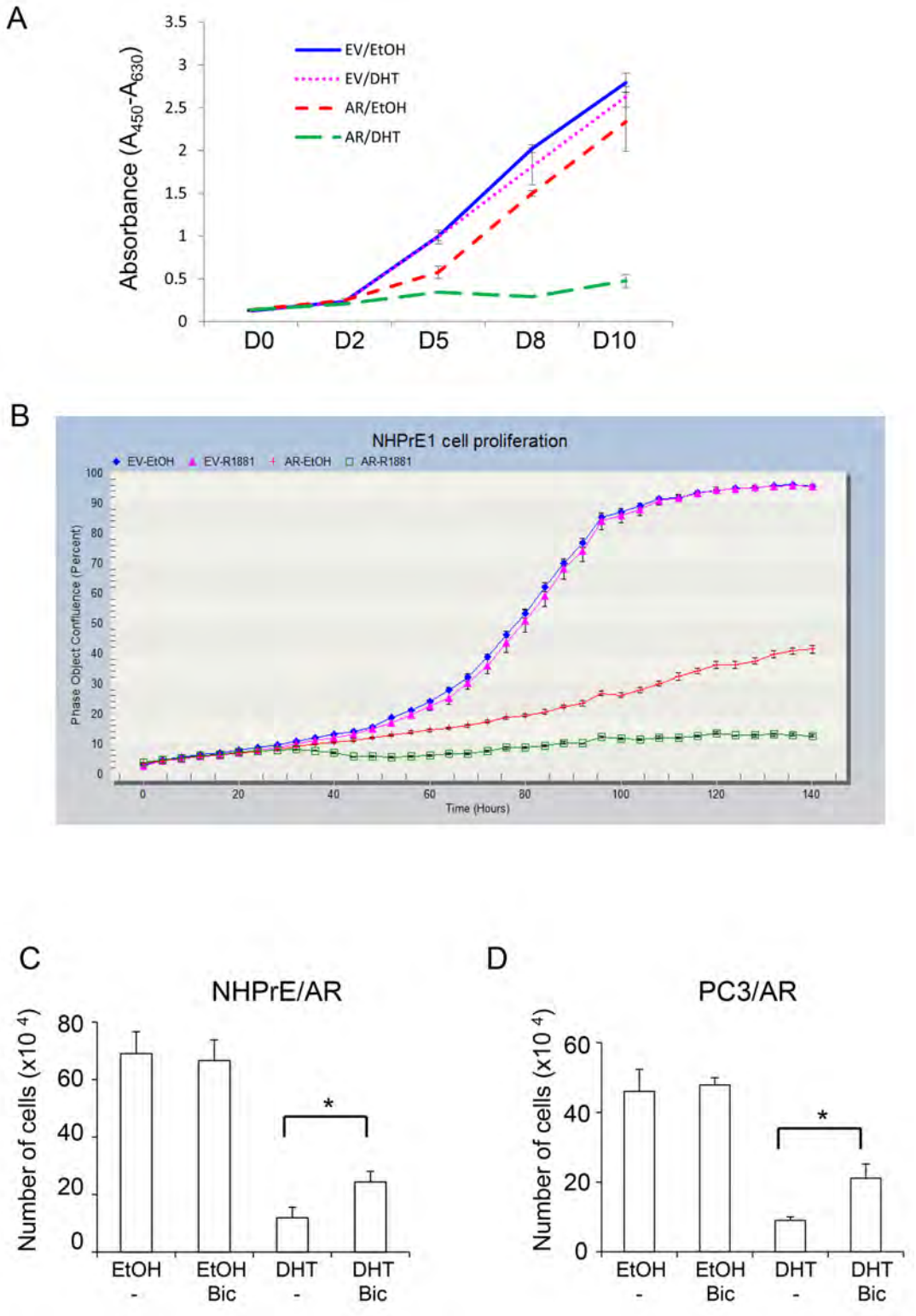


Figure 2: Androgen differentially regulated prostatic cell proliferation. The proliferation of NHPrE1 cells with or without AR expression was assessed by using both WST-1 **A.** and IncuCyte **B.** methods. NHPrE1/EV cells and NHPrE1/AR cells were cultured in the absence or presence of androgens (10 nM DHT or 1 nM R1881). Androgen treatment had negligible effects on the proliferation of empty vector control cells, but suppressed proliferation of AR-expressing NHPrE1 cells (panel B, $p<0.01$ from 36 hour onward, comparison between ethanol- and R1881- treated NHPrE1/AR cells). Overall, compared with NHPrE1/EV cells, NHPrE1/AR cells displayed suppressed cell proliferation. **C** and **D**, blocking AR attenuated androgen-induced proliferation inhibition. NHPrE1/AR (C) or PC3/AR cells (D) were cultured with or without androgen (10 nM DHT) in the presence or absence of 10 μM bicalutamide (Bic) for 5 days. While DHT suppressed the proliferation of NHPrE1/AR and PC3/AR cells, addition of bicalutamide attenuated this inhibitory effect of androgens. *$p<0.05$, t-test.

AR signaling inhibits the proliferation of PC3/AR cells [13, 14, 20–22]. In our study, treatment of PC3/AR cells with bicalutamide also induced a growth restoration effect (Figure 2D). Together, these results suggest that inhibition of cell proliferation by androgen signaling in NHPrE1/AR cells is mediated by AR.

Androgen differentially regulates MYC levels in prostatic cells

MYC is a nuclear protein that plays important roles in cell cycle regulation. MYC is often and/or mutated in cancer, especially in the prostate where it can play a role as an oncogene [23, 24]. Studies indicate that MYC is implicated in AR-mediated growth modulation of prostatic cells [12, 15, 16]. To determine whether MYC is also involved in the function of ectopically expressed AR in NHPrE1 cells, we examined the levels of MYC in NHPrE1/EV and NHPrE1/AR cells under DHT treatment. LNCaP and PC3/AR cells were used as controls. The results (Figure 3A) showed that levels of MYC were associated with androgenic modulation of proliferation of NHPrE1, LNCaP, and PC3 cells. DHT (10 nM) treatment resulted in a down-regulation of MYC in NHPrE1/AR as well as PC3/AR cells, correlating with the inhibitory effects of androgens in both cell types (Figure 2A and reference [13]). In contrast, DHT induced an up-regulation of MYC in LNCaP cells, a well-established androgen-dependent PCa cell line [10]. We also examined whether bicalutamide treatment could reverse androgen-mediated down-regulation of MYC in NHPrE1/AR cells. As shown in Figure 3B, DHT treatment caused a reduction of MYC in NHPrE1/AR cells, but the addition of bicalutamide restored MYC expression in NHPrE1/AR cells. In summary, the expression of MYC is associated with AR-mediated growth-inhibition of NHPrE1 cells.

Proteasomal degradation is one of the key mechanisms that regulates intracellular MYC levels [25]. To determine whether androgen treatment affects the stability of MYC protein, cycloheximide chase analyses were conducted using NHPrE1/AR cells cultured with or without androgen. The results showed that androgen treatment did not affect the turnover of MYC protein in these cells as MYC protein in NHPrE1/AR cells displayed a similar degradation pattern regardless of whether DHT was present or not (Figure 3C). Also, we assessed whether AR signaling affected the levels of MYC mRNA in these cells. As shown in Figure 3D, DHT treatment caused a reduction of MYC mRNA in both NHPrE1/AR and PC3/AR cells ($p<0.01$ and 0.05, respectively). Together, these results suggest that inducing AR signaling in NHPrE1 cells down regulates MYC mRNA level and subsequently decreases MYC protein expression, but does not alter proteasomal degradation of MYC.

Ectopic expression of AR promotes NHPrE1 cells to form invasive PCa *in vivo*

In order to study how ectopic expression of AR modulates the proliferation of NHPrE1 cells *in vivo*, we conducted tissue recombination-xenografting experiments. Epithelial cells were combined with prostate-inductive mesenchymal cells, grafted under the renal capsules of male mice and allowed to grow for 3 months [26]. We used rat urogenital sinus mesenchyme (UGM), which can induce some prostatic epithelial cells to form prostatic glandular structures [18, 27]. For the epithelia, we used NHPrE1/EV control cells and NHPrE1/AR cells. Previous research has shown that when recombined with UGM and grafted *in vivo,* NHPrE1 cells form glandular structures [18], thereby allowing us to study how ectopic expression of AR alters the cell behavior *in vivo* and how signals from prostatic stromal cells regulate the proliferation of NHPrE1 cells through stromal/epithelial interactions. Our results showed that while the growth of NHPrE1/EV grafts was grossly negligible (Figure 4A), NHPrE1/AR grafts formed large invasive tumors (Figure 4B). To trace the epithelial cells in the NHPrE1/UGM tissue recombinants, we used immunohistochemical staining for GFP that was also expressed in these cells. We that the epithelial cells in the grafts were indeed NHPrE1 cells and were not contaminated with rat urogenital sinus epithelial cells. As shown in Figures 4C-4N, GFP-positive cells were detected in one of ten NHPrE1/EV grafts (Figures 4E and 4H), and the histology of this graft showed prostate glandular structure (Figures 4C and 4F). In contrast, eight of ten NHPrE1/AR grafts showed positive GFP IHC staining (Figures 4K and 4N). The inductive UGM dictated NHPrE1/EV cells to form benign glandular structures (Figures 4C and 4F), whereas the NHPrE1/AR recombinants developed invasive carcinomas (Figures 4I and 4L). No distant metastases were observed in any graft-bearing mice.

Although previous studies have indicated that tissue recombinants derived from early passages of NHPrE1 cells showed mature glandular differentiation with positive staining for AR in the glandular epithelial cells [18], less complete differentiation within luminal epithelium that was not clearly tall columnar and more limited AR expression was observed in the epithelial cells of the NHPrE1/EV grafts (Figures 4D and 4G). As expected, UGM-derived stromal cells were positive for AR. In contrast to the limited epithelial AR expression in NHPrE1/EV grafts, grafts derived from NHPrE1/AR cells showed strong AR staining in epithelial cells (Figures 4J and 4M), indicating that these cells did not lose the AR transgene during the three month *in vivo* growth phase without drug selection pressure.

In the one NHPrE1/EV graft that grew, epithelial cells formed glandular structures

consisting of cytokeratin 8/18-positive luminal epithelial cells (Figures 5A and 5B) and p63-positive basal cells (Figures 5E and 5F). In contrast, the invasive carcinomas formed by the NHPrE1/AR grafts were weakly positive for cytokeratin 8/18 (Figures 5C and 5D) and strongly positive for p63, a prostate basal cell marker (Figures 5G and 5H). A high proportion of malignant cells in the NHPrE1/AR grafts showed nuclear immunoreactivity for the cell proliferation marker Ki67 (Figures 5K and 5L), but only a few positive nuclei were seen in the luminal epithelial cells from NHPrE1/EV grafts (Figures 5I and 5J). Interestingly, most basal cells of the NHPrE1/EV graft were positive for Ki67 (Figures 5I and 5J). Overall, more Ki67 positive cells (including both luminal and basal epithelium) were detected in NHPrE1/AR than NHPrE1/EV grafts (Table 1). Taken together, these results indicate that ectopic expression of AR promotes NHPrE1cells to form invasive PCa *in vivo*.

Expression of MYC and pSTAT3, but not FOXA1, is induced in NHPrE1/AR grafts

Previous research has shown that AR partners with various transcription factors to regulate distinct sets of genes involved in modulating the differentiation and proliferation of prostatic cells [28]. In an effort to explore the mechanisms that transform NHPrE1/AR cells to form invasive cancers *in vivo*, we examined the

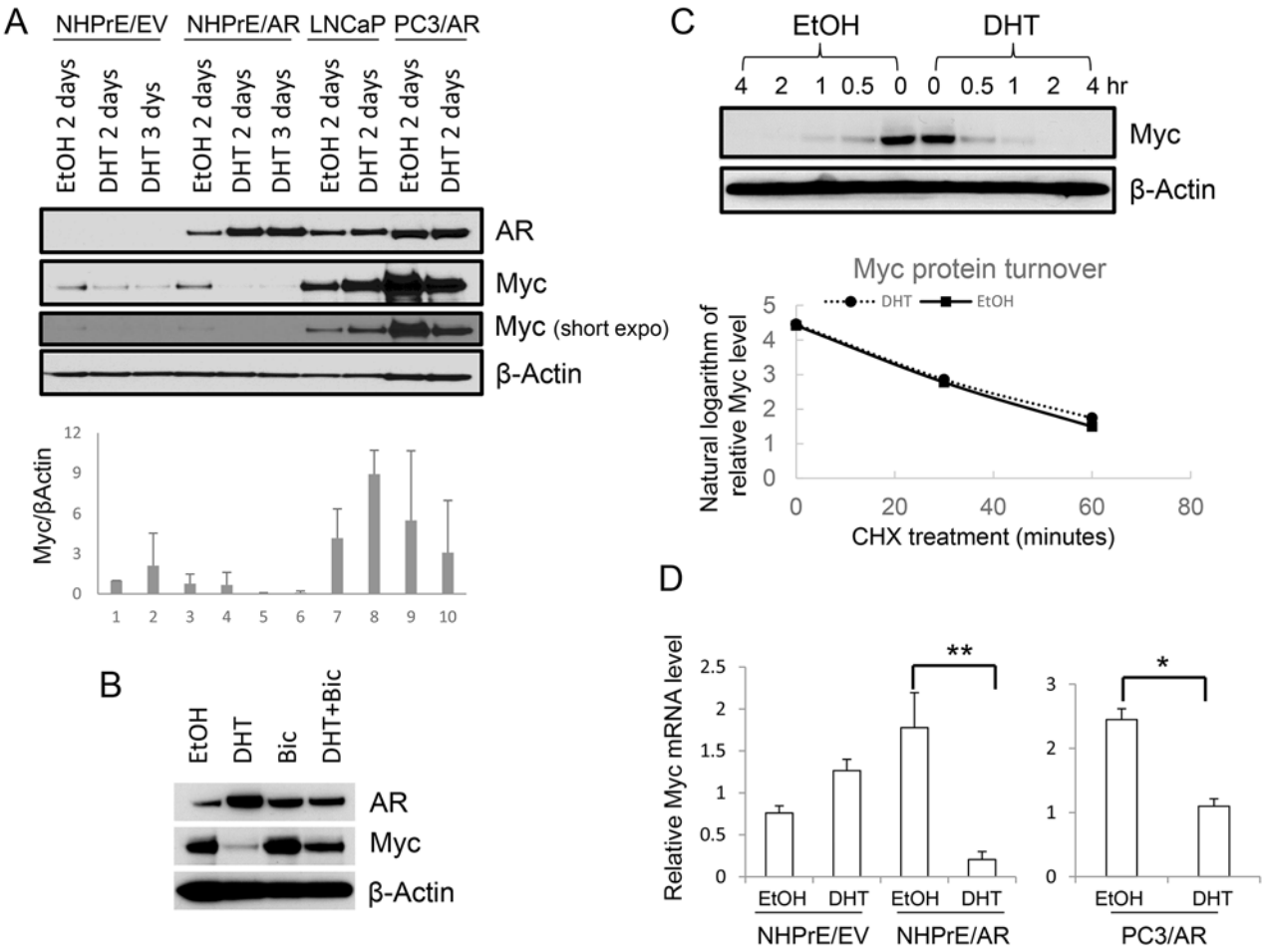


Figure 3: Androgen differentially regulated MYC expression. A. Western blot for AR and MYC in prostatic cells. Androgen (DHT, 10 nM) treatment resulted in up-regulation of MYC in LNCaP cells, but down-regulation of MYC in NHPrE1/AR and PC3/AR cells. Lower panel is the of MYC Western blot. **B.** Western blot for AR and MYC. NHPrE1/AR cells were cultured in androgen-depleted medium for 2 days with or without the addition of 10 nM DHT and/or 10 μM Bicalutamide (Bic). Bicalutamide treatment reversed androgen-mediated reduction of MYC. **C.** Analysis of MYC protein stability. Cycloheximide chase analyses were conducted using NHPrE1/AR cells to determine whether androgen treatment affected the turnover of MYC. NHPrE1/AR cells were treated with 50 μg/ml cycloheximide to block protein synthesis in the presence or absence of 10 nM DHT and harvested at different time points post treatment. Androgen treatment did not alter the stability of MYC protein in NHPrE1/AR cells. Lower panel is the semi-logarithm plot of MYC levels at different times of cycloheximide treatment. **D.** qRT-PCR to assess the levels of MYC mRNA in NHPrE1/EV, NHPrE1/AR, and PC3/AR cells. The expression of GAPDH was used to normalize the qPCRs. DHT treatment decreased the level of MYC mRNA in NHPrE1/AR and PC3/AR cells. *p<0.05, **p<0.01, t-test.

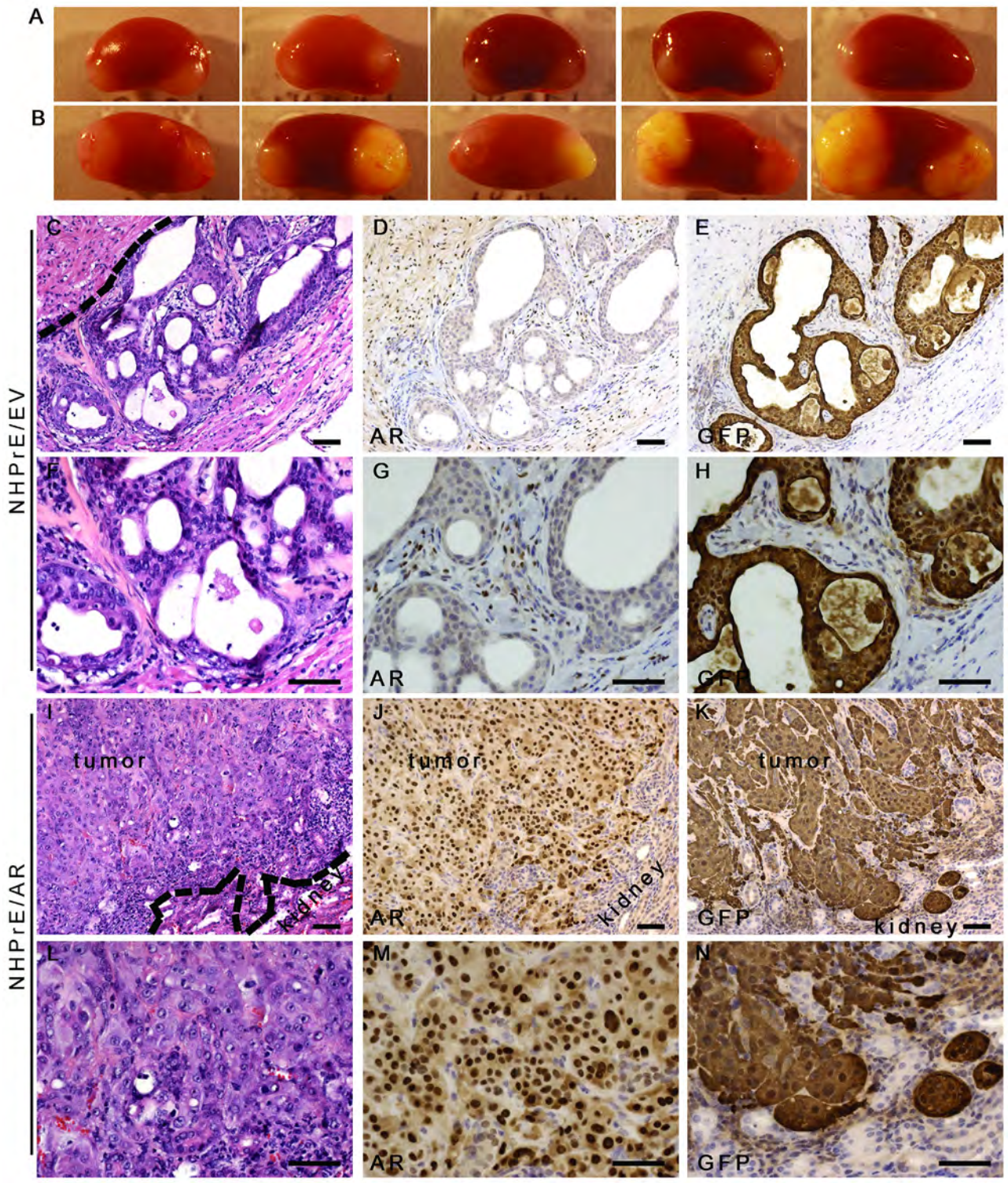


Figure 4: Ectopic-expression of AR transformed NHPrE1 cells *in vivo*. NHPrE1/EV or NHPrE1/AR cells were recombined with rat UGM and grafted *in vivo*. **A** and **B.** gross morphology of renal subcapsular grafts. A, grafts derived from empty vector control NHPrE1/EV cells showed limited growth; B, grafts derived from NHPrE1/AR cells grew extensively. **C-N.** H&E and IHC staining performed on serial sections derived from NHPrE1/EV (C-H) or NHPrE1/AR (I-N) grafts. F-H and L-N are higher pictures of C-E and I-K, respectively. Broken lines in panels C and I indicate the interface between the NHPrE1 grafts and host kidneys. While a clear boundary existed between the NHPrE1/EV graft and host kidney (C), NHPrE1/AR tumors focally invaded renal parenchyma (I-K). While epithelial cells in NHPrE1/AR grafts were positive for AR by IHC staining (J and M), epithelial cells in NHPrE1/EV graft showed little AR immunoreactivity (D and G). Stromal cells in NHPrE1/EV grafts (derived from rat UGM) were positive for AR staining (D and G). Epithelial cells in NHPrE1/EV grafts showed positive IHC staining for GFP and formed glandular structures (E and H); whereas GFP-tagged NHPrE1/AR cells (K and N) formed invasive carcinomas. Scale bars represent 25 μm.

expression of FOXA1, a well-established AR co-activator [29], as well as MYC and pSTAT3, two genes that are differentially recruited to the AR transcriptome [28], in tissue recombinants derived from NHPrE1/EV and NHPrE1/AR cells. We showed that FOXA1 was weakly expressed in a subpopulation of glandular epithelial cells within the NHPrE1/EV graft (Figure 6A). However, little or no FOXA1 expression was detected in the malignant cells of NHPrE1/AR grafts (Figure 6D), indicating a lack of induction of FOXA1 by stromal signals in NHPrE1/AR cells.

Our *in vitro* study indicated that expression of MYC was directly associated with proliferation of NHPrE1 cells. To study whether MYC is associated with tumorigenicity of NHPrE1 cells in *vivo*, we used IHC staining to assess the expression of MYC in grafts derived from NHPrE1/AR or empty vector control NHPrE1/EV cells. Our results showed that while MYC was only expressed in a few basal cells in NHPrE1/EV grafts (Figure 6B), many more MYC-positive cells were detected in the carcinomas that formed in NHPrE1/AR grafts (Figure 6E and Table 1). These data indicate that AR regulates MYC expression in NHPrE1/AR cells in a context-dependent manner: suppressing MYC expression and inhibiting cell proliferation in 2D *in vitro* culture, but elevating MYC expression and promoting carcinoma formation *in vivo*.

A possible explanation for the incongruous regulation of NHPrE1 proliferation by AR signaling *in vitro* and *in vivo* is the presence of stromal/epithelial communication within tissue recombinants. Since signal transducer and activator of transcription 3 (STAT3) is instrumental in several signaling pathways that mediate prostatic stromal/epithelial cell interactions [30], we examined activated pSTAT3 (Tyr-705) expression in grafts derived from NHPrE1/EV and NHPrE1/AR cells. As shown in Figures 6C & 6F, pSTAT3 is barely detectable in the epithelial cells of empty vector control grafts but numerous pSTAT3-positive cells were observed in NHPrE1/AR grafts (Figures 6C & 6F and Table 1), indicative of active STAT3 signaling in these grafts.

The presence of stromal cells restores proliferation of NHPrE1/AR cells

To determine the role of stromal cells in regulating the proliferation of NHPrE1 cells, stromal/epithelial co-culture experiments were conducted. The results showed that the presence of prostate stromal cells

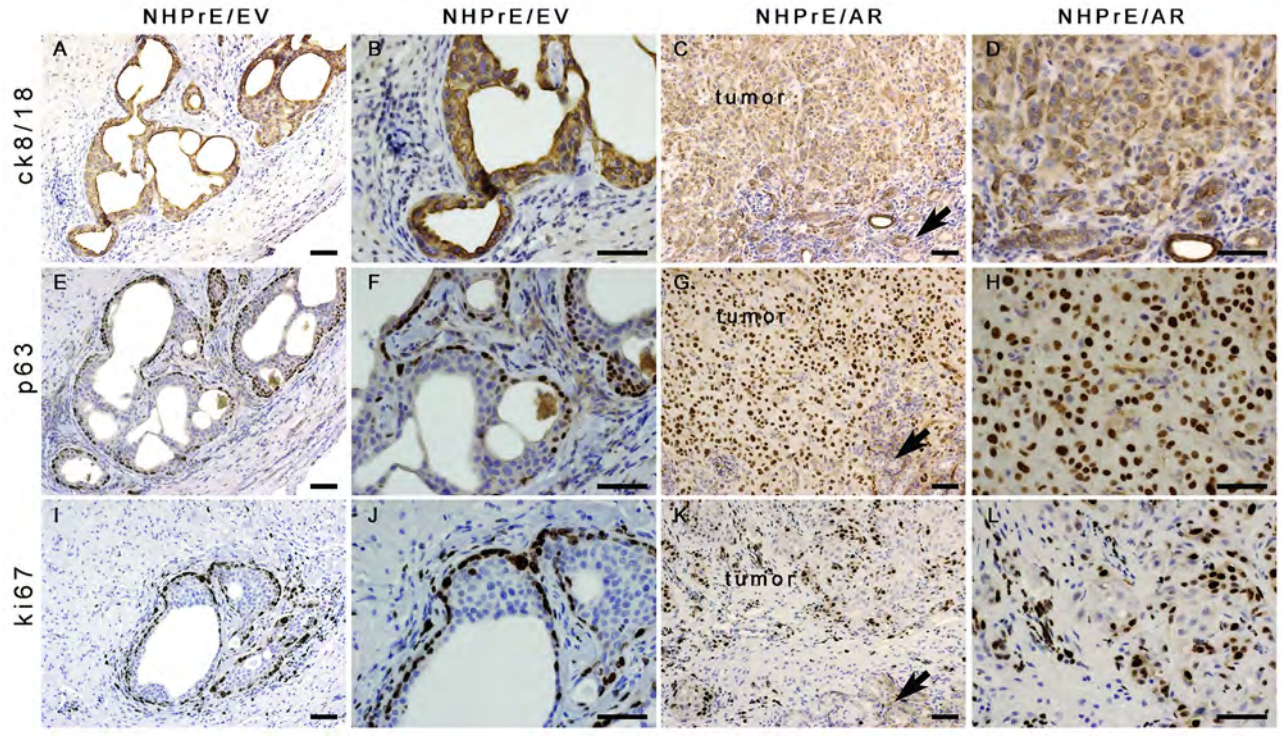


Figure 5: Histology of NHPrE1/EV and NHPrE1/AR grafts. IHC stains for cytokeratin 8/18 (ck8/18, luminal epithelial cell marker), p63 (basal epithelial cell marker), and ki67 (cell proliferation marker) were performed on serial sections derived from NHPrE1/EV **A, B, E, F, I**, and **J.** or NHPrE1/AR grafts **C, D, G, H, K**, and **L.** B, D, F, H, J, and L are higher photomicrographs of A, C, E, G, I, and K, respectively. Arrows in panels C, G, and K indicate host kidney. While NHPrE1/EV control cells formed glandular structures consisting of cytokeratin 8/18-positive luminal epithelial cells (A and B) and p63-positive basal cells (E and F), NHPrE1/AR cells formed invasive carcinomas that were positive for both cytokeratin 8/18 (C and D) and p63 IHC staining (G and H). Ki67 was barely detectable in luminal epithelial cells from NHPrE1/EV grafts (I and J), but was present in many basal cell nuclei in NHPrE1/EV grafts (I and J), as well as in malignant cells in NHPrE1/AR grafts (K and L). Scale bars represent 25 μm.

(PrSC) promoted the proliferation of NHPrE1/AR cells. , when NHPrE1/AR cells were co-cultured with PrSC cells, the inhibitory effect of androgens on cell proliferation was diminished (Figure 7A). These results suggest that factors secreted from prostatic stromal cells may stimulate proliferation of NHPrE1/AR cells. Given that pSTAT3 was induced in the NHPrE1/AR tissue recombinants, we hypothesized that the IL-6/STAT3 pathway, a well-established mechanism of stromal/epithelial communication [30], was involved in the crosstalk between NHPrE1/AR and stromal cells. To test this hypothesis, we assessed the levels of pSTAT3 (Tyr-705) in NHPrE1/EV and NHPrE1/AR cells cultured in the presence or absence of stromal cells. The results showed that co-culture with PrSC cells increased the levels of pSTAT3 in both NHPrE1/EV and NHPrE1/AR cells (Figures 7B-7D). However, co-culture with PrSC did not induce the expression of MYC in these cells (Figure 7B), suggesting that elevated MYC in NHPrE1/AR tissue recombinants may result from the presence of other cellular components in the tumor microenvironment. Also, we utilized an IL-6 neutralizing antibody to block IL-6 signaling in the co-culture system. The IL-6 levels in the cell culture supernatant were measured by ELISA to that addition of IL-6 neutralizing antibody effectively decreased IL-6 levels in the cell culture media (Table 2). While co-culture with stromal cells promoted proliferation of NHPrE1/AR cells, concomitant with an increase of IL-6 in cell co-culture media, addition of IL-6 neutralizing antibodies decreased IL-6 level in cell co-culture media and partially attenuated the restoration of cell proliferation induced by stromal cells (Figure 7E, $p<0.05$). These results suggest that IL-6 pathway is involved in PrSC/NHPrE1 communications. However, addition of IL-6 (25 ng/ml) to the cell culture medium failed to induce the cell proliferation of NHPrE1 cells (Figure 7F), indicating that IL-6 alone is not to promote the proliferation of NHPrE1/AR

Table 1: Quantification of immunostaining

	Ki67 % Mean (SD)	Myc % Mean (SD)	pSTAT3 % Mean (SD)
NHPrE1/EV	18.3	9.8	4.4
NHPrE1/AR	39.8 (5.3)	40.6 (2.6)	36.8 (15.8)

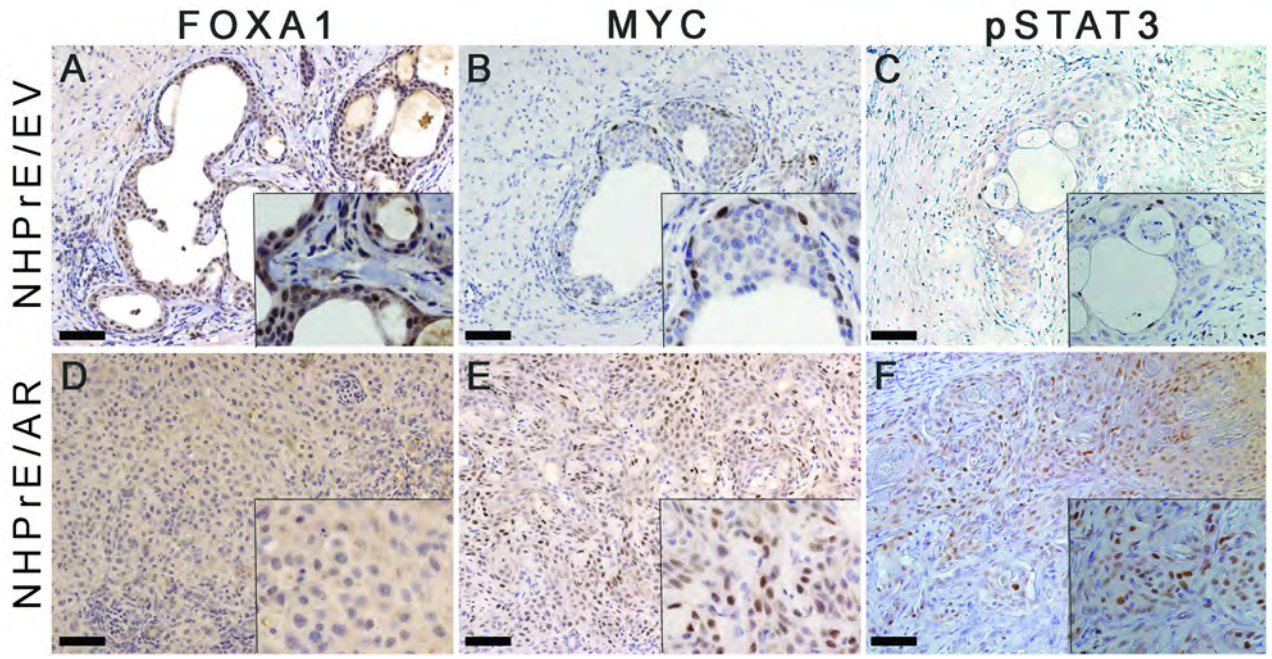


Figure 6: Expression of MYC and pSTAT3, but not FOXA1 was increases in NHPrE1/AR grafts. IHC staining for FOXA1, MYC, and pSTAT3 was performed on serial sections derived from NHPrE1/EV and NHPrE1/AR grafts. Insets in each panel are higher photomicrographs. While NHPrE1/EV grafts showed weak IHC staining for FOXA1 **A.** FOXA1 was not expressed in the majority of epithelial cells in NHPrE1/AR grafts **D.** While only a few basal cells in NHPrE/EV grafts displayed immunoreactivity MYC **B.** MYC was highly expressed in tumor cells from NHPrE1/AR grafts **E.** Malignant cells in some areas of NHPrE1/AR grafts were also positive for pSTAT3 **F.** whereas pSTAT3 levels were negligible in epithelial cells of NHPrE1/EV grafts **C.**

cells and additional signals from PrSC are indispensable for stimulating the proliferation of these cells. Further, we analyzed the relative levels of IL-6 mRNA in PrSC cells, as well as in PrSC cells co-cultured with NHPrE1/EV or NHPrE1/AR cells (Figure 7G). The results showed that when co-cultured with epithelia, PrSC cells produced more IL-6 than when cultured alone ($p<0.001$). A schematic illustration on the two-way stromal/epithelial communication was summarized in Figure 8. Together, these data suggest that stromal cells may help to restore proliferation of NHPrE1/AR cells by releasing IL-6 and possibly other pro-growth factors.

DISCUSSION

Although several studies have examined the growth-modulating effects of inducing AR signaling in prostatic cells *in vitro*, the *in vivo* effect of ectopic-expression of AR has not been well In this study, we chose NHPrE1 cells as a model system to ectopically express AR and study the *in vitro* and *in vivo* effects of inducing AR signaling in these cells. The capacity of NHPrE1cells to form glandular structures when recombined with inductive UGM enabled us to investigate how ectopic expression of AR changed prostatic histomorphology. We found that inducing AR signaling inhibited the proliferation of NHPrE1cells *in vitro*, but surprisingly promoted NHPrE1 cells to form invasive tumors *in vivo*.

In an effort to decipher the mechanisms that caused the differential proliferative responses of NHPrE1 cells to AR signaling *in vitro* and *in vivo*, we conducted stromal/epithelial cell co-cultures. Prostatic the major cellular components of the tumor microenvironment, were used in our co-culture study. Other cellular components, such as endothelium and immune cells, have yet been tested. Our results showed that the presence of prostate stromal cells (PrSC) diminishes the inhibitory effects of androgen, suggesting that factors secreted from PrSC stimulate proliferation of NHPrE1/AR cells. Furthermore, we explored the signaling pathways that might mediate this stromal/epithelial interaction and found that blocking IL-6 signaling partially attenuated the growth restoration

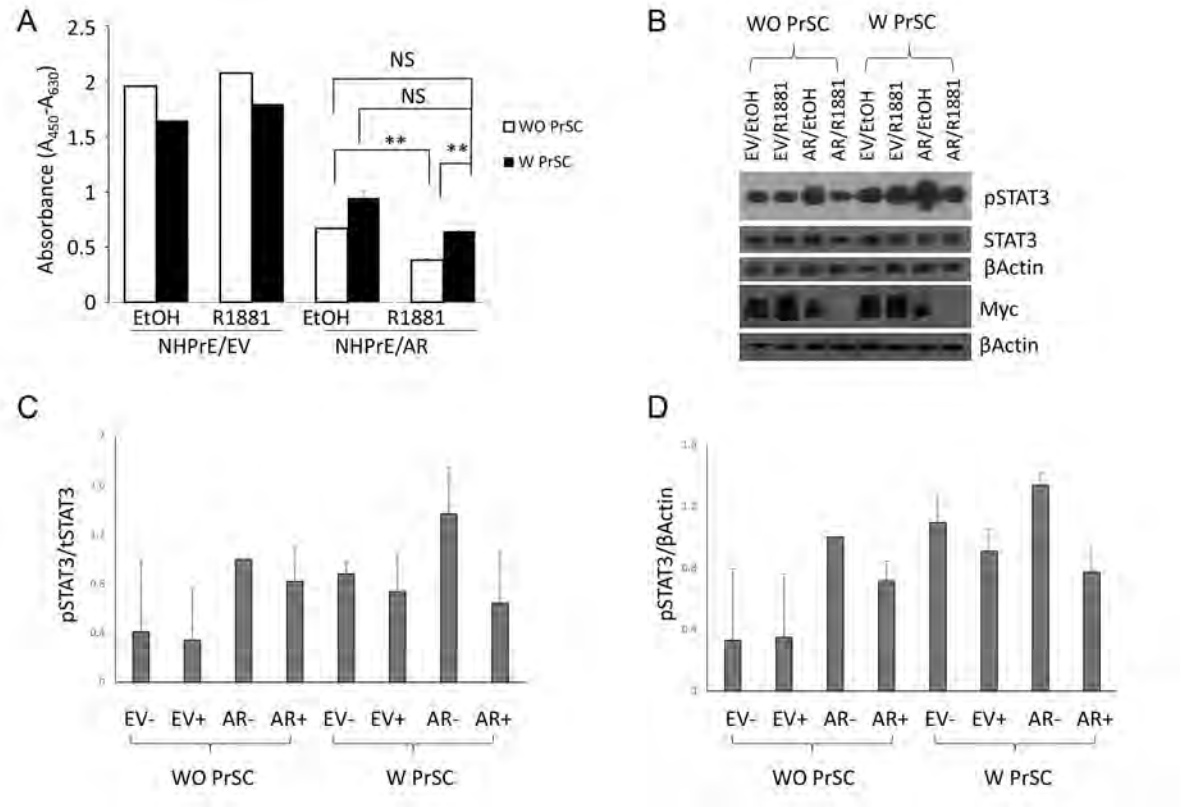


Figure 7: Stromal/epithelial interactions are involved in modulating the proliferation of NHPrE1 cells. A. WST-1 cell proliferation assay to assess proliferation of NHPrE1 cells in the presence or absence of prostatic stromal cells (PrSC). NHPrE1/EV and NHPrE1/AR cells were co-cultured with PrSC for 5 days. While androgen treatment (1 nM R1881) inhibited proliferation of NHPrE1/AR cells in the absence of PrSC, co-culture with PrSC stimulated proliferation of NHPrE1/AR cells and partially reversed the proliferation inhibitory effect of androgens seen *in vitro*. ** $p<0.01$, t-test. **B.** Western blots to assess levels of pSTAT3 in NHPrE1/EV or NHPrE1/AR cells cultured with or without PrSC for 2 days. Levels of pSTAT3 (Tyr-705) and total STAT3 in NHPrE1/EV and NHPrE1/AR cells were compared; beta-Actin served as loading control. Co-culture with PrSC increased the levels of pSTAT3 but not MYC in NHPrE1 cells. **C** and **D.** AT3 Western blot. The levels of pSTAT3 were normalized by total STAT3 (C) or by beta-Actin (D). (*Continued*)

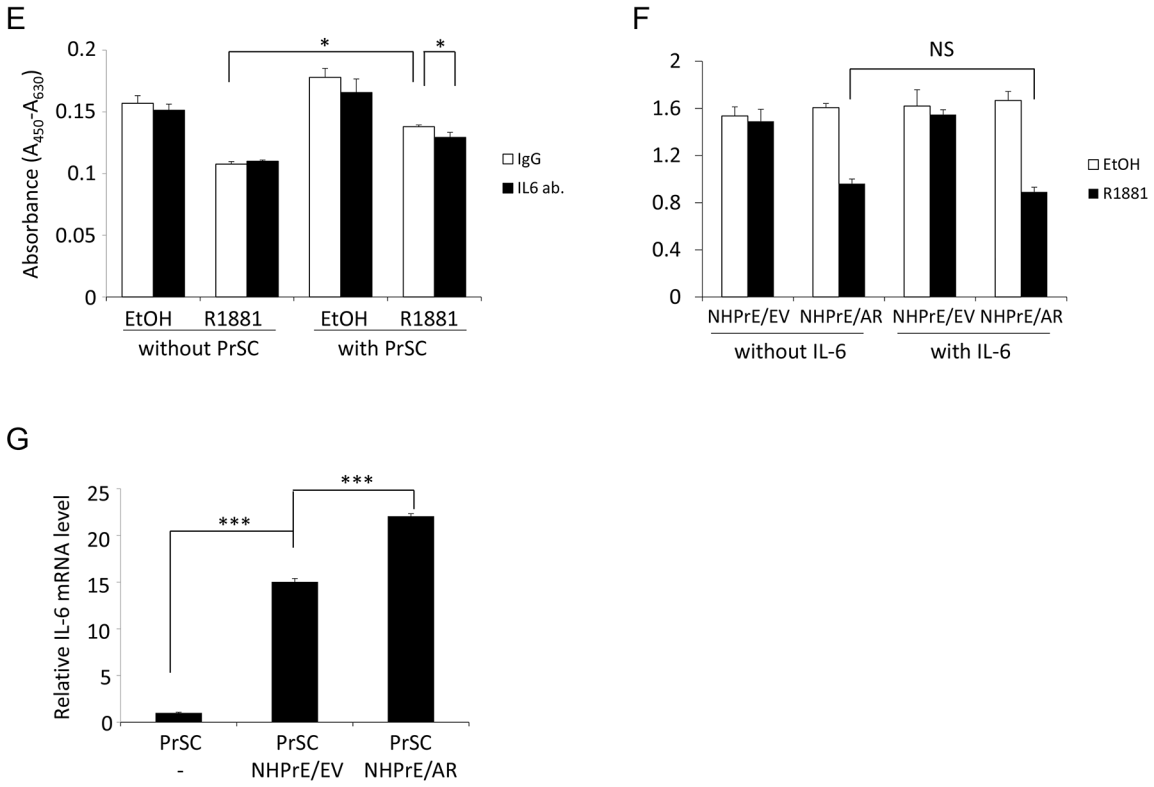


Figure 7: (*Continued*) Stromal/epithelial interactions are involved in modulating the proliferation of NHPrE1 cells. **E.** blocking IL-6 attenuated the stimulatory effect of PrSC on NHPrE1 cell proliferation. NHPrE1/AR were cultured with or without PrSC cells in the presence or absence of IL-6 neutralizing antibody for 3 days. Anti-IL-6 attenuated the proliferation stimulation effect of PrSC. * $p<0.05$, t-test. Similar trend was observed in additional independent experiments. **F.** WST-1 cell proliferation assay. NHPrE1 cells were cultured in the presence or absence of androgens (1 nM R1881) with or without the addition of IL-6 (25 ng/ml). Addition of IL-6 to the cell culture medium did not induce the proliferation of NHPrE1 cells. **G.** RT-qPCR to assess expression of IL-6 in PrSC cells. Prostate stromal cells (PrSC) were cultured in the presence or absence of NHPrE1/EV (N/EV) or NHPrE1/AR (N/AR) cells. Co-culture with prostate epithelial cells stimulated production of IL-6 mRNA in PrSC cells. ***$p<0.001$, t-test.

effect of stromal cells, indicating that IL-6/STAT3 is one of the mechanisms through which PrSC and NHPrE1/AR cells communicate. However, it was also noticed that blocking IL-6 led to only a small decrease in cell proliferation, indicating that other pathways are likely involved in the stroma and epithelia communication. Although co-culture with PrSC induced the level of pSTAT3 in NHPrE1 cells, the expression of MYC in these cells was hardly affected. This suggests that elevated MYC expression in the NHPrE1/AR tissue recombinants may result from signals from other cellular components, such as immune cells, of the tumor microenvironment. Our results also showed that prostate stromal cells expressed more IL-6 mRNA when they were co-cultured with NHPrE1 cells than when cultured alone, indicating that factors secreted from epithelial cells modulate gene expression in adjacent stromal cells. Further research is warranted to identify the factors that mediate the crosstalk from NHPrE1 to PrSC cells.

We found that inducing AR signaling inhibited the proliferation of NHPrE1 cells *in vitro*. This observation differs from a recent study in which androgens slightly promote the proliferation of NHPrE1 cells that have AR stably expressed [31]. We note that while the parental cells used in these two studies are the same, the generation of the AR expressing variants was performed separately with the CMV promoter driving expression in the cells used here and the EF1A promoter in the study reported by Austin et al. As a result these cell strains are not identical with different AR integration sites in the two NHPrE1-AR derivatives. This, along with the different gene expression levels elicited by the two promoters and somewhat different timing and culture conditions may explain the discrepancy observed in these studies.

AR expression was induced less strongly in epithelial cells of the NHPrE1/EV + rUGM tissue recombinants described here than was expected based upon previous studies [18]. The previous study was performed using castrated SCID mice pelleted with testosterone [18], whereas intact nude mice were used in this current study. The lower testosterone levels in the host mice may explain the lower level of induction of AR in the NHPrE1/EV control graft and the less complete differentiation of the epithelial structures illustrated here.

We also found that ectopic expression of AR promoted NHPrE1cell proliferation when recombined with UGM and grafted *in vivo*. This result appears to be at odds with previous *in vivo* studies showing that knockout of AR in prostate luminal epithelial cells results in increased cell proliferation, suggestive of a growth-inhibitory effect of AR signaling in these cells [32]. One explanation for this discrepancy is that the luminal epithelial cells of AR knockout prostates are fully differentiated, but NHPrE1 cells have progenitor features [18]. While recombination with UGM can instruct NHPrE1/EV cells to form benign prostate glandular structures, constitutive expression of AR in NHPrE1 cells alters their response to gland-organizing signals from UGM, resulting in the development of carcinomas. It was also noteworthy that these tumors retained expression of the basal cell marker p63, perhaps suggesting that their pathogenesis is somewhat different from that of prostate tumors in the general population. Additionally, previous studies have shown that androgenic modulation of prostatic growth is biphasic, i.e., androgens either stimulate or inhibit proliferation of prostatic cells depending on the developmental stage of the organ [33, 34]. Whereas androgens stimulate prostatic growth in the prepubertal period, most prostatic cells enter proliferative quiescence after sexual maturation, despite the continuous presence of androgen. Accumulating evidence indicates that AR signaling provides a mechanism to suppress the proliferation of these fully differentiated prostatic luminal epithelial cells [32]. In line with this notion, ectopic expression of AR in PC3 cells induces differentiation [13] and suppresses the growth of PC3/AR tumors *in vivo* [22]. In our case, the progenitor features of NHPrE1 cells may enable these cells to escape from AR-mediated suppression of proliferation.

Our observation that AR signaling differentially regulates prostatic cell proliferation *in vitro* and *in vivo* is in line with a recent study conducted by Neal and colleagues that showed that AR induces a distinct transcriptional program *in vivo* that is not observed in cultured cells [28]. Using a ChIP-Seq approach, they showed that in cultured cells, AR binding sites are associated with potential FOXA1 and NFI binding sites to regulate a set of differentiation-related genes; whereas *in vivo*, AR potentially partners with MYC, STAT, and E2F

Table 2: IL-6 concentration in the culture media of NHPrE1/AR cells

	Secreted IL-6 (pg/ml)			
	Without PrSC		With PrSC	
	EtOH Mean (SD)	R1881 Mean (SD)	EtOH Mean (SD)	R1881 Mean (SD)
IgG (Control)	226.8 (7.7)	100.0 (6.9)	537.7 (1.0)	298.5 (1.5)
IL-6 Blocking Antibody	2.3 (0.2)	2.6 (0.2)	2.6 (0.2)	2.6 (0.2)

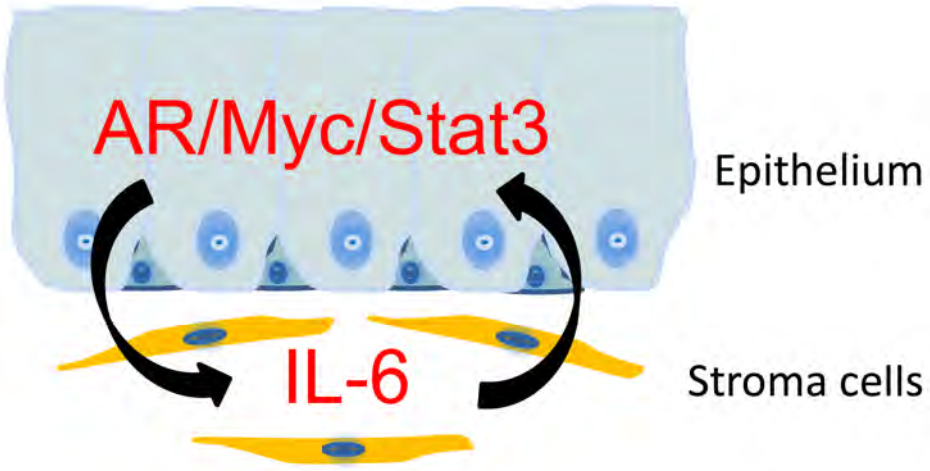


Figure 8: A schematic representation on the stromal/epithelial interaction in NHPrE1/AR tissue recombinants. NHPrE1 cells produce factors that induce the expression of IL-6 in prostate stroma. Prostate stromal cells promote the proliferation of NHPrE1 cells via a mechanism that involves the induction of MYC and pSTAT3 in NHPrE1/AR cells. The combined expression of AR, MYC, and pSTAT3 may transform NHPrE1 cells *in vivo*.

to control the expression of a different set of genes that may modulate cell proliferation [28]. MYC is an oncogene frequently altered in advanced stage PCa [23, 24]. Over-expression of MYC confers an androgen-independent PCa cell growth *in* vitro [35], and over-expression of MYC results in the development of invasive PCa *in* vivo [36]. The association of MYC expression with AR modulation of the proliferation of NHPrE1 cells and the high expression of MYC in NHPrE1/AR tissue recombinants further suggests involvement of MYC in transforming NHPrE1/AR cells *in vivo*. IL-6/STAT3 is involved in the communications between prostate tumor cells and the microenvironment. Moreover, STAT3 is an important modulator of AR signaling in the prostate [30]. The combination of the expression of MYC, STAT3, and AR in NHPrE1/AR grafts may reprogram the AR transcriptome and promote neoplastic transformation of these cells.

Our study also found, in contrast to the elevated expression of MYC and pSTAT3, that FOXA1 was not expressed in the malignant cells in NHPrE1/AR grafts. However, FOXA1 expression was detected in the glandular epithelial cells of the NHPrE1/EV tissue recombinant, likely the differentiation status of the cells, illustrated by the absence of basal cell markers. FOXA1 is a well-established AR co-activator [29], and previous studies have suggested that the AR/FOXA1 complex is involved in controlling differentiation-related genes instead of proliferation-related genes in prostatic cells. For example, studies have shown that FOXA1 interacts with AR to regulate the expression of prostate- genes such as PSA, PAP, and SBP [29]. More recent studies have shown that, as a pioneer transcription factor, FOXA1 recruits AR to the promoters of a set of genes that prostate differentiation. However, depletion of FOXA1 in PCa cells did not cause AR to lose all its binding sites; instead, the AR transcriptome was reprogrammed and new AR binding sites were found on the promoters of a distinct set of genes not observed in parental cells [37, 38]. These new AR target genes may be involved in promoting PCa progression. Consistent with the role of FOXA1 in regulating the differentiation of prostate epithelial cells, FOXA1 mutations are observed in advanced stage human PCa [39, 40] and inactivation of FOXA1 gene in prostate epithelial cells promotes prostatic hyperplasia in murine models [41]. Conversely, ectopic expression of FOXA1 inhibits the invasive capacity of PC3 and DU145 cells [40], thereby conferring a less aggressive phenotype in cells that represent advanced stage PCa. Taken together, these studies suggest that expression of FOXA1 restrains the AR transcriptome to genes related to differentiated function and that the lack of FOXA1 expression permits a switch in the AR transcriptome that results in enhanced cell proliferation. Therefore, in addition to the induction of MYC and pSTAT3, the lack of FOXA1 expression in NHPrE1/AR cells *in vivo* might be another contributor to, or indicator of, the transformation of these cells.

In conclusion, in this study, we found that AR signaling differentially regulated the proliferation of NHPrE1 cells *in vitro* and *in vivo* via mechanisms that involved prostate stromal/epithelial interactions.

MATERIALS AND METHODS

Cell culture

NHPrE1 cells [18] were maintained in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 1% penicillin/streptomycin (Gibco), 10 ng/ml epidermal growth factor (Sigma– Aldrich, St. Louis, MO), 1% insulin-transferrin-selenium (ITS) (Gibco), and 0.4% bovine pituitary extract (Atlanta Biologicals). NHPrE1 cells were transfected with EGFP-expressing plasmid; GFP-positive NHPrE1 cells were selected by cell sorting. To establish AR-expressing NHPrE1/GFP cells, CMV promoter-driven LNCX or LNCX-AR retroviral vector-based plasmids were transfected into Phoenix packaging cells (ATCC, Manassas, VA). Twenty-four hours later, culture media were collected and used to infect NHPrE1/GFP cells. The infection procedure was repeated twice. The transduced cells, stably expressing AR, were selected by culturing them in the presence of G418 (Sigma, St. Louis, MO, USA). G418 resistant cell populations were used in this study. American Type Culture Collection (ATCC) has authenticated NHPrE1 cells and no contamination from other type of cells was found. PC3/AR [21] and LNCaP (ATCC) cells were cultured in RPMI1640 supplemented with10% serum.

Cell proliferation assay

Both IncuCyte and WST-1 methods were used to assess the proliferation of NHPrE1/EV and NHPrE1/AR cells. For IncuCyte cell proliferation assay, NHPrE1/EV or NHPrE1/AR cells (1500 cells per well, 96-well plate) were cultured in DMEM medium containing 5% charcoal-stripped, heat inactivated serum (Atlanta Biologicals) without other additives to avoid the cross-activation of AR by exogenous growth factors. After the cells are attached, the cell culture medium were changed to DMEM-5% charcoal-stripped serum with or without the addition of androgens (1 nM R1881 or 0.1% ethanol) for up to 140 hours. Cell was monitored every 4 hours. For WST- assay (Roche Applied Science, Indianapolis, IN), NHPrE1/EV or NHPrE1/AR cells were cultured in DMEM-5% charcoal-stripped serum with or without the addition of 10 nM DHT. Cell culture medium were replenished daily when DHT was used due to its metabolic instability. WST-1 cell proliferation assay was conducted according to manufacturer's instruction. Ten μL WST-1

reagent was added to each well and incubated at 37°C for 1 hour. Absorbance at 440-450 nm (630 nm was used as reference wavelength) was measured using a microplate reader.

Co-culture of prostate stromal and epithelial cells

NHPrE1 cells were cultured with or without primary prostate stromal cells (PrSC) (Lonza, Williamsport, PA). NHPrE1/EV (empty vector) or NHPrE1/AR cells were seeded overnight in 24-well plates in DMEM media containing 5% charcoal-stripped serum. The next day, a WST-1 assay was conducted to assess for equal seeding of each cell line. PrSC cells were then seeded into cell culture inserts (0.4 μm pores) and co-cultured with NHPrE1 cells in DMEM media containing 5% charcoal-stripped serum supplemented with R1881 (1 nM) (Sigma) or ethanol for 5 days. Cell culture medium were replenished every other day. In some experiments, IL-6 neutralizing antibody concentration 2 μg/ml, R&D system, Minneapolis, MN) was added to block IL-6 signaling; goat IgG served as a negative control. WST-1 cell proliferation assays were conducted to assess the proliferation of NHPrE1 cells that were co-cultured with PrSC according to the manufacturer's instructions. For experiments where the expression of IL-6 in PrSC cells was examined, PrSC cells were seeded in 6-well plates and co-cultured with NHPrE1/EV or NHPrE1/AR cells for 2 days. RNA was extracted from PrSC cells and levels of IL-6 mRNA were assessed by quantitative (q)RT-PCR.

Western blot analysis

Protein lysates were prepared from prostatic cells as described previously [43]. Twenty-micrograms of total protein was loaded for electrophoresis. After transfer, membranes were blocked in 5% non-fat milk for one hour, incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA) at room temperature for one hour. ECL-Plus detection system (PerkinElmer, Waltham, MA) was used to visualize immunolocalization. Rabbit antibodies against MYC were purchased from Epitomics (Burlingame, CA), AR from Santa Cruz Biotechnology (Santa Cruz, CA), STAT3 and pSTAT3 from Cell Signaling Technology (Danvers, MA), and β-actin from Sigma.

Cycloheximide chase analysis

Cycloheximide chase analysis was conducted to determine the half-life of MYC in NHPrE1/AR cells in the presence or absence of androgen. Cells were treated with 50 μg/ml cycloheximide (Sigma) to block protein synthesis in the presence or absence of 10 nM DHT and harvested at 0, 0.5, 1, 2, and 4 hours post-treatment. Western blotting was performed using anti-MYC antibody and band intensities were measured by using Image J (NIH).

Histology and immunohistochemical staining

Immunohistochemistry (IHC) was conducted as described previously [27]. Tissues were in 10% buffered formalin overnight and processed to IHC stains were performed following routine and rehydration of 5 μm sections. Antigen retrieval was performed by microwaving slides for 20 min in boiling antigen-unmasking solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked with DAKO Peroxidase Blocking Reagent (DAKO, Carpinteria, CA) for 15 min. Sections were incubated with primary antibodies at 4°C overnight in a chamber. Antibodies used were: AR, p63, FOXA1, and GFP (Santa Cruz Biotechnology), MYC (Epitomics, Burlingame, CA), pSTAT3 (Cell Signaling Technology), and Ki67 (Abcam, Cambridge, MA). antibody binding was detected using the Vectastain Elite ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol with the DAKO DAB-Chromogen System (DAKO, Carpinteria, CA). Sections were counterstained with hematoxylin, dehydrated, and cover-slipped.

Tissue recombination-Xenografting

Tissue recombination experiments were conducted as described previously [27]. , 6×10^5 NHPrE1/EV or NHPrE1/AR cells were recombined with 3×10^5 rat UGM cells in 50 μl of neutralized type I rat tail collagen to make the tissue recombinants. recombinants were cultured overnight and then grafted beneath the renal capsules of adult male nude mice. Host mice were 3 months later by anesthetic overdose followed by cervical dislocation. Kidneys were excised, and grafts were dissected and processed for histology and immunohistochemistry. All the animal experiments were approved by the Institutional Animal Care and Use Committee.

Statistical analysis

All the experiments were conducted in triplicate and repeated at least once. Statistical was evaluated using a two-sided Student's t test and a p-value of 0.05 was considered statistically of immunohistochemistry staining was conducted using ImmunoRatio program.

ACKNOWLEDGMENTS

We thank Dr. Robert Matusik for advice on the research presented here.

CONFLICTS OF INTEREST

GRANT SUPPORT

This research was supported by DOD award (W81XWH-12-1-0212), LSUHSC Feist-Weiller Cancer Center Foundation Legacy Funds, and LSU Health Sciences Center Shreveport of Research Funds to X Yu, and grants supported by The National Natural Science Foundation of China and the Research Foundation for Jiangsu Specially-Appointed Professor (Sujiaoshi [2012] No. 34) to M Jiang.

REFERENCES

1. Huggins C: Effect of Orchiectomy and Irradiation on Cancer of the Prostate. Annals of surgery 1942, 115:1192-200.
2. Debes JD, Tindall DJ: Mechanisms of androgen-refractory prostate cancer. N Engl J Med 2004, 351:1488-90.
3. Wang Y, Hayward S, Cao M, Thayer K, Cunha G: Cell differentiation lineage in the prostate. Differentiation; research in biological diversity 2001, 68:270-9.
4. Shabsigh A, Chang DT, Heitjan DF, Kiss A, Olsson CA, Puchner PJ, Buttyan R: Rapid reduction in blood to the rat ventral prostate gland after castration: preliminary evidence that androgens prostate size by regulating blood to the prostate gland and prostatic endothelial cell survival. Prostate 1998, 36:201-6.
5. Shabsigh A, Tanji N, D'Agati V, Burchardt T, Burchardt M, Hayek O, Shabsigh R, Buttyan R: Vascular anatomy of the rat ventral prostate. Anat Rec 1999, 256:403-11.
6. Kurita T, Wang YZ, Donjacour AA, Zhao C, Lydon JP, O'Malley BW, Isaacs JT, Dahiya R, Cunha GR: Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. Cell Death Differ 2001, 8:192-200.
7. Wang Y, Sudilovsky D, Zhang B, Haughney PC, Rosen MA, Wu DS, Cunha TJ, Dahiya R, Cunha GR, Hayward SW: A human prostatic epithelial model of hormonal carcinogenesis. Cancer research 2001, 61:6064-72.
8. Placencio VR, Sharif-Afshar AR, Li X, Huang H, Uwamariya C, Neilson EG, Shen MM, Matusik RJ, Hayward SW, Bhowmick NA: Stromal transforming growth factor-beta signaling mediates prostatic response to androgen ablation by paracrine Wnt activity. Cancer research 2008, 68:4709-18.
9. Siwela AA, Tam WH: Ultrastructural changes in the prostate gland of a seasonally breeding mammal, the grey squirrel (Sciurus carolinensis, Gmelin). J Anat 1984, 138:153-62.
10. van Steenbrugge GJ, Groen M, van Dongen JW, Bolt J, van der Korput H, Trapman J, Hasenson M, Horoszewicz J: The human prostatic carcinoma cell line LNCaP and its derivatives. An overview. Urological research 1989, 17:71-7.
11. Zhau HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, Sang QA, Pathak SJ, Chung LW: Androgen-repressed phenotype in human prostate cancer. Proceedings of the National Academy of Sciences of the United States of America 1996, 93:15152-7.
12. Chuu CP, Kokontis JM, Hiipakka RA, Fukuchi J, Lin HP, Lin CY, Huo C, Su LC, Liao S: Androgen suppresses proliferation of castration-resistant LNCaP 104-R2 prostate cancer cells through androgen receptor, Skp2, and c-Myc. Cancer science 2011, 102:2022-8.
13. Yuan S, Trachtenberg J, Mills GB, Brown TJ, Xu F, Keating A: Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA. Cancer research 1993, 53:1304-11.
14. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ: Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 1997, 126:59-73.
15. Antony L, van der Schoor F, Dalrymple SL, Isaacs JT: Androgen receptor (AR) suppresses normal human prostate epithelial cell proliferation via AR/beta-catenin/TCF-4 complex inhibition of c-MYC transcription. The Prostate 2014, 74:1118-31.
16. Vander Griend DJ, Litvinov IV, Isaacs JT: Conversion of androgen receptor signaling from a growth suppressor in normal prostate epithelial cells to an oncogene in prostate cancer cells involves a gain of function in c-Myc regulation. International journal of biological sciences 2014, 10:627-42.
17. Lee SO, Tian J, Huang CK, Ma Z, Lai KP, Hsiao H, Jiang M, Yeh S, Chang C: Suppressor role of androgen receptor in proliferation of prostate basal epithelial and progenitor cells. J Endocrinol 2012, 213:173-82.
18. Jiang M, Strand DW, Fernandez S, He Y, Yi Y, Birbach A, Qiu Q, Schmid J, Tang DG, Hayward SW: Functional remodeling of benign human prostatic tissues in vivo by spontaneously immortalized progenitor and intermediate cells. Stem cells 2010, 28:344-56.
19. Furr BJ, Tucker H: The preclinical development of bicalutamide: pharmacodynamics and mechanism of action. Urology 1996, 47:13-25; discussion 9-32.
20. Litvinov IV, Antony L, Dalrymple SL, Becker R, Cheng L, Isaacs JT: PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor. The Prostate 2006, 66:1329-38.
21. Litvinov IV, Antony L, Isaacs JT: Molecular characterization of an improved vector for evaluation of the tumor suppressor versus oncogene abilities of the androgen receptor. The Prostate 2004, 61:299-304.

22. Niu Y, Altuwaijri S, Lai KP, Wu CT, Ricke WA, Messing EM, Yao J, Yeh S, Chang C: Androgen receptor is a tumor suppressor and proliferator in prostate cancer. Proceedings of the National Academy of Sciences of the United States of America 2008, 105:12182-7.

23. Koh CM, Bieberich CJ, Dang CV, Nelson WG, Yegnasubramanian S, De Marzo AM: MYC and Prostate Cancer. Genes Cancer 2010, 1:617-28. doi: 10.1177/1947601910379132.

24. Fleming WH, Hamel A, MacDonald R, Ramsey E, Pettigrew NM, Johnston B, Dodd JG, Matusik RJ: Expression of the c-myc protooncogene in human prostatic carcinoma and benign prostatic hyperplasia. Cancer research 1986, 46:1535-8.

25. Hann SR: Role of post-translational in regulating c-Myc proteolysis, transcriptional activity and biological function. Seminars in cancer biology 2006, 16:288-302.

26. Hayward SW, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR: Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. Differentiation; research in biological diversity 1998, 63:131-40.

27. Wang Y, Kasper S, Yuan J, Jin RJ, Zhang J, Ishii K, Wills ML, Hayward SW, Matusik RJ: Androgen-dependent prostate epithelial cell selection by targeting ARR(2)PBneo to the LPB-Tag model of prostate cancer. Laboratory investigation; a journal of technical methods and pathology 2006, 86:1074-88.

28. Sharma NL, Massie CE, Ramos-Montoya A, Zecchini V, Scott HE, Lamb AD, MacArthur S, Stark R, Warren AY, Mills IG, Neal DE: The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man. Cancer cell 2013, 23:35-47.

29. Gao N, Zhang J, Rao MA, Case TC, Mirosevich J, Wang Y, Jin R, Gupta A, Rennie PS, Matusik RJ: The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. Molecular endocrinology 2003, 17:1484-507.

30. Bishop JL, Thaper D, Zoubeidi A: The Multifaceted Roles of STAT3 Signaling in the Progression of Prostate Cancer. Cancers 2014, 6:829-59.

31. Austin DC, Strand DW, Love HL, Franco OE, Jang A, Grabowska MM, Miller NL, Hameed O, Clark PE, Fowke JH, Matusik RJ, Jin RJ, Hayward SW: NF-kappaB and androgen receptor variant expression correlate with human BPH progression. The Prostate 2016, 76:491-511.

32. Wu CT, Altuwaijri S, Ricke WA, Huang SP, Yeh S, Zhang C, Niu Y, Tsai MY, Chang C: Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. Proceedings of the National Academy of Sciences of the United States of America 2007, 104:12679-84.

33. Olea N, Sakabe K, Soto AM, Sonnenschein C: The proliferative effect of "anti-androgens" on the androgen-sensitive human prostate tumor cell line LNCaP. Endocrinology 1990, 126:1457-63.

34. Geck P, MV, Szelei J, Sonnenschein C, Soto AM: Androgen-induced proliferative quiescence in prostate cancer cells: the role of AS3 as its mediator. Proceedings of the National Academy of Sciences of the United States of America 2000, 97:10185-90.

35. Bernard D, Pourtier-Manzanedo A, Gil J, Beach DH: Myc confers androgen-independent prostate cancer cell growth. The Journal of clinical investigation 2003, 112:1724-31.

36. Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, Sawyers CL: Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer cell 2003, 4:223-38.

37. Jin HJ, Zhao JC, Wu L, Kim J, Yu J: Cooperativity and equilibrium with FOXA1 the androgen receptor transcriptional program. Nature communications 2014, 5:3972.

38. Yang YA, Yu J: Current perspectives on FOXA1 regulation of androgen receptor signaling and prostate cancer. Genes & Diseases 2015, 2:144-51.

39. Barbieri CE, Baca SC, Lawrence MS, Demichelis F, Blattner M, Theurillat JP, White TA, Stojanov P, Van Allen E, Stransky N, Nickerson E, Chae SS, Boysen G, et al. Exome sequencing recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nature genetics 2012, 44:685-9.

40. Jin HJ, Zhao JC, Ogden I, Bergan RC, Yu J: Androgen receptor-independent function of FoxA1 in prostate cancer metastasis. Cancer research 2013, 73:3725-36.

41. DeGraff DJ, Grabowska MM, Case TC, Yu X, Herrick MK, Hayward WJ, Strand DW, Cates JM, Hayward SW, Gao N, Walter MA, Buttyan R, Yi Y, et al. FOXA1 deletion in luminal epithelium causes prostatic hyperplasia and alteration of differentiated phenotype. Laboratory investigation; a journal of technical methods and pathology 2014, 94:726-39.

42. Uphoff CC, Drexler HG: Comparative PCR analysis for detection of mycoplasma infections in continuous cell lines. In Vitro Cell Dev Biol Anim 2002, 38:79-85.

43. Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ: Wnt/beta-catenin activation promotes prostate tumor progression in a mouse model. Oncogene 2011, 30:1868-79.

AR-positive prostate cancer 22Rv1 cells were inoculated into the tibiae of SCID mice. The host mice were treated with CXCR4 inhibitor (AMD3100) or Wnt/beta-Catenin inhibitor (ICG-001). The tumor growth and bone lesions were monitor by x-ray imaging. As shown in Figure 1, 22Rv1 generated mixed osteoclastic/osteoblastic bone lesions. Growing of these PCa cells caused lytic destruction to the cortical bone but stimulated new bone formation (new bony spikes grew outside of cortical bones). The lytic bone lesions and the new bone formation were analyzed by using microCT scanning (pages 3 to 8). Our analysis (Figure 2) showed that castration increased lytic bone lesions. CXCR4 inhibitor (AMD3100) and Wnt inhibitor (ICG-001) did not decreased lytic bone destruction in intact mice (CXCR4 inhibitor even increased lytic lesions). These inhibitors decrease lytic lesions in castrated mice, but did not affect the overall lytic lesions compared to intact non-treated mice.

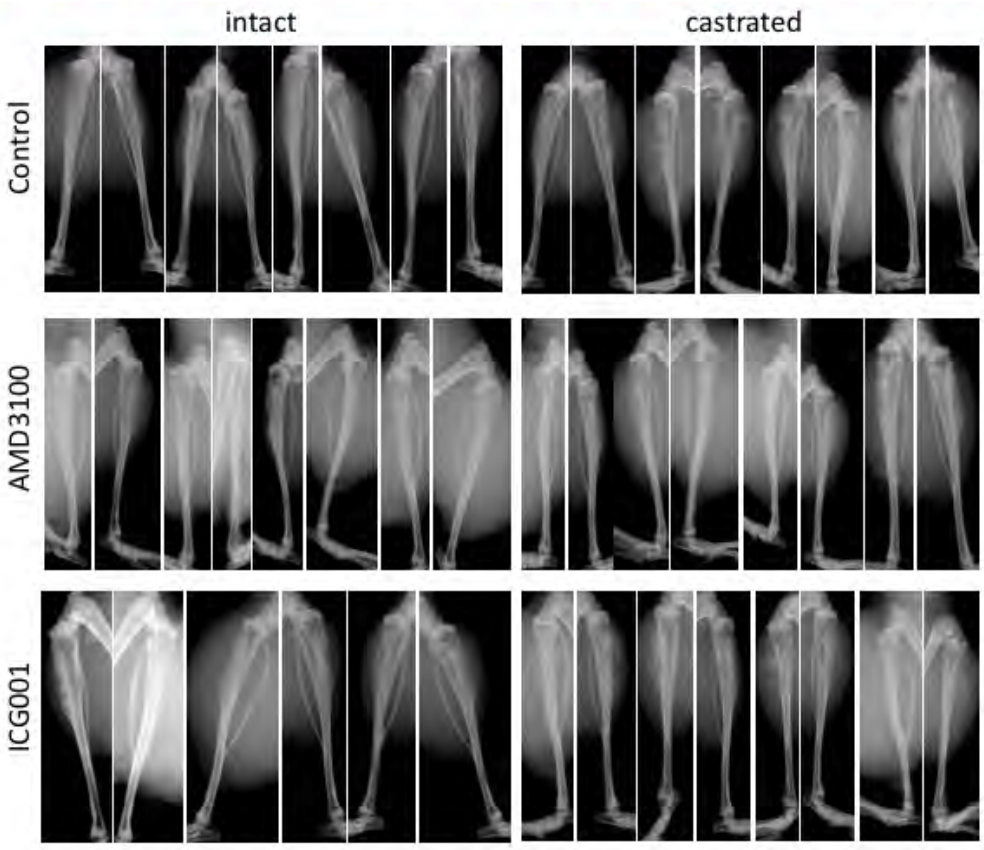


Figure 1. X-ray images. When injected into tibiae, 22Rv1 cells generated mixed osteoclastic and osteoblastic bone lesions. Treatment with CXCR4 inhibitor (AMD3100) or Wnt/beta-Catenin inhibitor (ICG-001) did not decrease the overall bone destruction.

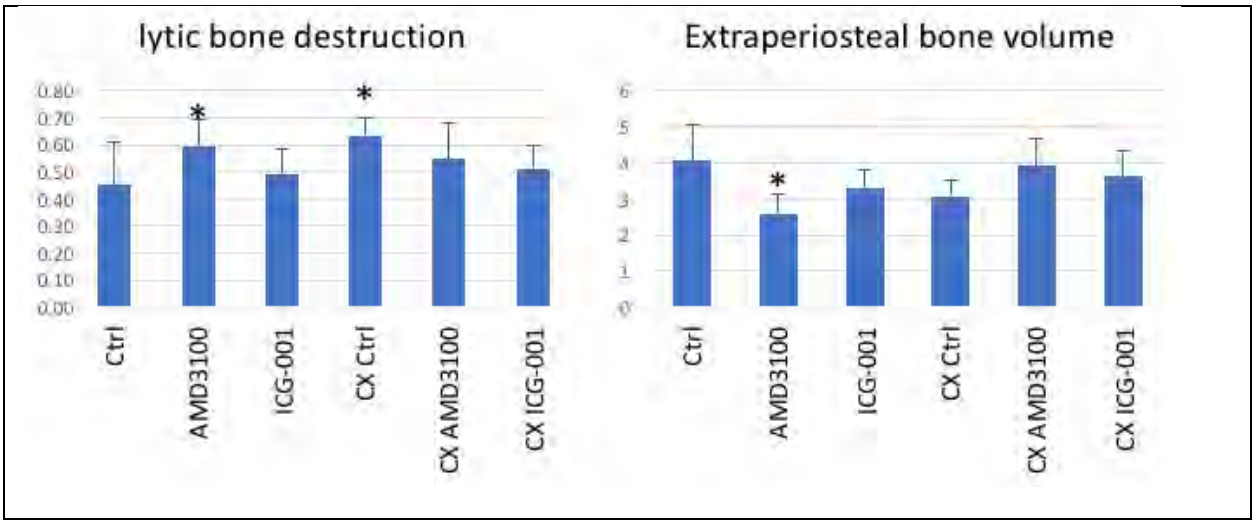


Figure 2. Summary of microCT results (images are in pages 3-8).

Left: lytic bone lesions

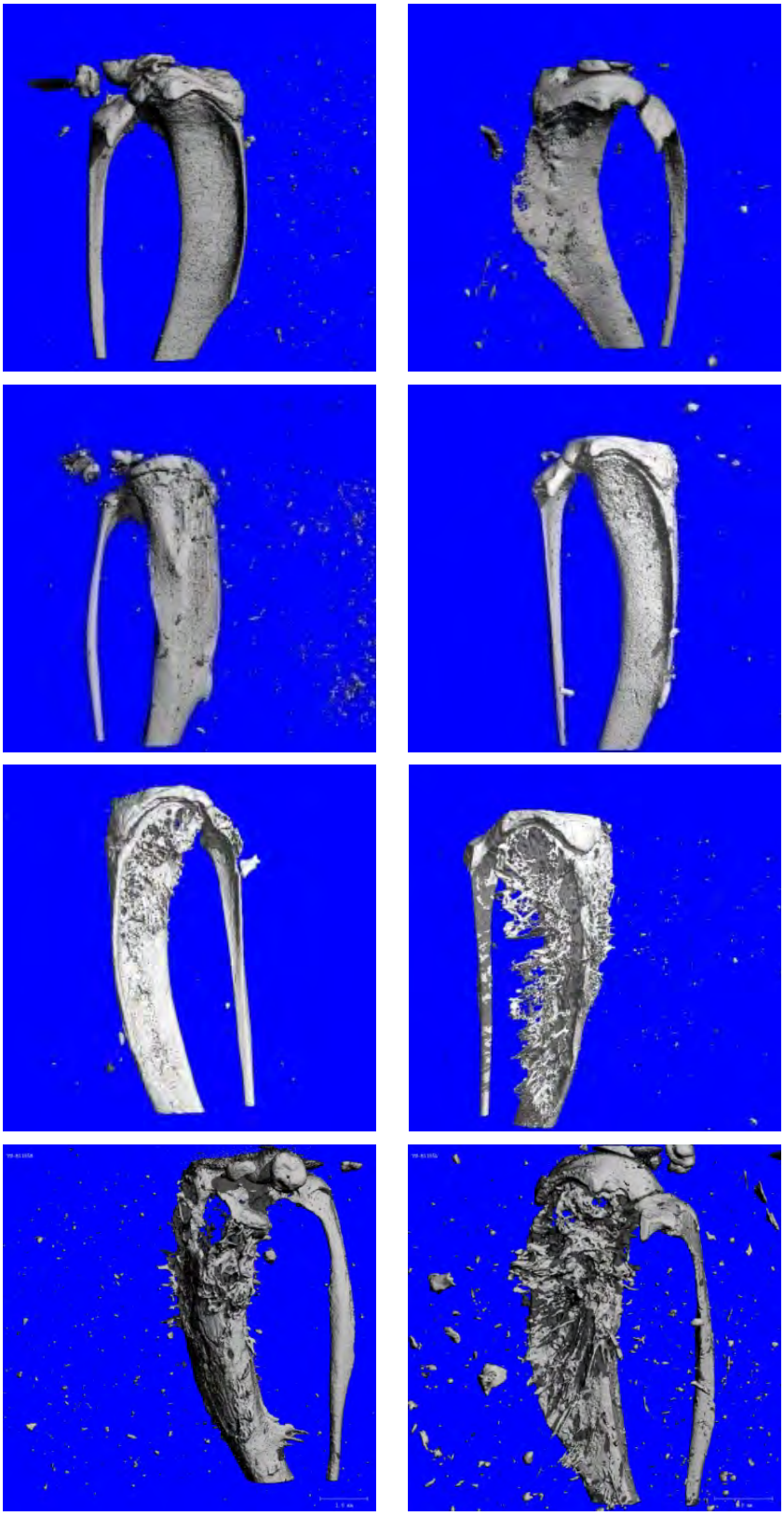
1. Surprisingly, castration increased lytic bone lesion
2. CXCR4 inhibitor also increased bone destruction in intact mice.
3. Wnt inhibitor did not affect bone destruction

Right: Osteoblastic bone lesion

1. Castration decreased new bone formation.
2. CXCR4 inhibitor decreased new bone formation in intact mice but not in castrated mice
3. Wnt inhibitor did not affect new bone formation.

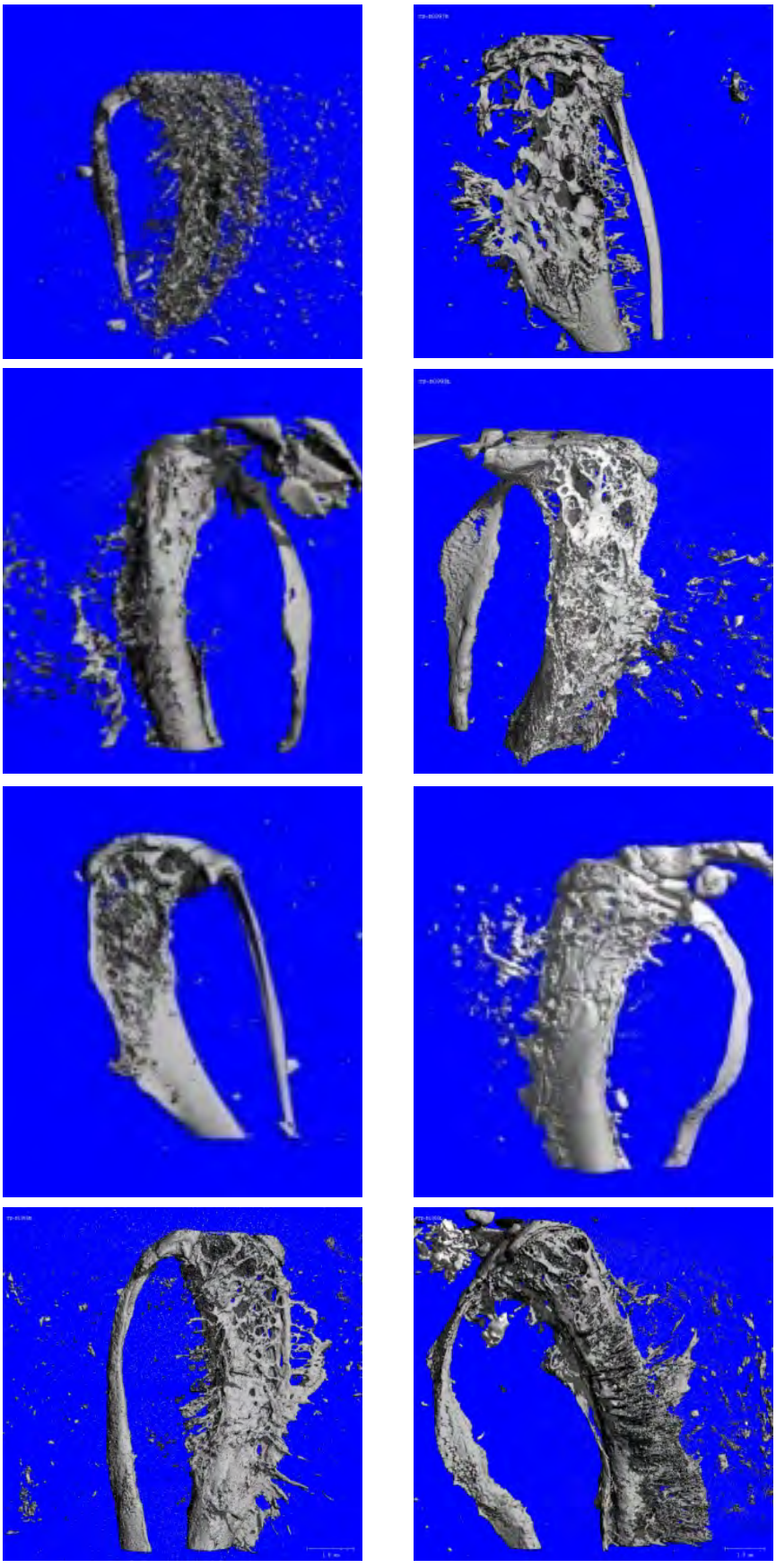
µCT images

Vehicle control



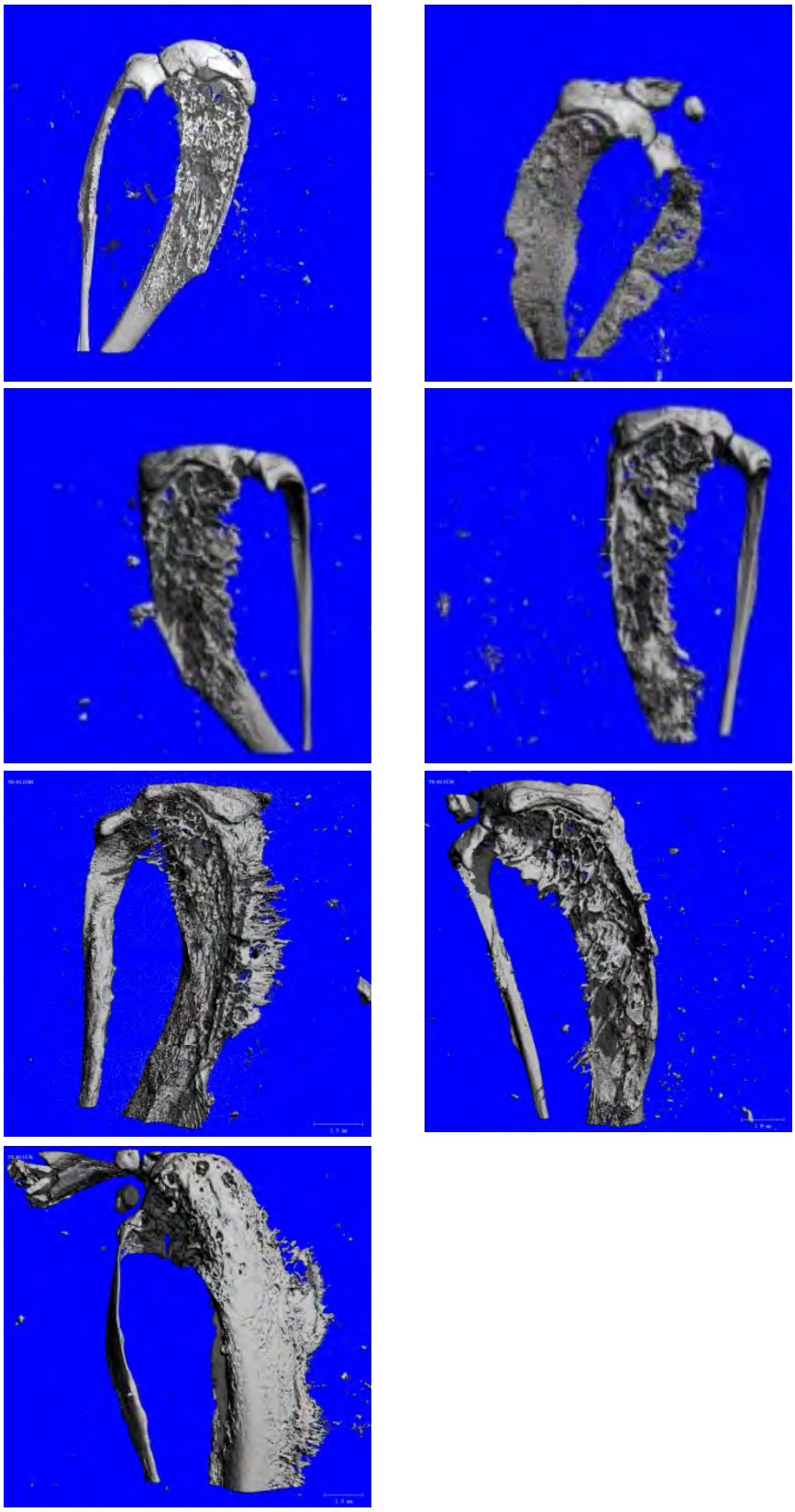
µCT images

CXCR4 inhibitor- AMD3100



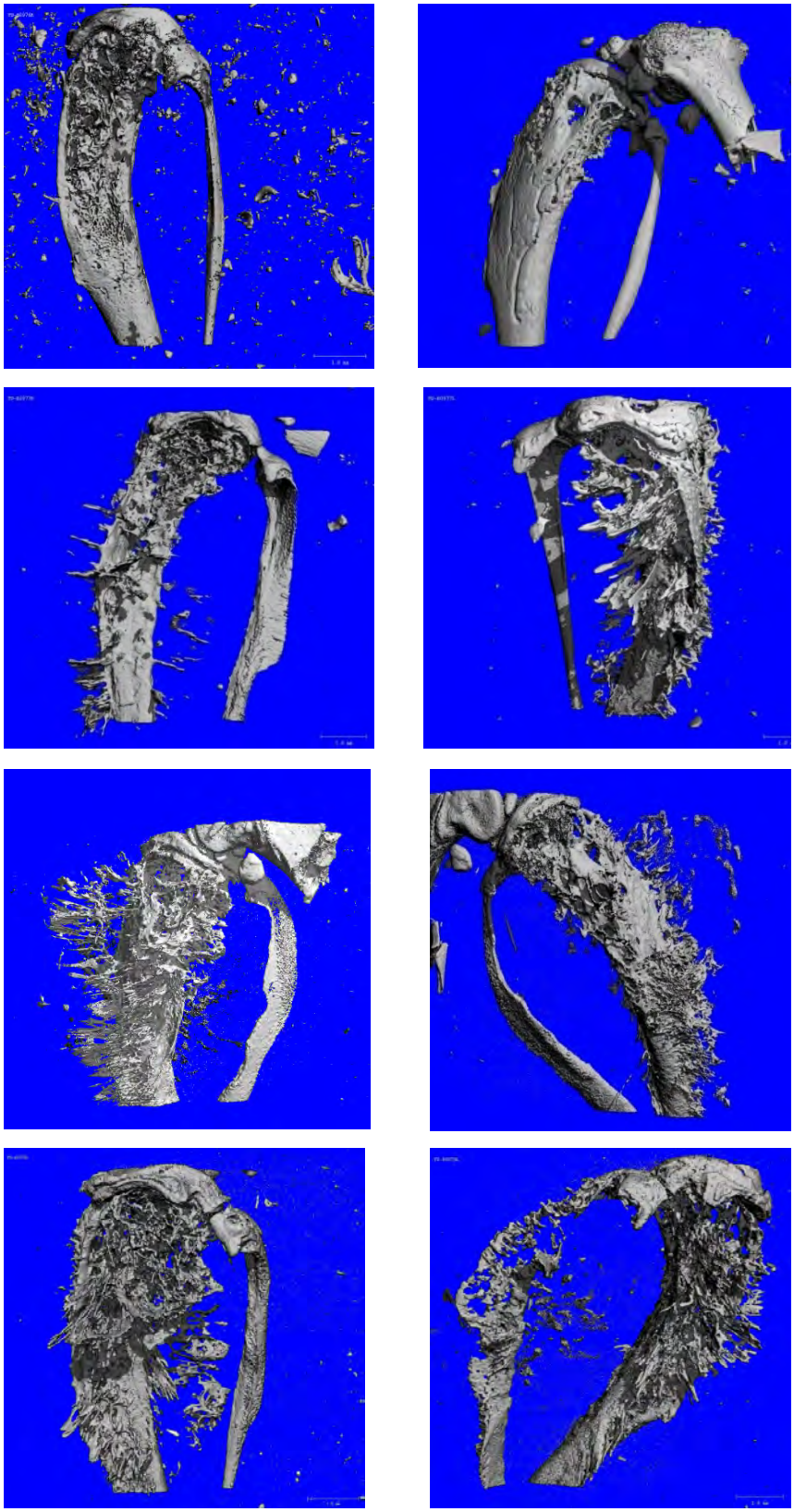
µCT images

Wnt/beta-Catenin inhibitor- ICG001



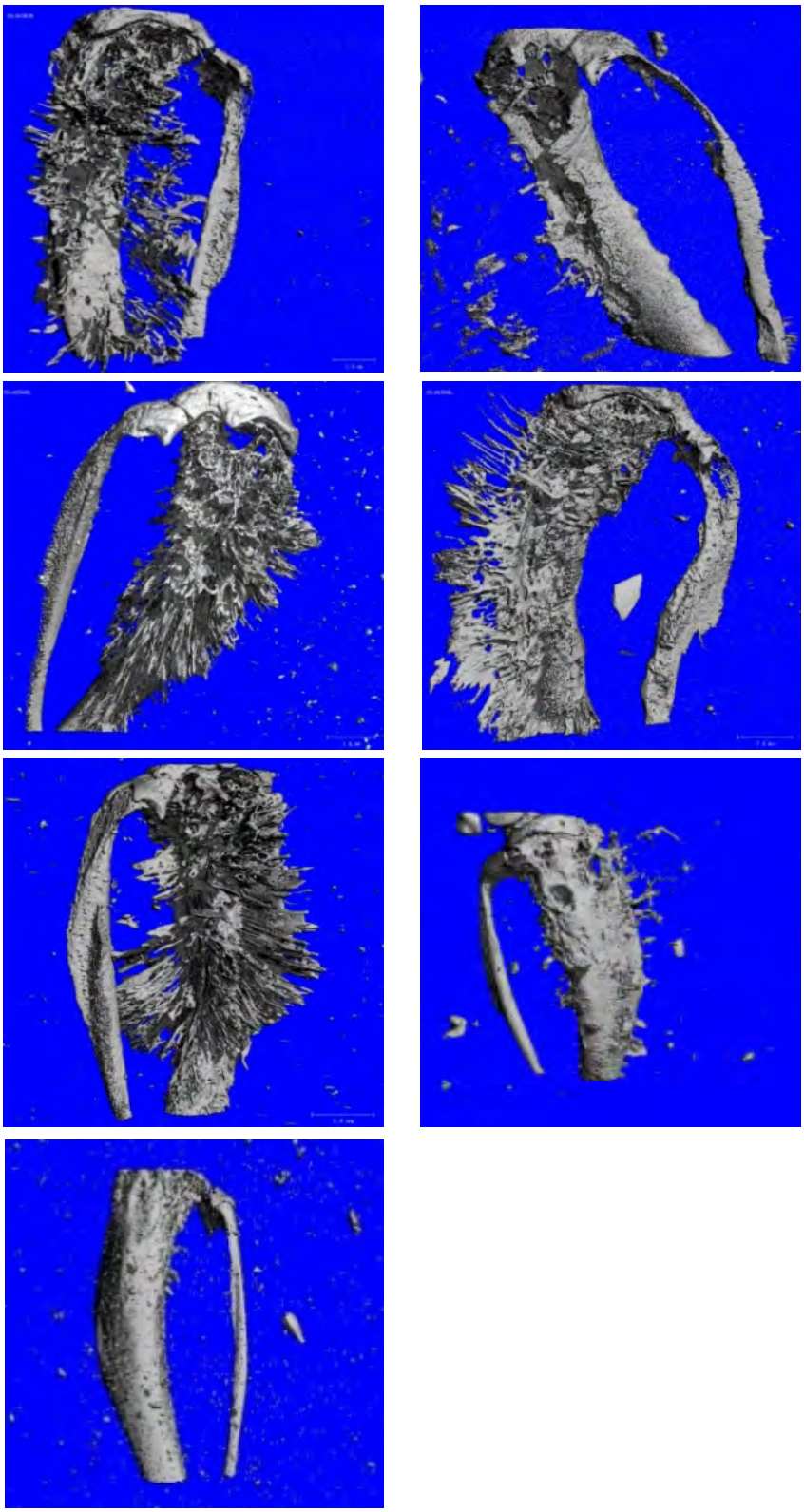
µCT images

Vehicle control, castrated



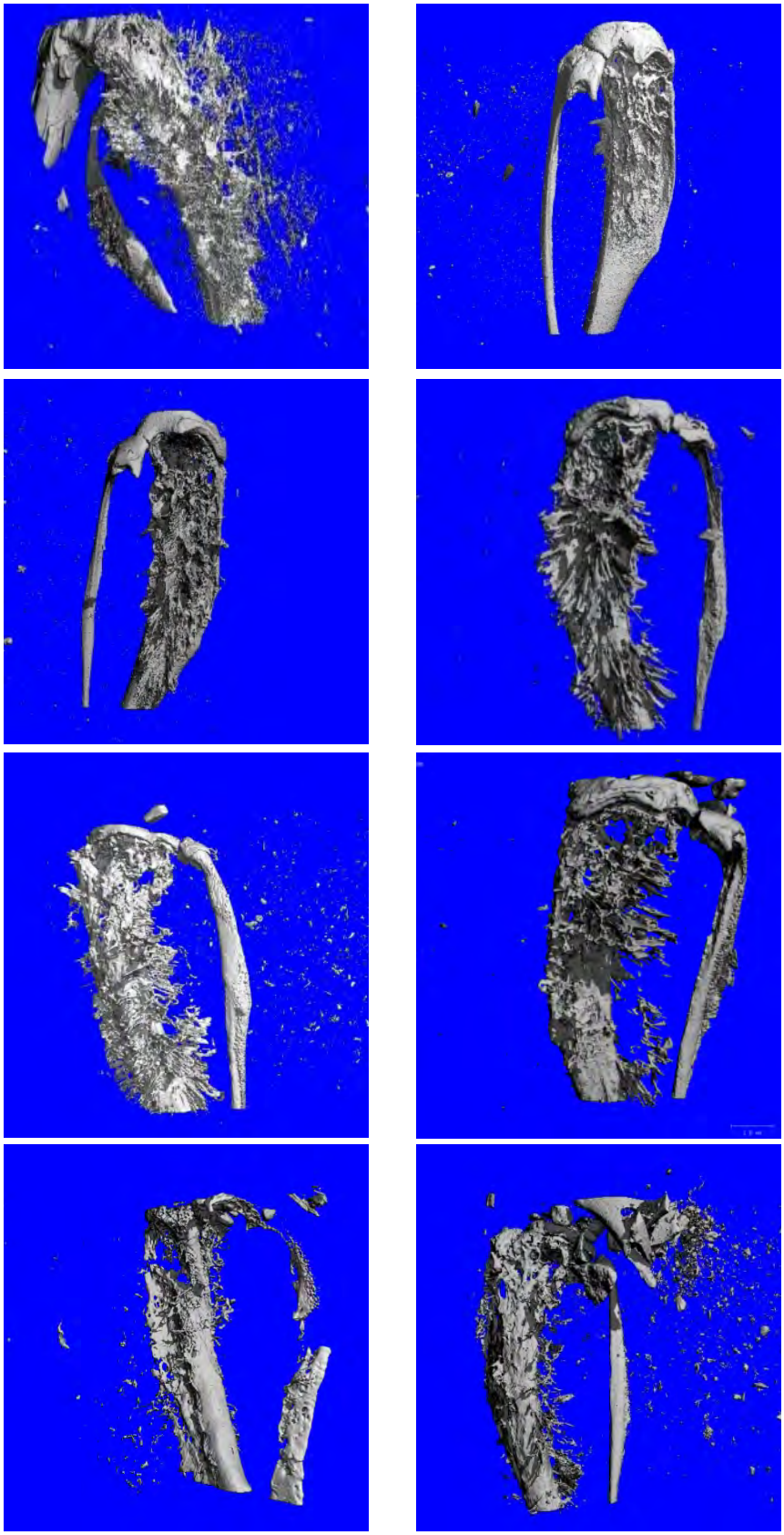
µCT images

AMD3100, castrated



µCT images

ICG-001, castrated



RESEARCH SUPPORT (current)

LSUHSC Offi of Res Seed Package (PI) 1/1/2015 – 12/31/2099
Prostate Cancer and Prostatic Disease
The aim is to identify molecular mechanisms involved in PCa progression

W81XWH-12-1-0212 (PI) 07/01/12 - 06/30/19
DOD no cost extension
Wnt/beta-Catenin, Foxa2, and CXCR4 controls prostate cancer progression
The aim is to study the functional implication of Wnt/beta-Catenin, Foxa2 and CXCR4 pathway in controlling prostate cancer metastasis and in the development of castration resistant prostate cancer.

R03CA212567 (PI) 12/1/2016 – 11/30/2019
NIH/NCI
The role of EZH2 in neuroendocrine prostate cancer
The aim is to study the implication of EZH2 in the development of NEPCa.

W81XWH-17-1-0417 (co-I, 10% effort) 07/01/17 - 06/30/20
DOD
The TLK1/NEK1 axis in prostate cancer
The aim is to study the involvement of TLK1 and NEK1 in castrate-resistant PCa progression.

R01CA226285 (PI) 7/01/2018 – 6/30/2023
NIH/NCI
Androgen Deprivation Activates Wnt/Beta-Catenin Signaling in Prostate Cancer
The aim is to study how Wnt/beta-Catenin pathway is activated and its involvement in NEPCa.

RESEARCH SUPPORT (completed)

Vanderbilt VICTR Funding (PI) 02/07/2008-02/06/2009
Wnt/beta-Catenin signaling in Castrate-Resistant Prostate Cancer
This research aims to study the involvement of Wnt/beta-Catenin signaling in CRPCa.

Vanderbilt VICTR Funding (PI) 09/02/2010-09/01/2011
Foxa2 Controls Prostate Cancer Metastasis
This study aims to identify genes regulated by Foxa2 that are involved in PCa metastasis.

R01 DK055748-14 Matusik (PI) 04/06/09 - 03/31/14
NIH/NIDDK
Control of Prostate-Specific Gene Expression
Our hypothesis is that by identifying the transcription factors (TFs) that control prostate-specific gene expression, we are also identifying TF that play a critical role in prostate development. We are identifying TFs in the androgen receptor and Foxa1 complex.

LSUHSCs Grant-In-Aid (PI) 7/1/2016 – 6/30/2017
Wnt/beta-catenin in NEPCa
The aim is to determine the molecular mechanisms involved in the development of NEPCa

LSUHSC FWCC Seed Package (PI) 10/1/2014 – 6/30/2018
Molecular mechanisms in CRPCa
The aim to identify the molecular mechanisms that promote castrate-resistant PCa.

CURRICULUM VITAE

Xiuping Yu
Associate Professor
Department of Biochemistry & Molecular Biology
Department of Urology
Louisiana State University Health Sciences Center
1501 Kings Hwy, Shreveport, LA 71103

EDUCATION

1987-1991	Bsc.	Nankai University, Tianjin, China
1991-1994	Msc.	Dalian Medical University, Dalian, China
1997-2000	Ph.D.	Dalian Medical University, Dalian, China

POSITIONS AND EMPLOYMENTS

1994-2000	Instructor and later Associate Professor, Dalian Medical University, Dalian, China
2000-2003	Postdoctoral Fellow, Medical College of Georgia, Augusta, GA
2003-2008	Postdoctoral Fellow, Vanderbilt University, Nashville, TN
2008-2014	Research Assistant Professor, Vanderbilt University, Nashville, TN
2014-present	Assistant Professor, Department of Biochemistry & Molecular Biology LSU Health Sciences Center, Shreveport, LA

JOURNAL REVIEWER

Neoplasia

Plos One

Oncogene

Oncotarget

Cancer Letters

The FASEB Journal

Nature Reviews Urology

Molecular Medicine Reports

Endocrine Related Cancer

ReDox Biology

Experimental and Therapeutic Medicine

Journal of Cellular Biochemistry

PEER-REVIEWED PUBLICATIONS

1. White RE, Han G, Maunz M, Dimitropoulou C, El-Mowafy AM, Barlow RS, Catravas JD, Snead C, Carrier GO, Zhu S, **Yu X**. Endothelium-independent effect of estrogen on Ca^{2+} activated K^{+} channels in human coronary artery smooth muscle cells. *Cardiovascular Research* 2002; 53: 650-661.

2. Ling J, Pi W, **Yu X**, Bengra C, Long Q, Jin H, Seyfang A, Tuan D. The ERV-9 LTR enhancer is not blocked by the HS5 insulator and synthesizes through the HS5 site non-coding, long RNAs that regulate LTR enhancer function. *Nucleic Acid Research* 2003; 31(5): 4582-4596.

3. Pi W, Yang Z, Wang J, Ruan L, **Yu X**, Ling J, Isales C. Conway S.J., Lin S. & Tuan D. The LTR enhancer of ERV-9 human endogenous retrovirus is active in oocytes and progenitor cells in transgenic zebrafish and humans. *Proceedings of the National Academy of Sciences of the United States of America* 2004; 101(3): 805-810.

4. Ling J, Ainol L, Zhang L, **Yu X**, Pi W, Tuan D. HS2 enhancer function is blocked by a transcriptional terminator inserted between the enhancer and the promoter. *J Biol Chem.* 2004 Dec 3; 279(49): 51704-13.

5. Gao N, Ishii K, Mirosevich J, Kuwajima S, Oppenheimer SR, Robert RL, Jiang M, **Yu X**, Shappell SB, Caprioli RM, Stoffel M, Hayward SW, Matusik RJ. Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. *Development.* 2005 Aug; 132 (15): 3431-43.

6. **Yu X**, Zhu X, Pi W, Ling J, Takeda Y, Tuan D. The long terminal repeat (LTR) of ERV-9 human endogenous retrovirus binds to NF-Y in the assembly of an active LTR enhancer complex NF-Y/MZF1/GATA-2. *J Biol Chem.* 2005 Oct 21; 280(42): 35184-94.

7. **Yu X,** Gupta A, Wang Y, Suzuki K, Mirosevich J, Orgebin-Crist MC, Matusik RJ. Foxa1 and foxa2 interact with the androgen receptor to regulate prostate and epididymal genes differentially. *Ann N Y Acad Sci.* 2005 Dec; 1061:77-93.

8. Han G, **Yu X**, Lu L, Li S, Ma H, Zhu S, Cui X, White RE. Estrogen receptor alpha mediates acute potassium channel stimulation in human coronary artery smooth muscle cells. *J Pharmacol Exp Ther.* 2006 Mar; 316(3): 1025-30.

9. Suzuki K, **Yu X**, Chaurand P, Araki Y, Lareyre JJ, Caprioli RM, Matusik RJ, Orgebin-Crist-MC. Epididymis-specific promoter-driven gene targeting: A transcription factor which regulates epididymis-specific gene expression. *Mol Cell Endocrinol.* 2006 May 16; 250 (1-2): 184-9.

10. **Yu X**, Suzuki K, Wang Y, Gupta A, Jin R, Orgebin-Crist MC, Matusik R. The role of forkhead box A2 to restrict androgen-regulated gene expression of lipocalin 5 in the mouse epididymis *Mol Endocrinol.* 2006 Oct; 20(10): 2418-31.

11. Suzuki K., **Yu X**, Chaurand P., Araki Y., Lareyre J., Caprioli R., Orgebin-Crist MC., Matusik RJ. Epididymis-specific lipocalin promoters *Asian J Androl*. 2007 Jul; 9(4): 515-21. PMID: 17589789

12. Matusik RI, Jin RJ, Sun Q, Wang Y, **Yu X**, Gupta A., Nandana S, Case TC, Paul M, Mirosevich J, Oottamasathien S, Thomas J. Prostate epithelial cell fate *Differentiation*. 2008 Jul; 76 (6): 682-98. PMID: 18462434

13. Jin RJ, Lho Y, Connelly L, Wang Y, **Yu X**, Saint Jean L, Case TC, Ellwood-Yen K, Sawyers CL, Bhowmick NA, Blackwell TS, Yull FE, Matusik RJ. The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth *Cancer Res*. 2008 Aug 15;68(16):6762-9. PMID: 18701501

14. **Yu X**, Wang Y, Jiang M, Bierie B, Roy-Burman P, Shen MM, Taketo MM, Wills M, Matusik RJ. Activation of beta-Catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. *Prostate.* 2009 Feb 15; 69(3): 249-62. PMID: 18991257

15. Degraff DJ, **Yu X**, Sun Q, Matusik RJ. The role of Foxa proteins in the regulation of androgen receptor activity. In: Tindall DJ, Mohler JL, eds. Androgen Action in Prostate Cancer. New York: Springer Science and Business Media; 2009.

16. Sun Q, **Yu X**, Degraff DJ, Matusik RJ. Upstream stimulatory factor 2, a novel FoxA1-interacting protein, is involved in prostate-specific gene expression. *Mol Endocrinol.* 2009 Dec; 23 (12): 2038-47. PMID: 19846536

17. Zhang J, Gao N, Degraff DJ, **Yu X**, Sun Q, Case TC, Kasper S, Matusik RJ. Characterization of cis elements of the probasin promoter necessary for prostate-specific gene expression. *Prostate.* 2010 Jun 15; 70(9): 934-51. PMID: 20209642

18. Jiang M, Fernandez S, Jerome WG, He Y, **Yu X**, Cai H, Boone B, Yi Y, Magnuson MA, RoyBurman P, Matusik RJ, Shappell SB, Hayward SW. Disruption of PPARgamma signaling results in mouse prostatic intraepithelial neoplasia involving active autophagy. *Cell Death Differ.* 2010 Mar; 17(3): 469-81. PMID: 19834493

19. **Yu X**, Wang Y, DeGraff DJ, Wills ML, Matusik RJ. Wnt/β-catenin activation promotes prostate tumor progression in a mouse model. *Oncogene*. 2011 Apr 21; 30(16): 1868-79. PMID: 21151173

20. DeGraff DJ, Clark PE, Cates JM, Yamashita H, Robinson VL, **Yu X**, Smolkin ME, Chang SS, Cookson MS, Herrick MK, Shariat SF, Steinberg GD, Frierson HF, Wu XR, Theodorescu D, Matusik RJ. Loss of the urothelial differentiation marker FOXA1 is associated with high grade, late stage bladder cancer and increased tumor proliferation. PLoS One. 2012; 7(5): e36669. PMCID: 3349679

21. Gupta A, **Yu X**, Case T, Paul M, Shen MM, Kaestner KH, Matusik RJ. Mash1 expression is induced in neuroendocrine prostate cancer upon the loss of Foxa2. *Prostate*. 2013 May; 73(6): 582-9: PMID: 23060003

22. Li B, Lu W, Yang Q, **Yu X**, Matusik RJ, Chen Z. Skp2 regulates androgen receptor through ubiquitin-mediated degradation independent of Akt/mTOR pathways in prostate cancer. *Prostate*, 74:421-32, 2014 PMID: 24347472.

23. Grabowska MM, DeGraff DJ, **Yu X**, Jin RJ, Chen Z, Borowsky AD, and Matusik RJ. Mouse Models of Prostate Cancer: Picking the best model for the question. *Cancer Metastasis Rev*, 2014 Sep; 33(2-3): 377-97. PMID: 24452759.

24. DeGraff DJ, Grabowska MA, Case T, **Yu X**, Herrick MK, Hayward W, Strand DW, Cates JM, Hayward SW, Gao N, Walter MA, Buttyan R, Yi Y, Kaestner KH, and Matusik RJ. Foxa1

deletion in luminal epithelium causes prostatic hyperplasia and alteration of differentiated phenotype. *Lab Invest*, 2014 Jul; 94(7): 726-39. PMID: 24840332.

25. Grabowska MM, Elliott AD, DeGraff DJ, Anderson PD, Anumanthan G, Yamashita H, Sun Q, Friedman DB, Hachey DL, **Yu X**, Sheehan JH, Ahn J-M, Raj G, Piston DW, Gronostajski RM, Matusik RJ. NFI Transcription Factors Interact with FOXA1 to Regulate Prostate Specific Gene Expression. *Mol Endocrinol*, 2014 Jun; 28(6): 949-64. PMID: 24801505

26. Jin R, Yamashita H, **Yu X**, Wang J, Franco OE, Wang Y, Hayward SW, Matusik RJ. Inhibition of NF-kappa B signaling restores responsiveness of castration-resistant prostate cancer cells to anti-androgen treatment by decreasing androgen receptor-variant expression. *Oncogene*. 2014 Sep 15. Doi 10.1038/onc.2014.302. [Epub ahead of print] PMID: 25220414

27. Valkenburg KC, **Yu X**, De Marzo AM, Spiering TJ, Matusik RJ, Williams BO. Activation of Wnt/β-catenin signaling in a subpopulation of murine prostate luminal epithelial cells induces high grade prostate intraepithelial neoplasia. *Prostate*. 2014 Nov;74(15):1506-20. PMID: 25175604

28. **Yu X***, Cates JM, Morrissey C, You C, Grabowska MM, Zhang J, DeGraff DJ, Strand DW, Franco OE, Lin-Tsai O, Hayward SW, Matusik RJ*. SOX2 expression in the developing and adult prostate, as well as in benign and malignant pathological states, *corresponding authors, *Prostate Cancer and Prostatic Dis*, 2014 Dec;17(4):301-9. PMID: 25091041

29. **Yu X**, Matusik RJ, Jin R RNASEL R4620 mutation in prostate cancer. *Gene Cell Tissue*. 2015 January; 2(1): e24411

30. Yang S, Jiang M, Grabowska MM, Li J, Connelly Z, Zhang J, Hayward SW, Cates JM, Han G, **Yu X*** Androgen receptor differentially regulates the proliferation of prostatic epithelial cells *in vitro* and *in vivo* *corresponding author, *Oncotarget.* 2016 Oct 25;7(43):70404-70419 PMID: 27611945

31. Connelly Z, Yang S, Chen F, Yeh Y, Khater N, Jin R, Matusik R, **Yu X*** Foxa2 Activates the Transcription of Androgen Receptor Target Genes in Castrate Resistant Prostatic Tumors *corresponding author. *American Journal of Clinical and Experimental Urology*, 2018 Oct 20;6(5):172-181

32. Singh V, Jaiswal PK, Ghosh I, Koul HK, **Yu X**, De Benedetti A. Targeting the TLK1/NEK1 DDR axis with Thioridazine suppresses outgrowth of Androgen Independent Prostate tumors. Int J Cancer. 2019 Feb 9. doi: 10.1002/ijc.32200. [Epub ahead of print]

33. Singh V, Jaiswal PK, Ghosh I, Koul HK, **Yu X**, De Benedetti A. The TLK1-Nek1 axis promotes prostate cancer progression. Cancer Lett. 2019 Jul 1;453:131-141. doi: 10.1016/j.canlet.2019.03.041. Epub 2019 Mar 27.