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14. ABSTRACT

The goal of the present proposal is to investigate the mechanism by which TCF4 regulates neuroendocrine differentiation (NED) and induces enzalutamide resistance in CRPC. In treating patients with CRPC, enzalutamide, the second-generation AR antagonist, has been considered a cornerstone of care. However, clinical benefits are limited to a median time of 4.8 months because resistance to enzalutamide inevitably emerges. Based on a body of preliminary data, we hypothesize that the transcription factor TCF4 is induced by AR-V7 and mediates, in part, enzalutamide resistance in CaP cells via the neuroendocrine marker, PTHrP. To test this hypothesis, two specific aims and four major tasks have been proposed. Of these objectives, major task 1 that focused on the role of AR and AR-V7 with TCF4 has been completed. We have found that the knockdown of AR-V7 reverses neuroendocrine differentiation and TCF4 expression. In addition, AR response element has been identified. Finally, overexpression of TCF4 has demonstrated neuroendocrine differentiation in vivo.

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1. Introduction

The goal of the present proposal is to investigate the mechanism by which the transcription factor TCF4 regulates neuroendocrine differentiation (NED) and induces enzalutamide resistance in castration-resistant prostate cancer (CRPC). In treating patients with CRPC, enzalutamide, the second-generation AR antagonist, has been considered a cornerstone of care. However, clinical benefits are limited to a median time of 4.8 months because resistance to enzalutamide inevitably emerges. In our preliminary RNA sequence study, it was found that the expression levels of NE markers such as chromogranin A and parathyroid hormone related peptide (PTHrP) were highly elevated in an enzalutamide-resistant human prostate cancer (CaP) cell line when compared to the parental cell line. After analyzing the promoters of the NED related genes, we found that TCF4, a transcription factor that has not been linked to CaP previously, mediated NED in response to enzalutamide treatment and was elevated in the enzalutamide-resistant CaP cell line. Importantly, the NED marker, PTHrP, mediated enzalutamide resistance in tissue culture. When tissues obtained from men who died of metastatic CaP were examined, a positive correlation was found between the expression levels of TCF4 and PTHrP. Inducible TCF4 overexpression also caused enzalutamide-resistance in mouse xenograft model. Importantly, enzalutamide stimulated the expression of the AR splice variant, ARV7, which then removed the suppressive effect of AR on TCF4 and increased its expression levels. Based on these observations, we hypothesize that TCF4 is induced by AR-V7 and mediates, in part, enzalutamide resistance in CaP cells via the neuroendocrine marker, PTHrP. To test this hypothesis, we propose the following specific aims:
Specific Aim 1: To investigate the mechanism of EnzR induced by AR-V7/TCF4/PTHrP axis:
Specific Aim 2: To investigate the clinical implications of AR-V7/TCF4/PTHrP axis in CaP.

2. Key Words: TCF4, castration resistant prostate cancer, enzalutamide, neuroendocrine differentiation, AR-V7.

3. Accomplishments

3A. Major Goals
There were two specific aims and four major tasks as follows
Specific Aim 1: To investigate the mechanism of enzalutamide resistance induced by AR V7/TCF4/PTHrP axis:
Major Task 1: To study the mechanism of Enz resistance mediated by TCF4 via AR-V7 in tissue culture
Major Task 2: To study the role of β-catenin in induction of PTHrP by TCF4

Specific Aim 2: To investigate the clinical implications of ARV7/TCF4/PTHrP axis in CaP.
Major Task 1: To analyze AR-V7, TCF4, and PTHrP expression levels in enzalutamide-resistant human CaP tissues.
Major Task 2: To investigate the therapeutic potential of blocking TCF4/PTHrP axis in enzalutamide resistant CaP cells.

3B. Accomplished goals
Major task 1 has been accomplished and a manuscript is in preparation. The results are as follows.

3B1. Enzalutamide-resistant human CaP cell line exhibits NED

To investigate the mechanism of enzalutamide resistance in CaP cells, an enzalutamide-resistant human CaP cell line was initially generated by continuously treating LNCaP with 10 μM enzalutamide in RPMI-1640 supplemented with 10% charcoal stripped fetal bovine serum (cFBS). After three months, cells began to proliferate consistently and were designated as LNCaP-EnzR. Simultaneously, LNCaP cultured chronically under cFBS was also generated (LNCaP-cFBS). With

these cells, RNAseq followed by an unsupervised data analysis was carried out. When compared to the LNCaP-cFBS, there was no obvious differences in AR signaling related gene expression levels. However, NED markers such as chromogranin A (CHGA, ChgA), neuron-specific enolase (ENO2, NSE), and PTHrP (PTHLP) were significantly higher in LNCaP-EnzR (Fig 1A). A similar pattern of increased NED was observed in LNCaP-cFBS when compared to the parental line maintained in the standard media (RPMI1640/10% FBS) (Fig 1B). To validate these observations, we treated the human prostate cancer cell lines LNCaP and 22Rv1 with increasing concentrations of enzalutamide (0-10 μM) under an androgen-deprived condition (RPMI-1640/10% cFBS) for 48 hours. The results demonstrated that enzalutamide increased expression levels of ChgA, NSE, and PTHrP protein (Fig 1C) and mRNA (Fig 1D) in a concentration-dependent manner.

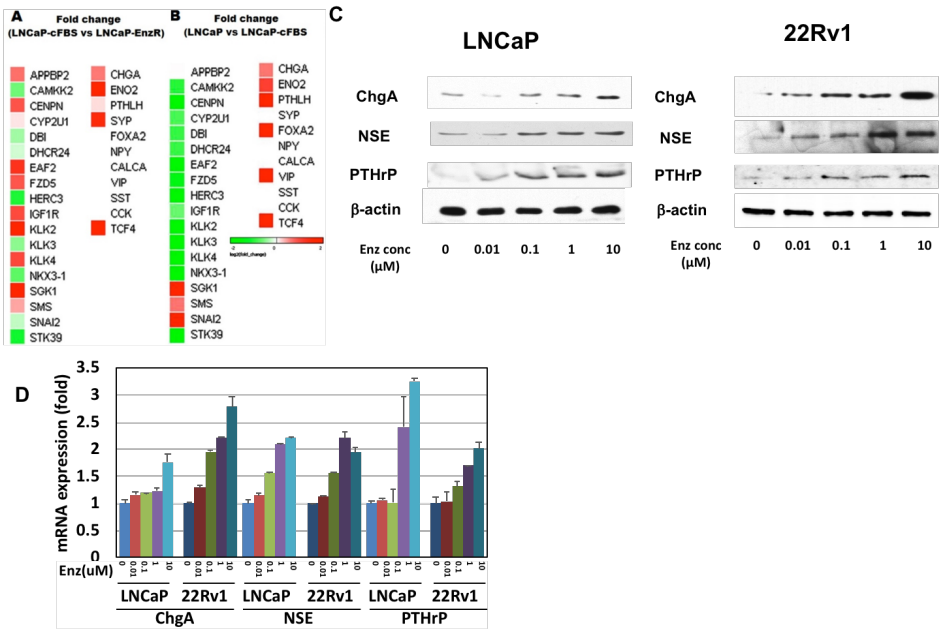

Figure 1. TCF4 mediates NED in human prostate cancer cell lines. **A.** Unsupervised comparison of transcriptome of LNCaP cultured in FBS vs charcoal-stripped FBS (cFBS) was carried out. **B.** Comparison of transcriptome between LNCaP-cFBS and LNCaP-Enz (resistant to enzalutamide). Markers of NED (ChgA, NSE, and PTHrP) were significantly increased in LNCaP-EnzR. **C.** Human prostate cancer cell lines LNCaP and 22Rv1 were treated with increasing concentrations of enzalutamide (0-10 μM) under an androgen-deprived condition (RPMI-1640/10% cFBS) for 48 hours. Immunoblot demonstrated that NED markers (ChgA, NSE, and PTHrP) were induced by enzalutamide. **D.** QPCR demonstrated that NED marker (ChgA, NSE, and PTHrP) mRNA expression levels increased also after treatment with enzalutamide at the indicated concentrations (0-10 μM) for 48 hours.

3B2. TCF4 mediates NED and enzalutamide resistance in human CaP cell lines

Based on the observation that the mRNA levels of NED markers are induced by enzalutamide and increased in LNCaP-EnzR, we hypothesized that the anti-androgen may regulate a common transcription factor that regulates NED. To test this concept, we carried out a bioinformatics-based analysis of the ChgA, NSE, and PTHrP promoters to identify common transcription factors that potentially bind to all three NED markers (alggen, http://alggen.lsi.upc.es/). This effort identified consensus sequences for binding of five transcription factors within the promoters of NED markers: TCF4, POU2F2 (Oct2.1), MRF-2, LCR-F1, and MBF-1 (EDF-1). Quantitative PCR (qPCR) confirmed that mRNA levels of TCF4 and POU2F2 were significantly higher in LNCaP-EnzR when compared to the parental cell line (data not shown). This QPCR result was consistent with our transcriptome analysis that showed increased TCF4 expression levels in LNCaP-EnzR (Fig 1A). To assess the role of TCF4 and POU2F2 on NED, three androgen-responsive human CaP cell lines (LNCaP, 22Rv1, and VCaP) were transiently transfected with TCF4 and POU2F2. Only cells overexpressing TCF4 demonstrated significant increase in the mRNA levels of the NED markers ChgA, NSE, and PTHrP (Fig 2A). In addition, it was found that TCF4-expressing cells were more resistant to enzalutamide 10 μM treatment as the cell number was nearly double that of the control after three days of culture (Fig 2B). This resistance was not merely due to differentiation as TCF4-expressing cells continued to proliferate in the presence of enzalutamide. When the two transcription factors were knocked down in

LNCaP and 22Rv1 with shRNA, enzalutamide no longer induced the expression of ChgA, NSE, and PTHrP proteins only when TCF4 expression was blocked (Fig 2C). The kinetics of enzalutamide-induced TCF4 and NED markers expression revealed that the increase in TCF4 mRNA preceded that of the NED markers by approximately eight hours in tissue culture (Fig 2D). These results collectively demonstrate that TCF4 mediates the expression of NED markers in human CaP cell lines.

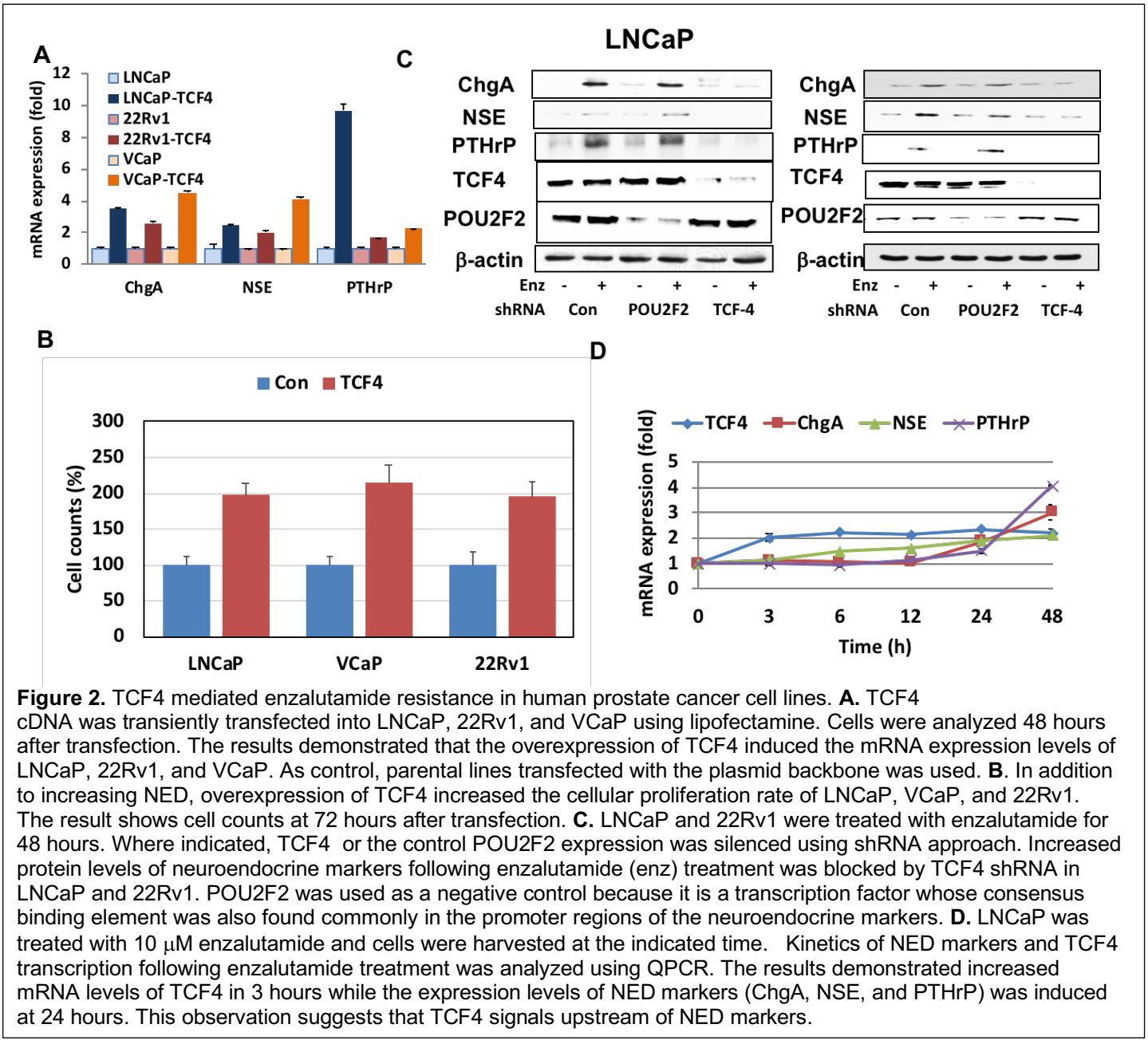

Figure 2. TCF4 mediated enzalutamide resistance in human prostate cancer cell lines. **A.** TCF4 cDNA was transiently transfected into LNCaP, 22Rv1, and VCaP using lipofectamine. Cells were analyzed 48 hours after transfection. The results demonstrated that the overexpression of TCF4 induced the mRNA expression levels of LNCaP, 22Rv1, and VCaP. As control, parental lines transfected with the plasmid backbone was used. **B**. In addition to increasing NED, overexpression of TCF4 increased the cellular proliferation rate of LNCaP, VCaP, and 22Rv1. The result shows cell counts at 72 hours after transfection. **C.** LNCaP and 22Rv1 were treated with enzalutamide for 48 hours. Where indicated, TCF4 or the control POU2F2 expression was silenced using shRNA approach. Increased protein levels of neuroendocrine markers following enzalutamide (enz) treatment was blocked by TCF4 shRNA in LNCaP and 22Rv1. POU2F2 was used as a negative control because it is a transcription factor whose consensus binding element was also found commonly in the promoter regions of the neuroendocrine markers. **D.** LNCaP was treated with 10 μM enzalutamide and cells were harvested at the indicated time. Kinetics of NED markers and TCF4 transcription following enzalutamide treatment was analyzed using QPCR. The results demonstrated increased mRNA levels of TCF4 in 3 hours while the expression levels of NED markers (ChgA, NSE, and PTHrP) was induced at 24 hours. This observation suggests that TCF4 signals upstream of NED markers.

3B3. Blocking TCF4/PTHrP partially reverses enzalutamide resistance in human CaP cell lines

To examine the biological significance of TCF4 and NED induction on enzalutamide resistance, we generated two additional human CaP cell lines that are resistant to enzalutamide (22Rv1-EnzR and VCaP-EnzR). Then, of the NED markers, we focused on PTHrP as a potential mediator of enzalutamide resistance because this peptide has been demonstrated to mediate castration resistance (18, 19). Consistent with the published data reporting that TCF4 is a binding partner of β-catenin (20, 21), pretreatment with the β-catenin degradation activator, XAV939 (22) at 10 μM five minutes prior to enzalutamide treatment, abrogated PTHrP mRNA induction by enzalutamide in all three human CaP cell lines (Fig 3A). In addition to the ligand, we assessed whether enzalutamide

altered the expression of the PTHrP receptor, PTH1R. The result, shown in Fig 3B, revealed that 10 μM enzalutamide significantly increased mRNA levels in LNCaP, VCaP, and 22Rv1. Next, TCF4 and PTHrP were blocked in all three enzalutamide-resistant cell lines using the reported inhibitors – PKF118-310 and $PTHrP_{(7-34)}$ (23, 24). Both PKF118-310 and $PTHrP_{(7-34)}$ significantly decreased the cellular proliferation of all three cell lines in a concentration-dependent manner over a three-day period. Between the two inhibitors, $PTHrP_{(7-34)}$ had a more moderate effect. In the three enzalutamide-resistant cell lines (LNCaP-EnzR, 22Rv1-EnzR, and VCaP-EnzR), PKF118-310 again inhibited the cellular proliferation in a concentration-dependent manner up to 50 μM after 3 days (Fig 3C). Similarly, $PTHrP_{(7-34)}$ treatment also decreased cell count of LNCaP-EnzR, 22Rv1-EnzR, and VCaP-EnzR in a concentration-dependent manner up to 1 mM after 3 days. To assess whether PFK118-310 and $PTHrP_{(7-34)}$ affected enzalutamide sensitivity, we next cultured the three enzalutamide-resistant cell lines with a fixed concentration of PKF118-310 (5 μM) or $PTHrP_{(7-34)}$ (10 μM) and varying concentrations of enzalutamide (0-10 μM). The results demonstrated that both PKF118-310 and $PTHrP_{(7-34)}$ reversed enzalutamide resistance in LNCaP-EnzR, 22Rv1-EnzR, and VCaP-EnzR (Fig 3D). It should be noted that enzalutamide still exhibited a concentration-dependent inhibitory effect on all three enzalutamide-resistant cell lines, demonstrating that these cells have a relative and not an absolute resistance to enzalutamide.

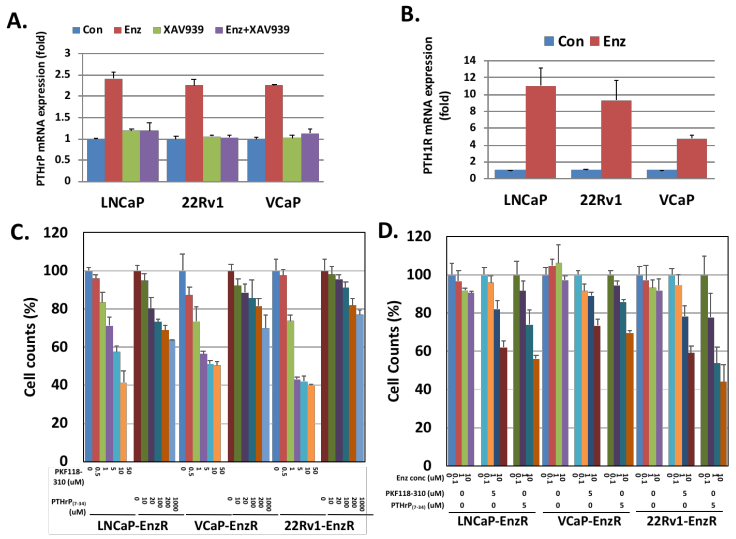

Figure 3. Effect of blocking TCF4/β-catenin (PKF118-310) or PTHrP ($PTHrP_{(7-34)}$) on enzalutamide resistant prostate cancer cells. **A.** LNCaP, 22Rv1, and VCaP were treated with enzalutamide (10 μM) and/or XAV939 (10 μM), β-catenin degradation activator for 48 hours. XAV939 treatment was carried out 5 min prior to the addition of enzalutamide. PTHrP mRNA induction after 48 hours of treatment with 10 μM enzalutamide was completely blocked by XAV939, β-catenin inhibitor in LNCaP, 22Rv1 and VCaP. **B.** LNCaP, 22Rv1, and VCaP were treated with 10 uM enzalutamide for 48 hours. The PTHrP receptor, PTH1R, mRNA level significantly increased after enzalutamide treatment in all three cell lines. **C.** Three enzalutamide-resistant human prostate cancer cell lines (LNCaP-EnzR, 22Rv1-EnzR, and VCaP-EnzR) were treated with 10 μM enzalutamide and increasing concentrations of PKF118-310 and $PTHrP_{(7-34)}$ as indicated. After 48 hours, viable cells were counted. In the presence of 10 μM Enz, PKF118-310 or $PTHrP_{(7-34)}$ increased enzalutamide sensitivity in the enzalutamide resistant prostate cancer cell lines, LNCaP-EnzR, VCaP-EnzR, and 22Rv1-EnzR. **D.** Enzalutamide-resistant human prostate cancer cell lines were treated with a fixed concentration of PKF118-310 (5 μM) or $PTHrP_{(7-34)}$ (10 μM) and varying concentrations of enzalutamide (0-10 μM). After 48 hours, viable cells were counted. In the presence of 5 μM of PKF118-310 or 10 μM of $PTHrP_{(7-34)}$, enzalutamide inhibited cellular proliferation in a concentration-dependent manner.

Because TCF4 has been linked to Wnt signaling, we next examined the effect of typical canonical and non-canonical Wnts [Wnt1, 3b, 5a, and 5b (10 ng/ml)] on NED. The results demonstrated no significant effect of on the expression levels of ChgA, NSE, and PTHrP mRNA after 48 hours (data not shown). These results suggest that Wnts likely do not regulate NED in our experimental conditions and that the effect of TCF4 on NED is likely independent of the Wnt signaling pathway.

3B4. In human CaP tissues, PTHrP and TCF4 co-localize and are associated with metastasis

To clinically validate these observations, we next carried out immunofluorescence (IF) microscopy on the CRPC tissue microarray (TMA) obtained from the University of Michigan's rapid autopsy program. Although this CRPC TMA was established prior to the formal approval of enzalutamide by the United

States Food and Drug Administration, co-localization of PTHrP and TCF4 was frequently observed in CRPC tissues (Fig 4A). PTHrP and TCF4 expression had positive correlation (Fig 4B). Furthermore, metastatic CaP was found to express higher levels of TCF4 and PTHrP (Fig 4C and D, respectively) when compared to localized CaP and benign prostate hyperplasia. Human kidney tissues were used as a positive control. As negative controls, tissues were stained only with the secondary antibody conjugated with FITC or red fluorescence protein (RFP).

3B5. TCF4 has an oncogenic function

To assess the effect of TCF4 *in vivo*, we established a doxycycline-inducible TCF4-expressing cell line, LNCaP-TCF4. After screening multiple clones, the one with the highest induction level of TCF4 on treatment with 1 μg/ml of tetracycline was selected and further characterized. When LNCaP-TCF4 was injected into flanks of Rag2-/-, γc-/- immunodeficient mice and TCF4 expression was induced by doxycycline following surgical castration, a relative resistance to enzalutamide treatment was observed over a six-week period (Fig 5A). There was no change in histology among the harvested tumors regardless of the grouping (Fig 5B). Immunofluorescence microscopy demonstrated that enzalutamide treatment or TCF4 induction (doxy) increased the expression of the NED marker, PTHrP (Fig 5C). In addition, co-localization of TCF4 and PTHrP was confirmed. Subsequently, immunoblot confirmed the immunofluorescence microscopy results in that increased TCF4 and PTHrP proteins were observed in groups treated with either enzalutamide or doxycycline (Fig 5D). QPCR demonstrated an increase in TCF4 and PTHrP mRNA levels upon treatment with

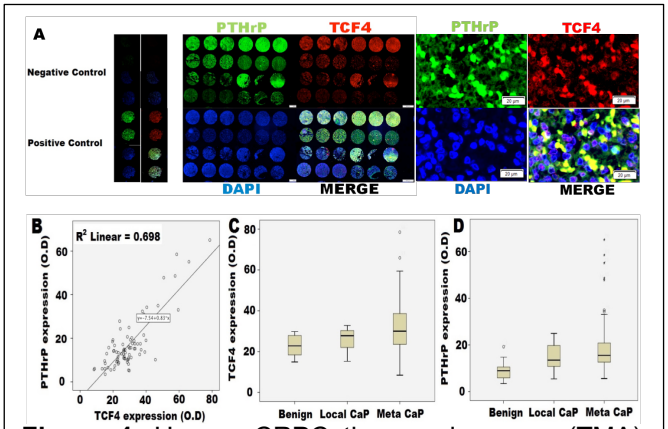

Figure 4. Human CRPC tissue microarray (TMA) analysis. TMA was obtained from the rapid autopsy program at the University of Michigan. This array contains 51 CRPC samples as well as 16 benign prostate tissues and 12 localized prostate cancer tissues for controls. **A.** Immunofluorescence microscopy demonstrated a consistent co-localization of TCF4 (red) and PTHrP (green) in CRPC tissues. **B.** There was a correlation between PTHrP and TCF4 expression. **C.** Protein expression levels of TCF4 and **D.** PTHrP in patients with localized CaP (Local CaP) and metastatic CaP (Meta CaP). TCF4 and PTHrP protein levels were higher in CaP when compared with benign and increased even further in metastatic group when compared with localized CaP group. Benign n=16, Local CaP n=12,

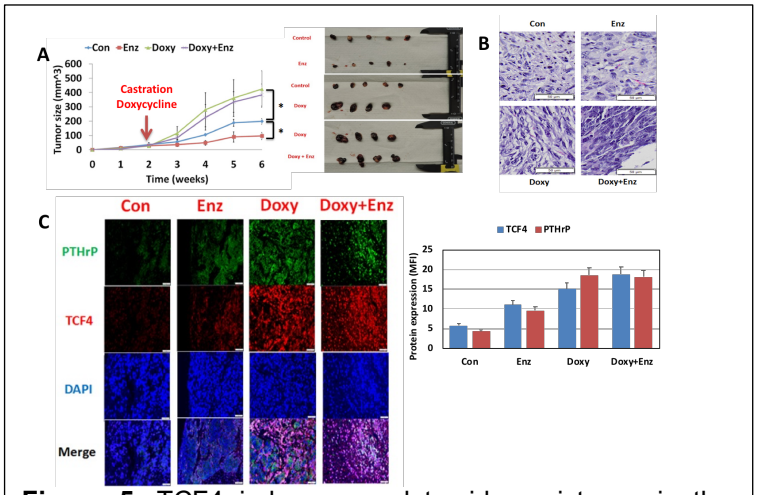

Figure 5. TCF4 induces enzalutamide resistance in the human prostate cancer cell line, LNCaP. LNCaP transfected with tetracycline-inducible TCF4 plasmid (LNCaP-TCF4) was injected into the flanks of twenty Rag2-/-, γc-/- immunodeficient mice. When tumors reached an average size of 3 mm, the mice were divided into four groups of five each. Where indicated, 10 mg/kg enzalutamide was delivered orally daily. In the designated groups, TCF4 was delivering doxycycline via the drinking water. At the end of the indicated duration, all tumors were harvested and analyzed for protein and mRNA expression. **A.** When TCF4 expression was induced with doxycycline (Doxy), tumor growth rate increased when compared to the control group. In the absence of TCF4 induction, enzalutamide treatment slowed tumor growth rate. However, enzalutamide treatment had no demonstrable inhibitory effect in TCF4-induced doxycycline group. Con = LNCaP-TCF4 without doxycycline. Enz = enzalutamide. **B.** H&E staining. There was no difference among all groups. **C.** Immunofluorescence staining for PTHrP (green), TCF4 (red) with DAPI (blue) staining. Increased TCF4 and PTHrP protein levels were observed following the induction of TCF4 with doxycycline. Directly supporting the tissue culture data, enzalutamide treatment also increased protein levels of TCF4 and PTHrP. Error bars indicate average ± SE and * p-value<0.05.

enzalutamide as well as doxycycline (data not shown). Collectively, these results suggest that TCF4 renders CaP cells more aggressive.

3B6. TCF4 inhibitor and PTHrP antagonist inhibit the proliferation of enzalutamide resistant prostate cancer cells *in vivo*

To study the therapeutic potential of targeting the TCF4/PTHrP axis, we carried out an *in vivo* study with LNCaP-EnzR in mice. After establishing tumor xenografts, all animals underwent a bilateral orchiectomy and were administered 10 mg/kg of enzalutamide via oral gavage daily. To predesignated groups, 0.85 mg/kg of PKF-118-310, 0.2 mg/kg of $PTHrP_{(7-34)}$, or both in combination were delivered daily. At the end of seven weeks, the results demonstrated that PKF118-310 and $PTHrP_{(7-34)}$ dramatically when combined with enzalutamide decreased the tumor xenograft growth dramatically compared to that of enzalutamide monotherapy (control) over a seven-week period (Fig 6A). However, there were no synergistic effect between PKF118-310 and PTHrP antagonist, $PTHrP_{(7-34)}$. Again, H&E staining showed no significant changes in the histology of the treated xenografts (Fig 6B). Immunofluorescence microscopy confirmed the co-localization of TCF4 and PTHrP while MFI measurement demonstrated that PKF118-310 treatment decreased PTHrP protein levels (Fig 6C). However, $PTHrP_{(7-34)}$ treatment again had no effect on TCF4 expression. Also consistent with the mechanism of action of PKF118-310 in which the interaction between TCF4 and B-catenin is disrupted, PKF118-310 treatment did not alter the expression levels of TCF4. These observations collectively support our concept that TCF4 is an upstream signaling molecule of PTHrP. Supporting the immunofluorescence MFI result, immunoblot revealed a decrease in PTHrP protein levels following PKF118-310 but not $PTHrP_{(7-34)}$ treatment (Fig 6D) levels. Again, no obvious changes in TCF4 protein level was seen with either PFK118-310 or $PTHrP_{(7-34)}$ administration. Result of the mRNA quantitation for TCF4 and PTHrP was consistent with that of immunoblot (data not shown).

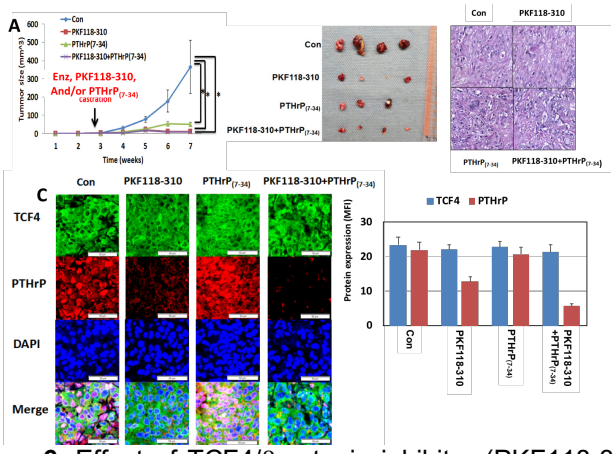

Figure 6. Effect of TCF4/β-catenin inhibitor (PKF118-310) and PTHrP antagonist ($PTHrP_{(7-34)}$) in enzalutamide-resistant prostate cancer. After injection of LNCaP-EnzR into the flanks of forty Rag2-/-, γ_c-/- immunodeficient mice, all mice were surgically castrated divided into four groups of ten each. Animals in predesignated groups were treated daily with PKF118-310 (0.85 mg/kg intraperitoneal) and/or $PTHrP_{(7-34)}$ (0.2 mg/kg subcutaneous). All mice were administered daily 10 mg/kg enzalutamide orally. **A.** Treatment of PKF118-310 and/or $PTHrP_{(7-34)}$ with 10 mg/kg enzalutamide decreased tumor growth compare with vehicle treatment control group (con). **B.** H&E staining. There was no difference among all groups. **C.** Immunofluorescence staining for TCF4 (green), PTHrP (red) with DAPI (blue) staining. Consistent with its mechanism of action, PFK118-310 treatment decreased PTHrP protein levels. However, there was no effect on TCF4 levels. In contrast, $PTHrP_{(7-34)}$ had no demonstrable effect on the protein levels of both TCF4 and PTHrP. Treatment of PKF118-310 decreased PTHrP protein. Error bars indicate average ± SE and * p-value<0.05.

4. Impact

Thus far, we have investigated the mechanism of enzalutamide resistance in CaP. After establishing multiple cell lines that are resistant to enzalutamide, NED was identified as a significant event through RNAseq. Subsequently, the transcription factor TCF4 was found to regulate NED which in turn, rendered the cells resistant to enzalutamide in part via PTHrP. In vivo studies confirmed the critical role of TCF4 in enzalutamide resistance and NED. Collectively, these observations demonstrate that enzalutamide stimulates TCF4 transcription which then leads to NED and treatment resistance. More

importantly, the in vivo studies suggest that TCF4 may potentially be a new therapeutic target in patients with CRPC.

5. Changes/Problems

None.

6. Products

None.

7. Participants and other collaboration organizations

Name:	Isaac Kim
Project Role:	*PI*
Nearest person month worked:	2
Contribution to Project:	*Dr. Kim managed the overall project.*

Name:	Geun Taek Lee
Project Role:	Co-investigator
Nearest person month worked:	10
Contribution to Project:	*Dr. Lee executed all the studies described in this report.*

8. Special reporting requirements

Not applicable.

9. Appendices

None.