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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

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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-

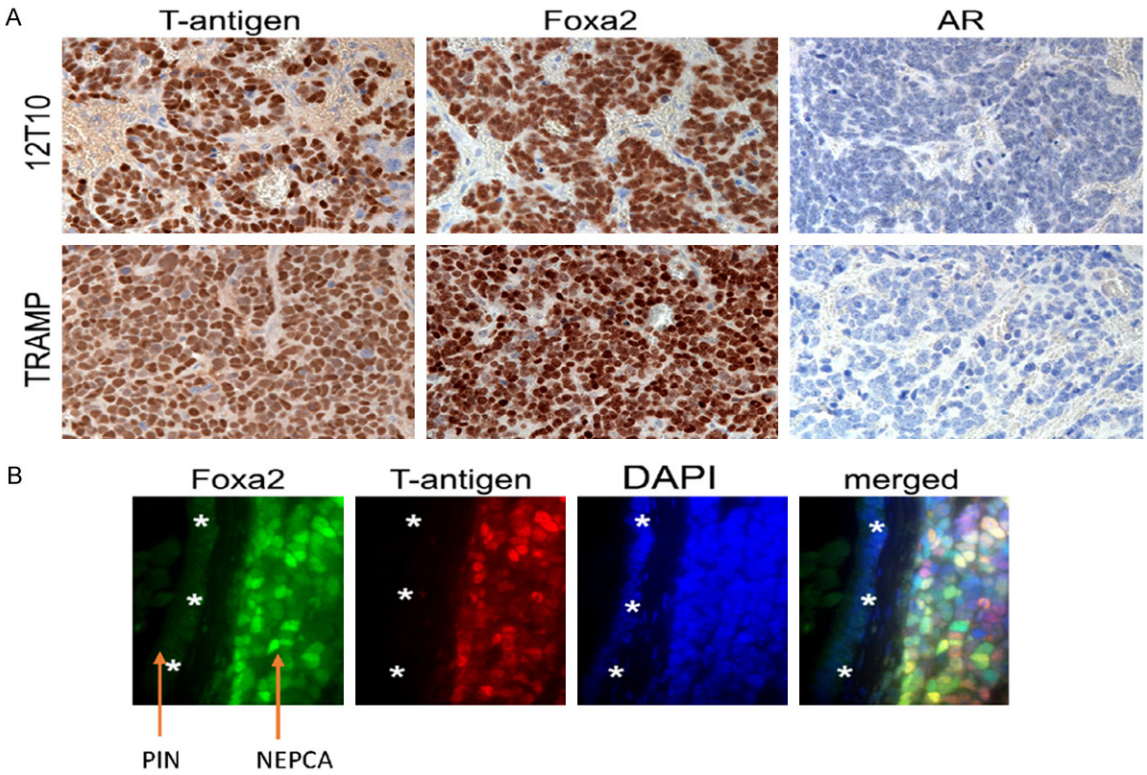

Figure 1. T-antigen and Foxa2 are co-expressed in prostatic tumors of transgenic mice. A. Immunohistochemistry staining of T-antigen, Foxa2, and AR conducted on serial sections derived from prostatic tumors of 12T-10 LADY and TRAMP mice. While nuclear AR is not expressed in the cancer cells, the expression 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

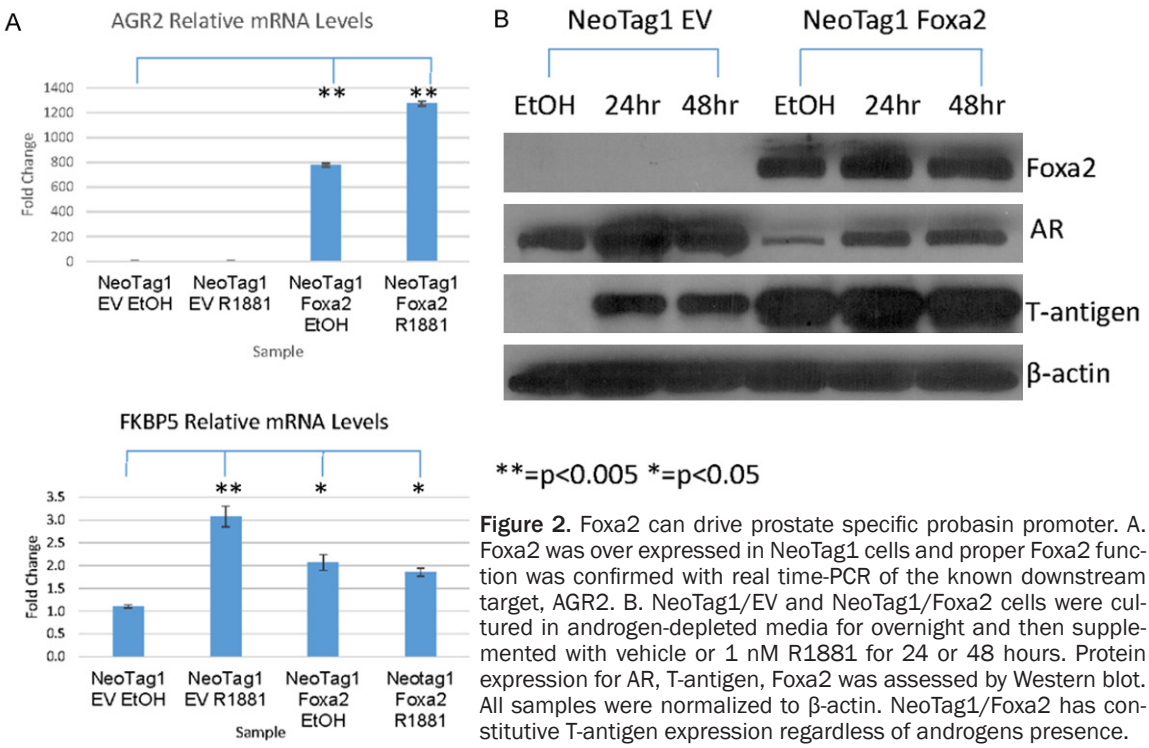

Figure 2. Foxa2 can drive prostate specific probasin promoter. A. Foxa2 was over expressed in NeoTag1 cells and proper Foxa2 function was confirmed with real time-PCR of the known downstream target, AGR2. B. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and 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

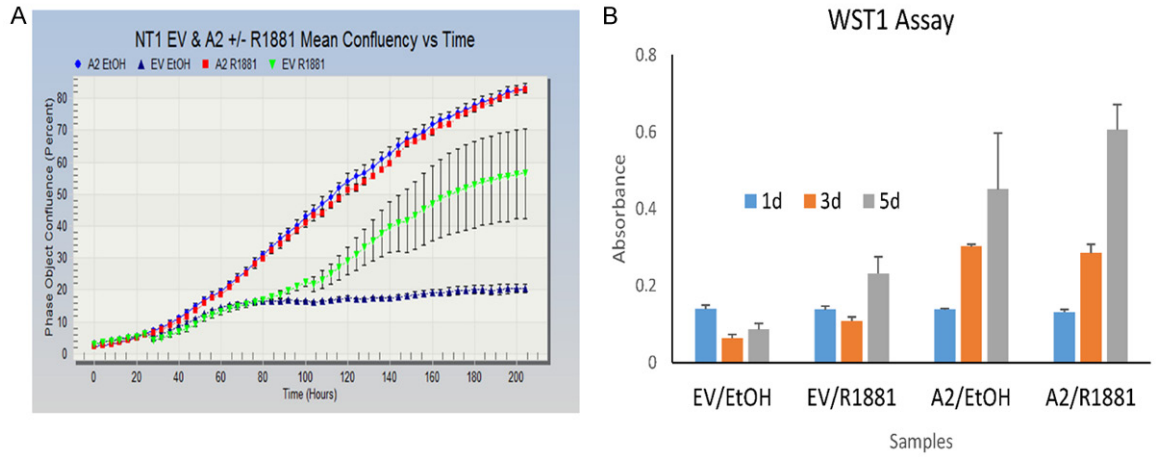

Figure 3. Cell proliferation assays. A. The proliferation of NeoTag1/EV and NeoTag1/Foxa2 was assessed using IncuCyte Zoom method. One thousand cells were seeded in each well of 96-well plate (time 0 hour) in cell culture media containing 10% fetal bovine serum. 24 hours later, the cells were cultured 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

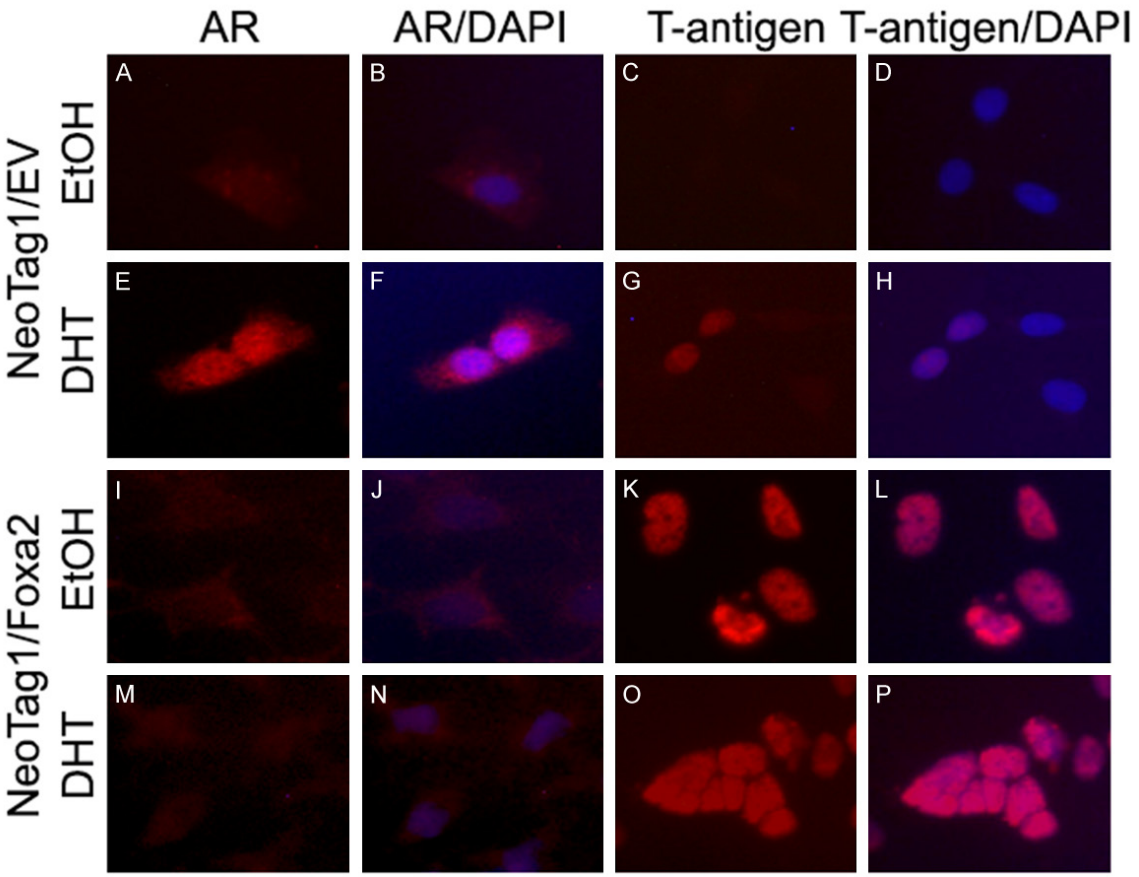

Figure 4. Decreased nuclear androgen receptor in NeoTag1/Foxa2 cells. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and then supplemented with ethanol or 1 nM R1881 for 2 hours. In the NeoTag1/EV cells, 2-hour androgen treatment provoked androgen receptor nuclear 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

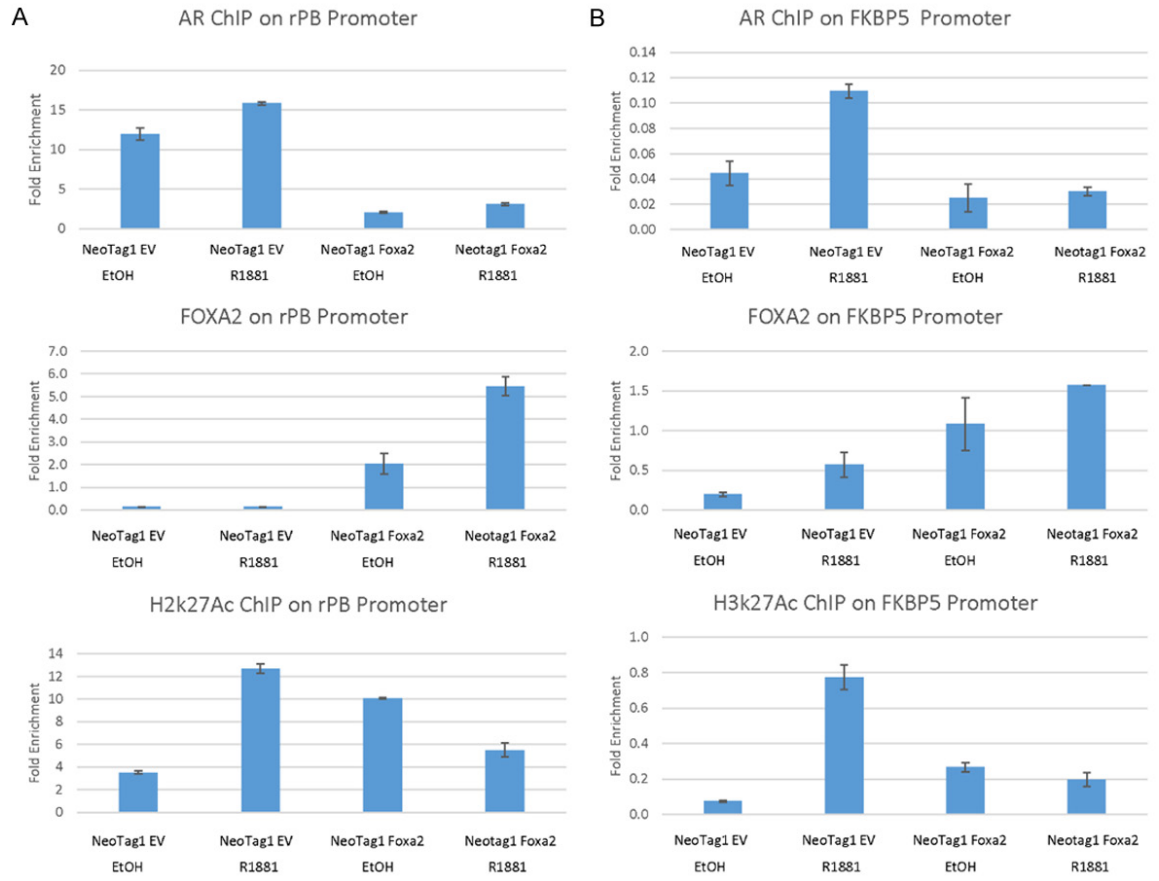

Figure 5. Foxa2 retains active chromatin state in the absence of androgens. NeoTag1/EV and NeoTag1/Foxa2 cells were cultured in androgen-depleted media for overnight and then supplemented with ethanol or 1 nM R1881 for two hours. Cells were then fixed and Chromatin Immunopreciptation was conducted. A. 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.

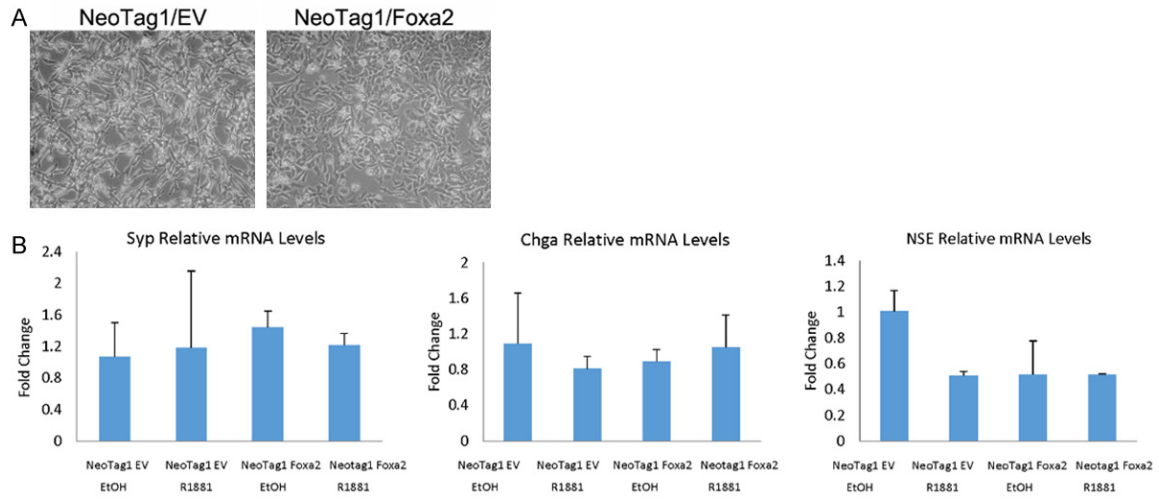

Figure 6. Ectopic expression of Foxa2 failed to induce NE differentiation. A. cell morphology of Neotag1/EV and NeoTag1/Foxa2 cells. B. qRT-PCR. The mRNA levels of NEPCa markers including synaptophysin (Syp), chromogranin A (Chga), and NSE were assessed by qRT-PCR. Over-expression of Foxa2 did not cause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

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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

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