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Lin28 Induces Resistance to Anti-Androgens Via Promotion of AR Splice Variant Generation

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Abstract

BACKGROUND—Prostate cancer (PCa) is androgen-dependent initially and progresses to a castration-resistant state after androgen deprivation therapy. Treatment options for castration-resistant PCa include the potent second-generation anti-androgen enzalutamide or CYP17A1 inhibitor abiraterone. Recent clinical observations point to the development of resistance to these therapies which may be mediated by constitutively active alternative splice variants of the androgen receptor (AR).

METHODS—Sensitivity of LNCaP cells overexpressing Lin28 (LN-Lin28) to enzalutamide, abiraterone, or bicalutamide was compared to that of control LN-neo cells using cell growth assays, proliferation assays using MTT, anchorage-dependent clonogenic ability assays and soft agar assays. Ability of LN-Lin28 cells to maintain AR activation after treatment with enzalutamide, abiraterone, or bicalutamide was tested using immunofluorescence, Western blotting, ChIP assays, and qRT-PCR. Importance of Lin28 in enzalutamide resistance was assessed by the downregulation of Lin28 expression in C4-2B and 22Rv1 cells chronically treated with enzalutamide. Requirement for sustained AR signaling in LN-Lin28 cells was examined by the downregulation of either full length AR or AR-V7 using siRNA.

RESULTS—We show that Lin28 promotes the development of resistance to currently used targeted therapeutics by enhancing the expression of AR splice variants such as AR-V7. PCa cells overexpressing Lin28 exhibit resistance to treatment with enzalutamide, abiraterone, or bicalutamide. Downregulation of Lin28 resensitizes enzalutamide-resistant PCa cells to enzalutamide treatment. We also show that the upregulation of splicing factors such as hnRNPA1 by Lin28 may mediate the enhanced generation of AR splice variants in Lin28-expressing cells.

CONCLUSIONS—Our findings suggest that Lin28 plays a key role in the acquisition of resistance to AR-targeted therapies by PCa cells and establish the importance of Lin28 in PCa progression.

Keywords

Lin28; anti-androgens; AR variants; resistance

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INTRODUCTION

Prostate cancer (PCa) is the most common cancer among men in the US and other Western countries [1]. Androgen signaling driven by the androgen receptor (AR) plays a central role in PCa tumorigenesis and hence the standard-of-care treatment for PCa is androgen deprivation by a variety of strategies such as luteinizing hormone-releasing hormone agonists, anti-androgens, estrogens, orchiectomy, and drugs preventing both intratumoral and adrenal androgen production [2]. Almost all PCa tumors eventually develop castration resistance and exhibit re-activation of androgen signaling and/or overexpression and/or reprogramming of AR signaling. Reactivation of AR signaling is a hallmark of PCa progression and is also associated with patient response to therapy. Constitutive AR signaling is mediated by various mechanisms including AR overexpression, mutations in the AR, interactions with other signaling pathways, and increased generation of alternative splice variants lacking the ligand-binding domain [3]. The next generation anti-androgen enzalutamide [4] and androgen synthesis inhibitor abiraterone [5] have been approved for the treatment of patients prior to or after chemotherapy. These therapies, though effective initially, rapidly result in the development of resistance to the agent and progression of disease [6]. Hence it is critically important to understand the mechanisms leading to the development of resistance to these therapies in order to devise combination strategies that achieve a better and more sustained response to treatment.

The Lin28 proteins are evolutionarily conserved RNA binding proteins that regulate several important cellular functions associated with development, glucose metabolism, differentiation, and pluripotency [7,8]. LIN28 binds to the terminal loops of *Let-7* miRNA family precursors and inhibits their processing into mature miRNAs [9]. By blocking *Let-7* biogenesis, Lin28 leads to derepression of known oncogenic targets of *Let-7* such as *Myc* [10], *Ras* [11], *HMGA2* [12], and cyclins [13]. Moreover, Lin28 has been shown to promote glycolytic metabolism in tissues and cancer cells, and thus is a central regulator of cellular bioenergetics [14,15]. It is a predominantly cytoplasmic protein that associates with RNA in stress granules, P-bodies, and polysomes [16]. In embryonic stem cells, LIN28 enhances the translation of mRNAs important for cell growth via the recruitment of RNA helicase A to polysomes in a *Let-7*-independent manner [17]. In mature cells, LIN28 binds to and enhances the translation of mRNAs for several metabolic enzymes, thereby increasing glycolysis and oxidative phosphorylation, thus driving tissue repair by reprogramming cellular metabolism [14]. In both human embryonic stem cells and somatic cells, LIN28 has been shown to regulate splicing factor abundance [18]. Consistent with Lin28's impact on alternative splicing factors, the up-regulation of Lin28a in somatic HEK293 cells caused dramatic changes in alternative splicing patterns.

Increased Lin28 expression was reported in various carcinomas and was associated with poor patient prognosis [7,19]. Lin28 has profound pleiotropic effects on both the proliferative and metabolic machinery of tumor cells. The strong effect of Lin28 on cell progression and proliferation and its frequent reactivation in multiple cancers supports the role of Lin28 as a potential oncogene [20]. Lin28 is expressed in all grades of prostatic carcinomas and in some PCa cell lines, but not in normal prostate tissue [21]. Our previous

studies demonstrated that Lin28 is highly expressed in prostate tumors compared to their matched benign counterparts and that Lin28 expression is correlated positively with that of the AR and c-Myc in PCa [22,23]. In this report, we studied the role of Lin28 in the development of resistance to currently used therapeutics such as enzalutamide, abiraterone or bicalutamide. We show that Lin28 regulates PCa cell sensitivity to androgen-signaling inhibitors by promoting the generation of and altering the ratio of AR splice variants to full length AR.

MATERIALS AND METHODS

Cell Lines and Reagents

LNCaP, C4-2B, CWR22Rv1, and VCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were cultured in RPMI containing either 10% complete FBS or 10% charcoal-dextran-stripped FBS (CS-FBS) and penicillin/streptomycin. LNCaP passage numbers <20 were used throughout the study. 22Rv1 and C4-2B cells resistant to enzalutamide (22Rv1-Enza-R and C4-2B-Enza-R) were generated as described previously [24,25]. Antibodies against AR (441; mouse monoclonal) and Tubulin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies against Lin28 were purchased from Abcam (San Francisco, CA). Antibodies against AR-V7 splice variant were obtained from Precision Antibody (Columbia, MD). Sso Fast™ Eva Green qPCR Supermix was from Bio-Rad. All other reagents were of analytical grade and obtained from local suppliers.

Cell Growth Assays

Plasmid and plasmid-based shRNA transfections were performed using the Attractene transfection reagent (Qiagen). Oligonucleotide siRNA transfections were performed using the Lipofectamine 2000 transfection reagent (Invitrogen). Viable cell numbers were determined using a Coulter cell counter (Beckman Coulter). Cell proliferation assays using MTT were performed as described previously [26].

Western Blot Analysis

Cells were lysed in high salt buffer containing 50 mM Hepes pH 7.9, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM Na Vanadate, 1 mM NaF and protease inhibitor cocktail (Roche) as described earlier [27]. Total protein was estimated using the Coomassie Protein Assay Reagent (Pierce). Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBST (1× PBS+0.1% Tween-20) and probed with the indicated primary antibodies in 1% BSA. The signal was detected by ECL (Millipore) after incubation with the appropriate HRP-conjugated secondary antibodies.

Real-Time Quantitative Rt-pcr

Total RNAs were extracted using TriZOL reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using Sso Fast™ Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions and as described previously [28]. Each reaction

was normalized by co-amplification of actin. Triplicates of samples were run on a Bio-Rad CFX-96 real-time cyclers.

Measurement of PSA

PSA levels were measured in the culture supernatants using an ELISA kit (United Biotech Inc, Mountain View, CA) according to the manufacturer's instructions and as described previously [29].

Chromatin Immunoprecipitation Assay (ChIP)

DNA-protein complexes in the cells were cross-linked with 1% formaldehyde for 10 min at 37°C. Cells were washed and harvested and cell lysates prepared by sonication at 4°C. 10 µg of the total protein was immunoprecipitated with anti-AR antibodies. Isotype matched IgG was used as control. The immunoprecipitated protein-DNA complexes were washed, eluted, the cross linking was reversed with NaCl for 4 hr at 65°C, and subjected to proteinase K digestion at 37°C overnight. Bound DNA was extracted with phenol: chloroform:isoamylalcohol, and precipitated with the addition of ammonium acetate, glycogen and 100% ethanol. Dissolved DNA was subjected to PCR with primers spanning either the proximal or the distal enhancer AREs of the PSA promoter. The primer sequences used were:

PSA promoter: (Proximal) ARE-I/II: Forward: 5' -
CCTAGATGAAGTCTCCATGAGCTACA-3'

ARE-I/II: Reverse: 5' -GGGAGGGAGAGCTAGCACTTG-3'

(Distal) ARE-III: Forward: 5' -CAT GTT CAC ATT AGT ACA CCT TGC C-3'

ARE-III: Reverse: 5' -TCT CAG ATC CAG GCT TGC TTA CTG TC-3'

Immunofluorescence

LN-neo and LN-Lin28 cells were used to assess the extent of AR nuclear translocation after treatment with vehicle, enzalutamide, abiraterone, or bicalutamide. 5×10^3 cells were plated in 6-well chamber slides and treated with 20 µM each of enzalutamide, abiraterone, or bicalutamide. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100, and incubated with 1% BSA to block non-specific binding. Cells were incubated with anti-AR monoclonal antibodies (441, Santa Cruz Biotechnology) overnight, localization of the intracellular AR protein was visualized with secondary antibodies conjugated to Alexa Fluor 594 (Abcam), and nuclei were visualized with mounting medium containing DAPI.

Clonogenic Assays

Anchorage-dependent clonogenic ability assays were performed as described previously [30]. Briefly, LN-neo and LN-Lin28 cells were treated with vehicle or 20 µM each of enzalutamide, abiraterone, or bicalutamide for 24 hr. Cells were trypsinized and replated at low densities (1000 cells/dish) in 10 cm culture plates. The plates were incubated at 37°C in RPMI1640 containing 10% FBS and were left undisturbed for 14 days. At the end of the

experiment, cells were fixed with methanol, stained with 0.05% Crystal Violet, and the numbers of colonies were counted.

Soft-Agar Colony Formation Assays

Anchorage-independent colony formation assays were performed as described previously [31]. LN-neo and LN-Lin28 cells were treated with vehicle or 20 μ M each of enzalutamide, abiraterone, or bicalutamide for 24 hr. After treatment, cells were plated in 0.35% agarose overlying a 1.2% agar layer. Cells were fed twice a week with complete RPMI1640 with 10% FBS and were incubated at 37°C for 2 weeks. At the end of the experiment, colonies were stained with 0.05% Crystal Violet and counted under a microscope.

Statistical Analyses

Data are shown as means $\pm$ SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for the comparison of means. $P < 0.05$ was considered significant.

RESULTS

Lin28 Induces Resistance to Therapeutics Targeting Androgen-Signaling

Our previous studies showed that Lin28 is overexpressed in prostate cancer and activates the androgen receptor [22]. LNCaP cells stably expressing Lin28 (LN-Lin28) exhibited higher growth rate and higher clonogenic ability compared to control LN-neo cells. In this study, we examined whether prostate cancer cells expressing Lin28 exhibit altered sensitivity to anti-androgens such as enzalutamide, abiraterone, or bicalutamide. LN-neo and LN-Lin28 cells were treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide for 48 hr and cell survival was analyzed. As shown in Figure 1A, treatment with enzalutamide, abiraterone, or bicalutamide decreased cell survival of LN-neo cells by ~60% after 48 hr treatment, while having no effect on cell survival of LN-Lin28 cells (LN-Lin28 cl#10). To account for clone-specific effects, we tested another clone of LN-Lin28 cells (LN-Lin28 cl#4). As shown in Figure 1B, there were no significant differences between the number of LN-Lin28 cells surviving treatment with enzalutamide, abiraterone or bicalutamide and those treated with vehicle. Insets in Figure 1A and B show the expression levels of Lin28 in both stable clones. These results indicate that prostate cancer cells expressing Lin28 may exhibit resistance to anti-androgen treatment. All subsequent experiments were performed using the LN-Lin28 cl#10. To test whether cytotoxicity was a factor in reducing cell survival, we assessed proliferation indices of LN-neo and LN-Lin28 cells treated with 0, 5, 10, or 20 μ M enzalutamide or abiraterone for 24, 48, or 72 hr. As shown in Figure 1C and D, 20 μ M enzalutamide or abiraterone (highest doses used) did not exert cytotoxicity on either LN-neo or LN-Lin28 cells. Next, we tested whether treatment with AR-targeted therapeutics affects the clonogenic ability of LN-Lin28 cells. We performed anchorage-dependent colony formation assays using LN-neo and LN-Lin28 cells treated with vehicle, enzalutamide, abiraterone or bicalutamide (20 μ M each) for 24 hr. As shown in Figure 2A, the number of colonies formed by LN-neo cells decreased by ~50% when treated with enzalutamide, abiraterone, or bicalutamide compared to treatment with vehicle. On the other hand, colony formation ability of LN-Lin28 cells was not affected by treatment with enzalutamide,

abiraterone, or bicalutamide. We also performed anchorage-independent soft agar colony formation assays using LN-neo and LN-Lin28 cells treated with vehicle, enzalutamide, abiraterone, or bicalutamide for 24 hr. As shown in Figure 2B, LN-Lin28 cells formed significantly higher numbers of colonies in soft agar compared to LN-neo cells, which is consistent with our previous observations that Lin28 confers a growth and survival advantage on prostate cancer cells. Moreover, the ability of LN-Lin28 cells to form colonies in soft agar was not impaired significantly by treatment with enzalutamide, abiraterone, or bicalutamide, demonstrating that Lin28 may induce resistance to therapy in prostate cancer cells.

Lin28 Induced AR Activation is Not Inhibited by Treatment With Targeted Therapeutics

We showed previously that Lin28 activates the androgen receptor [22]. Since our results show that prostate cancer cells expressing Lin28 are resistant to treatment with enzalutamide, abiraterone, or bicalutamide, we tested whether Lin28-induced activation of AR is affected by treatment with these agents. We analyzed the nuclear translocation of the AR in LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide or abiraterone. Treatment with enzalutamide or abiraterone abolished the nuclear translocation of AR in LN-neo cells while not affecting the nuclear translocation of AR in LN-Lin28 cells (Fig. 3A). The nuclear translocation of FL AR as well as AR variants was maintained in LN-Lin28 cells treated with enzalutamide or abiraterone (Fig. 3A, middle panel). These results were confirmed using immunofluorescence assays (Fig. 3B) and indicate that while enzalutamide or abiraterone succeed in inhibiting nuclear translocation of AR in prostate cancer cells lacking expression of Lin28, they fail to do so in prostate cancer cells expressing Lin28. Further, we tested whether recruitment of AR to the promoters of target genes is affected by treatment with enzalutamide, abiraterone, or bicalutamide in LN-Lin28 cells using ChIP assays. As shown in Figure 3C, the recruitment of AR to AREI/II (left panel) and AREIII (right panel) in the PSA promoter was almost entirely (>90%) abolished by treatment with enzalutamide, abiraterone, or bicalutamide, while the recruitment of AR to the PSA promoter was reduced minimally by <30% in LN-Lin28 cells, indicating that prostate cancer cells expressing Lin28 can sustain the activation of AR even during treatment with next generation therapeutics. Next, we tested whether the transactivation of target gene promoters by AR is affected in LN-neo and LN-Lin28 cells using qRT-PCR and ELISA assays. As shown in Figure 3D, the transcript levels of PSA (left panel) and NKX3.1 (right panel), two classical target genes of the AR, were reduced upon treatment with enzalutamide, abiraterone, or bicalutamide in LN-neo cells but were not affected significantly in LN-Lin28 cells. These results were also confirmed by measuring the secretion of PSA using ELISA. As shown in Figure 3E, the secretion of PSA by LN-neo cells was reduced by ~60% after treatment, while secretion of PSA by LN-Lin28 cells was not affected significantly by treatment with enzalutamide, abiraterone, or bicalutamide. Collectively, these results demonstrate that sustained AR activation induced by Lin28 may mediate the resistance to enzalutamide, abiraterone, or bicalutamide exhibited by prostate cancer cells expressing Lin28.

Downregulation of Lin28 Resensitizes Enzalutamide-Resistant Cells to Enzalutamide

Our findings suggest that the upregulation of Lin28 may mediate the acquisition of resistance to anti-androgens such as enzalutamide. Hence, we analyzed the expression levels of Lin28 in enzalutamide-resistant C4-2B-Enza-R and 22Rv1-Enza-R cells (generated by chronic exposure to enzalutamide) compared to levels in parental C4-2B and 22Rv1 cells, respectively. As shown in Figure 4A, both C4-2B-Enza-R and 22Rv1-Enza-R cells express higher levels of Lin28 protein, suggesting that upregulation of Lin28 may be one of the mechanisms of resistance in these cells. Interestingly, C4-2B-Enza-R and 22Rv1-Enza-R cells also express higher levels of both full length AR and the AR-V7 splice variant. AR-V7 has been implicated in the acquired resistance to enzalutamide in several studies [25,32,33]. Hence, we examined the effects of downregulation of Lin28 on the sensitivity/resistance of enzalutamide-resistant prostate cancer cells to enzalutamide, by transfecting two shRNAs against Lin28 into C4-2B, C4-2B-Enza-R; and 22Rv1, 22Rv1-Enza-R cells followed by treatment with 20 μ M enzalutamide. As shown in Figure 4B and C, downregulation of Lin28 enhanced the sensitivity of C4-2B-Enza-R and 22Rv1-Enza-R cells to enzalutamide treatment. Next, we analyzed the protein levels of Lin28 in C4-2B-Enza-R and 22Rv1-Enza-R cells transfected with shRNA against Lin28 to confirm the downregulation of Lin28 (Fig. 4D and E). The downregulation of Lin28 reduced the expression levels of both full length AR and AR-V7, indicating that suppression of Lin28 reduces inducible and constitutive AR signaling and thereby resensitizes enzalutamide-resistant cells to enzalutamide.

Downregulation of AR Signaling Resensitizes Lin28 Expressing Cells to Enzalutamide

Our results showed that LNCaP cells stably expressing Lin28 (LN-Lin28) express higher levels of both full length AR and AR-V7 (Fig. 5A). Our previous studies and others demonstrated that AR-V7 plays an important role in the development of resistance to enzalutamide. Hence, we tested whether the downregulation of either FL AR or AR-V7 resensitizes LN-Lin28 cells to enzalutamide treatment. LN-neo control and LN-Lin28 cells were transfected with siRNAs specific against either FL AR or AR-V7 and treated with 20 μ M enzalutamide. Cell survival was analyzed after 48 hr. The results showed that the suppression of expression of either FL AR or AR-V7 reduced the survival of LN-Lin28 cells treated with enzalutamide (Fig. 5B), indicating that the upregulation of both FL AR and AR-V7 is essential for the resistance to enzalutamide exhibited by LN-Lin28 cells. We reported previously that two splicing factors of the heterogeneous ribonucleoprotein family (hnRNPA1 and hnRNPA2) play important roles in the generation of AR splice variants such as AR-V7 in PCa cells [34]. As we observed higher levels of AR-V7 in LN-Lin28 cells compared to control LN-neo cells, we analyzed the expression levels of hnRNPA1 and hnRNPA2 in LN-Lin28 cells by Western blotting. As shown in Figure 5C, we observed higher levels of both hnRNPA1 and hnRNPA2 in LN-Lin28 cells compared to the control cells. These observations led us to test whether the downregulation of hnRNPA1 or hnRNPA2 affects the sensitivity of LN-Lin28 cells to enzalutamide. We transfected LN-neo and LN-Lin28 cells with siRNAs against either hnRNPA1 or hnRNPA2, treated them with 20 μ M enzalutamide, and analyzed cell survival after 48 hr. As shown in Figure 5D, downregulation of hnRNPA1 or hnRNPA2 leading to lower levels of AR-V7 increased the sensitivity of LN-Lin28 cells to enzalutamide, indicating that Lin28 induces the generation of AR-V7 via increase in levels of splicing factors such as hnRNPA1 and/or hnRNPA2.

These observations confirm the central role of Lin28 in altering AR signaling, and demonstrate that Lin28 modulates the ability of PCa cells to respond to enzalutamide treatment by increasing the generation of constitutively active AR splice variants such as AR-V7.

DISCUSSION

Lin28 is known as a reprogramming factor and a marker for induced pluripotent stem cells [35]. Effects of Lin28 can be mediated via Let-7-dependent or Let-7-independent pathways. Suppression of maturation of the Let-7 family miRNAs is one of the principal functions of Lin28, and in addition, it can regulate the translation of cellular mRNAs as an RNA-binding protein. Our previous studies indicated that Lin28 promotes PCa tumorigenesis by inducing AR activation [22]. We have demonstrated that the expression levels of AR and Lin28 correlate positively with each other in PCa and that increased expression of Lin28 marks castration-resistant progression of PCa. In this study, we analyzed the ability of Lin28 to modulate the resistance of PCa cells to treatment with enzalutamide, abiraterone, or bicalutamide. Our findings clearly demonstrate that Lin28 modulates the sensitivity of PCa cells to enzalutamide by altering the ratio of constitutively active AR variants to full length AR. Treatment with enzalutamide, abiraterone, or bicalutamide did not suppress Lin28-induced AR activation. Overexpression of Lin28 increased while downregulation of Lin28 decreased PCa cell survival upon treatment with enzalutamide. Overexpression of Lin28 enhanced the generation of AR variants such as AR-V7 while downregulation of Lin28 suppressed the levels of AR variants. Our results also show that Lin28 may regulate the generation of AR variants by altering the levels of splicing factors such as hnRNPA1. Suppression of expression of either hnRNPA1 or AR-V7 resensitized Lin28-expressing PCa cells to enzalutamide treatment. Taken together, these findings provide a mechanistic rationale for the potential targeting of Lin28 to combat PCa progression.

Generation of constitutively active splice variants of AR that are insensitive to anti-androgen therapy is one of the principal mechanisms through which PCa cells by-pass growth inhibition after treatment with next generation anti-androgens such as enzalutamide. Generation of AR-V7 has been shown in several studies to be responsible for the development of resistance to enzalutamide [24,25,32]. Indeed, the presence of AR-V7 in circulating tumor cells has been validated to predict patient response to enzalutamide or abiraterone treatment [33,36]. These findings attest to the importance of AR alternative splice variants in PCa. Even though the central role of AR splice variants has been established and is even being proposed to be used as a biomarker in a clinical setting, understanding of the factors/mechanisms which regulate the generation of AR variants remains rudimentary. Our previous studies showed that a regulatory circuit involving NF- κ B2/p52, c-Myc and hnRNPA1 may regulate the levels of AR variants [25,34]. In this report, we identified a novel role for Lin28 in the regulation of AR-V7 Levels, possibly involving hnRNPA1. HnRNPA1 belongs to the heterogeneous nuclear protein family composed of >20 splicing regulators. Expression of hnRNPA1 is enhanced in several human malignancies [37] and deregulated splicing due to altered levels of splicing factors can lead to tumorigenesis [38,39]. Our previous findings indicate that hnRNPA1 levels are enhanced in PCa and that recruitment of hnRNPA1 to the AR pre-mRNA is amplified in enzalutamide-

resistant cells [34]. HnRNPA1 and c-Myc exhibit reciprocal regulation of each other's expression [40]. Previous studies also showed that NF-kappaB and c-Myc are involved in the transcriptional regulation of Lin28 [41]. Taken together, these studies illustrate the importance of the NF-kappaB:c-Myc:Lin28 pathway in PCa. Our current results build upon these findings and show that Lin28 plays a major role in enzalutamide resistance by enhancing the expression of hnRNPA1 and thereby promoting the generation of constitutively active AR splice variants such as AR-V7.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin.* 2015; 65(1):5–29. [PubMed: 25559415]
2. Wyatt AW, Gleave ME. Targeting the adaptive molecular landscape of castration-resistant prostate cancer. *EMBO Mol Med.* 2015; 7(7):878–894. [PubMed: 25896606]
3. Farooqi A, Sarkar F. Overview on the complexity of androgen receptor-targeted therapy for prostate cancer. *Cancer Cell Int.* 2015; 15(1):7. [PubMed: 25705125]
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(5928):787–790. [PubMed: 19359544]
5. Sidaway P. Prostate cancer: Abiraterone treatment improves overall survival in patients with mCRPC. *Nat Rev Urol.* 2015; 12(3):120–120.
6. Vaishampayan UN. Sequences and combinations of multifaceted therapy in advanced prostate cancer. *Curr Opin Oncol.* 2015; 27(3):201–208. [PubMed: 25811344]
7. Zhou J, Ng S-B, Chng W-J. LIN28/LIN28B: An emerging oncogenic driver in cancer stem cells. *Int J Biochem Cell Biol.* 2013; 45(5):973–978. [PubMed: 23420006]
8. Kumar RM, Cahan P, Shalek AK, Satija R, Jay DaleyKeyser A, Li H, Zhang J, Pardee K, Gennert D, Trombetta JJ, Ferrante TC, Regev A, Daley GQ, Collins JJ. Deconstructing transcriptional heterogeneity in pluripotent stem cells. *Nature.* 2014; 516(7529):56–61. [PubMed: 25471879]
9. Thornton JE, Gregory RI. How does Lin28 Let-7 control development and disease? *Trends Cell Biol.* 2012; 22(9):474–482. [PubMed: 22784697]
10. Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, Petrelli NJ, Dunn SP, Krueger LJ. MicroRNA Let-7a downregulates MYC and reverts MYC-induced growth in burkitt lymphoma cells. *Cancer Res.* 2007; 67(20):9762–9770. [PubMed: 17942906]
11. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS Is regulated by the let-7 microRNA family. *Cell.* 2005; 120(5):635–647. [PubMed: 15766527]
12. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and hmga2 enhances oncogenic transformation. *Science.* 2007; 315(5818):1576–1579. [PubMed: 17322030]
13. Chang H-M, Martinez NJ, Thornton JE, Hagan JP, Nguyen KD, Gregory RI. Trim71 cooperates with microRNAs to repress Cdkn1a expression and promote embryonic stem cell proliferation. *Nat Commun.* 2012; 3:923. [PubMed: 22735451]

14. Shyh-Chang N, Zhu H, Yvanka de Soysa T, Shinoda G, Seligson Marc T, Tsanov Kaloyan M, Nguyen L, Asara John M, Cantley Lewis C, Daley George Q. Lin28 enhances tissue repair by reprogramming cellular metabolism. *Cell*. 2013; 155(4):778–792. [PubMed: 24209617]
15. Ma X, Li C, Sun L, Huang D, Li T, He X, Wu G, Yang Z, Zhong X, Song L, Gao P, Zhang H. Lin28/let-7 axis regulates aerobic glycolysis and cancer progression via PDK1. *Nat Commun*. 2014; 5:5212. [PubMed: 25301052]
16. Guo J, Li Z-c, Feng Y-h. Expression and activation of the reprogramming transcription factors. *Biochem a Biophys Res Commun*. 2009; 390(4):1081–1086.
17. Jin J, Jing W, Lei X-X, Feng C, Peng S, Boris-Lawrie K, Huang Y. Evidence that lin28 stimulates translation by recruiting RNA helicase A to polysomes. *Nucleic Acids Res*. 2011; 39(9):3724–3734. [PubMed: 21247876]
18. Wilbert Melissa L, Huelga Stephanie C, Kapeli K, Stark Thomas J, Liang Tiffany Y, Chen Stella X, Yan Bernice Y, Nathanson Jason L, Hutt Kasey R, Lovci Michael T, Kazan H, Vu Anthony Q, Massirer Katlin B, Morris Q, Hoon S, Yeo Gene W. LIN28 Binds Messenger RNAs at GGAGA Motifs and Regulates Splicing Factor Abundance. *Mol Cell*. 2012; 48(2):195–206. [PubMed: 22959275]
19. Tu H-C, Schwitalla S, Qian Z, LaPier GS, Yermalovich A, Ku YC, Chen S-C, Viswanathan SR, Zhu H, Nishihara R, Inamura K, Kim SA, Morikawa T, Mima K, Sukawa Y, Yang J, Meredith G, Fuchs CS, Ogino S, Daley GQ. LIN28 cooperates with WNT signaling to drive invasive intestinal and colorectal adenocarcinoma in mice and humans. *Genes Dev*. 2015; 29(10):1074–1086. [PubMed: 25956904]
20. Büsling I, Slack FJ, Großhans H. Let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med*. 2008; 14(9):400–409. [PubMed: 18674967]
21. Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, O’Sullivan M, Lu J, Phillips LA, Lockhart VL, Shah SP, Tanwar PS, Mermel CH, Beroukhir R, Azam M, Teixeira J, Meyerson M, Hughes TP, Llovet JM, Radich J, Mullighan CG, Golub TR, Sorensen PH, Daley GQ. Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet*. 2009; 41(7):843–848. [PubMed: 19483683]
22. Tummala R, Nadiminty N, Lou W, Zhu Y, Gandour-Edwards R, Chen H-W, Evans CP, Gao AC. Lin28 promotes growth of prostate cancer cells and activates the androgen receptor. *Am J Pathol*. 2013; 183(1):288–295. [PubMed: 23790802]
23. Nadiminty N, Tummala R, Lou W, Zhu Y, Zhang J, Chen X, deVere White RW, Kung H-J, Evans CP, Gao AC. MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells. *J Biol Chem*. 2012; 287(2):1527–1537. [PubMed: 22128178]
24. Liu C, Lou W, Zhu Y, Nadiminty N, Schwartz CT, Evans CP, Gao AC. Niclosamide inhibits androgen receptor variants expression and overcomes enzalutamide resistance in castration-resistant prostate cancer. *Clin Cancer Res*. 2014; 20:3198–3210. [PubMed: 24740322]
25. Nadiminty N, Tummala R, Liu C, Yang J, Lou W, Evans CP, Gao AC. NF- κ B2/p52 induces resistance to enzalutamide in prostate cancer: Role of androgen receptor and Its variants. *Mol Cancer Ther*. 2013; 12(8):1629–1637. [PubMed: 23699654]
26. Hamid ARAH, Verhaegh GW, Smit FP, van Rijt-van de Westerlo C, Armandari I, Brandt A, Sweep FCGJ, Sedelaar JPM, Schalken JA. Dutasteride and enzalutamide synergistically suppress prostate tumor Cell proliferation. *J Urol*. 2015; 193(3):1023–1029. [PubMed: 25242390]
27. Nadiminty N, Lou W, Lee SO, Lin X, Trump DL, Gao AC. Stat3 activation of NF-kappaB p100 processing involves CBP/p300-mediated acetylation. *Proc Natl Acad Sci USA*. 2006; 103(19):7264–7269. [PubMed: 16651533]
28. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung H-J, Evans CP, Zhou Q, Gao AC. Aberrant activation of the androgen receptor by NF-kB2/p52 in prostate cancer cells. *Cancer Res*. 2010; 70(8):3309–3319. [PubMed: 20388792]
29. Lee SO, Chun JY, Nadiminty N, Lou W, Gao AC. Interleukin-6 undergoes transition from growth inhibitor associated with neuroendocrine differentiation to stimulator accompanied by androgen receptor activation during LNCaP prostate cancer cell progression. *Prostate*. 2007; 67(7):764–773. [PubMed: 17373716]

30. Nadiminty N, Chun JY, Lou W, Lin X, Gao AC. NF- κ B2/p52 enhances androgen-independent growth of human LNCaP cells via protection from apoptotic cell death and cell cycle arrest induced by androgen-deprivation. *Prostate*. 2008; 68(16):1725–1733. [PubMed: 18781579]
31. Nadiminty N, Tummala R, Lou W, Zhu Y, Shi X-B, Zou JX, Chen H, Zhang J, Chen X, Luo J, deVere White RW, Kung H-J, Evans CP, Gao AC. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. *PLoS ONE*. 2012; 7(3):e32832. [PubMed: 22479342]
32. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KAT, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. *Cancer Res*. 2013; 73(2):483–489. [PubMed: 23117885]
33. Antonarakis ES, Lu C, Wang H, Lubner B, Nakazawa M, Roeser JC, Chen Y, Mohammad TA, Chen Y, Fedor HL, Lotan TL, Zheng Q, De Marzo AM, Isaacs JT, Isaacs WB, Nadal R, Paller CJ, Denmeade SR, Carducci MA, Eisenberger MA, Luo J. ARV7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med*. 2014; 371(11):1028–1038. [PubMed: 25184630]
34. Nadiminty N, Tummala R, Liu C, Lou W, Evans CP, Gao AC. NF- κ B2/p52:c-Myc:HnRNPA1 pathway regulates expression of androgen receptor splice variants and enzalutamide sensitivity in prostate cancer. *Mol Cancer Ther*. 2015; 14(8):1884–1895. [PubMed: 26056150]
35. Viswanathan SR, Daley GQ. Lin28: A microRNA regulator with a macro role. *Cell*. 2010; 140(4):445–449. [PubMed: 20178735]
36. Steinestel J, Lueddeke M, Arndt A, Schnoeller TJ, Lennerz JK, Wurm C, Maier C, Cronauer MV, Steinestel K, Schrader AJ. Detecting predictive androgen receptor modifications in circulating prostate cancer cells. *Oncotarget*. 2015
37. Carpenter B, MacKay C, Alnabulsi A, MacKay M, Telfer C, Melvin WT, Murray GI. The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. *Biochim Biophys Acta*. 2006; 1765(2):85–100.
38. David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: Pathways and programs unhinged. *Genes Dev*. 2010; 24(21):2343–2364. [PubMed: 21041405]
39. Ghigna C, Valacca C, Biamonti G. Alternative splicing and tumor progression. *Curr Genomics*. 2008; 9(8):556–570. [PubMed: 19516963]
40. David CJ, Chen M, Assanah M, Canoll P, Manley JL. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature*. 2010; 463(7279):364–368. [PubMed: 20010808]
41. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF- κ B, Lin28, Let-7 MicroRNA, and IL6 Links Inflammation to cell transformation. *Cell*. 2009; 139(4):693–706. [PubMed: 19878981]

Fig. 1.

PCa cells overexpressing Lin28 exhibit resistance to treatment with enzalutamide, abiraterone, or bicalutamide. **A:** LN-neo parental cells and LN-Lin28 cells were subjected to treatment with 20 μ M each of enzalutamide, abiraterone, or bicalutamide and cell survival was measured by counting cell numbers after 48 hr. Inset shows the expression of Lin28 in LN-Lin28 cells. Cell survival of LN-neo cells was suppressed by all three agents, which failed to suppress LN-Lin28 cell survival significantly. **B:** Similar experiments were performed using LN-neo and LN-Lin28 CI#4 cells and cell survival was measured by

counting cell numbers after 48 hr. Inset shows the expression of Lin28 in LN-Lin28 Cl#4 cells. Cell survival of LN-neo cells was suppressed by all three agents, which failed to suppress LN-Lin28 Cl #4 cell survival significantly. Cell proliferation assays were performed using MTT in LN-neo and LN-Lin28 cells treated with 0, 5, 10, and 20 μ M enzalutamide (**C**) or abiraterone (**D**). The observed reduction in cell numbers was due to the suppression of cell survival and not due to cytotoxicity induced by enzalutamide or abiraterone.

Fig. 2.

PCa cells overexpressing Lin28 exhibit resistance to treatment with enzalutamide, abiraterone, or bicalutamide. **A:** Quantitative assessment of the number of colonies formed in anchorage-dependent clonogenic assay of LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide for 24 hr and plated at a density of 1000 cells/dish. Numbers of colonies were counted after 2 weeks. Ability of LN-neo cells to form colonies was inhibited significantly by all three therapeutics, whereas ability of LN-Lin28 cells to form colonies was not inhibited. **B:** Quantitative assessment of the number of colonies formed in anchorage-independent soft-agar colony formation assay of LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide for 24 hr and plated in 0.35% agarose overlying a 1.2% agar layer. Numbers of colonies were counted after 2 weeks. Ability of LN-neo cells to form anchorage-independent colonies in soft agar was inhibited significantly by all three compounds, whereas ability of LN-Lin28 cells to form colonies was not inhibited.

Fig. 3.

LN-Lin28 cells sustain Lin28-induced AR activation when treated with enzalutamide, abiraterone, or bicalutamide. AR nuclear translocation was assessed by (A) Western blotting and (B) immunofluorescence assays in LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide. AR nuclear translocation was inhibited by all three agents in LN-neo cells while they failed to inhibit AR nuclear translocation in LN-Lin28 cells. C: Recruitment of AR to AREI/II (left panel) and AREIII (right panel) regions in PSA promoter was assessed by ChIP assays in LN-neo and LN-Lin28 cells treated with

20 μ M each of enzalutamide, abiraterone, or bicalutamide. Binding of AR to both regions was inhibited by all three in LN-neo cells, while AR recruitment was unimpaired in LN-Lin28 cells treated with these agents. **D:** Activation of the expression of AR target genes PSA (left panel) and NKX3.1 (right panel) was assessed by qPCR in LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide. Expression levels of both PSA and NKX3.1 were inhibited by all three agents in LN-neo cells, while they failed to suppress expression of PSA and NKX3.1 in LN-Lin28 cells. **E:** Secretion of PSA by LN-neo and LN-Lin28 cells was measured using ELISA in LN-neo and LN-Lin28 cells treated with 20 μ M each of enzalutamide, abiraterone, or bicalutamide. PSA secretion by LN-neo cells was inhibited by all three therapeutics, but not in LN-Lin28 cells.

Fig. 4. Lin28 is required for the acquired resistance of enzalutamide-resistant PCa cells to enzalutamide. **A:** Enzalutamide-resistant C4-2B-Enza-R and 22Rv1-Enza-R cells exhibit higher levels of Lin28, FL AR and AR-V7 compared to the parental C4-2B and 22Rv1 cells, respectively. C4-2B-Enza-R (**B**) and 22Rv1-Enza-R (**C**) cells were transfected with two different shRNAs against Lin28, were treated with 20 μ M enzalutamide and cell survival was assessed. Suppression of Lin28 expression in enzalutamide-resistant cells resensitized them to enzalutamide treatment. Suppression of Lin28 expression by shRNA reduced the expression levels of both FL AR and AR-V7 in C4-2B-Enza-R (**D**) and 22Rv1-Enza-R (**E**) cells.

Fig. 5.

Lin28 induces AR-V7 expression through upregulation of hnRNPA1. **A:** LN-Lin28 cells express higher levels of both FL AR and AR-V7. **B:** Survival of LN-neo and LN-Lin28 cells transfected with specific siRNAs against either FL AR or AR-V7 was assessed after treatment with 20 μ M enzalutamide. Downregulation of either FL AR or AR-V7 expression resensitized LN-Lin28 cells to treatment with enzalutamide. **C:** LN-Lin28 cells express high levels of the splicing factors, hnRNPA1 and hnRNPA2. **D:** Expression of hnRNPA1 or hnRNPA2 was downregulated using specific siRNAs in LN-neo and LN-Lin28 cells and the cells were subjected to treatment with 20 μ M enzalutamide. Suppression of expression of hnRNPA1 enhanced the sensitivity of LN-Lin28 cells to enzalutamide treatment. Panels below B and D show the immunoblots confirming the downregulation of FL AR, AR-V7, hnRNPA1 or hnRNPA2 after transfection with siRNA.