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Original Article

NRG1 secreted by cancer-associated fibroblasts contributes to enzalutamide resistance in prostate cancer cells

Chunyu Wang*, Hongwen Cao*, Peng Sun*, Lei Chen, Yigeng Feng, Renjie Gao

*Surgical Department I (Urology Department), LONGHUA Hospital Shanghai University of Traditional Chinese Medicine, No. 725 Wanping Road South, Xuhui District, Shanghai 200032, China. *Equal contributors.*

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Abstract: While androgen deprivation therapy (ADT) continues to be a fundamental aspect of prostate cancer treatment, the development of castration-resistant prostate cancer (CRPC) emphasizes the necessity for a more profound understanding of the tumor microenvironment (TME). Normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFs) were isolated and characterized from normal control and prostate cancer specimens, respectively. PC3 and DU145 cells, and the corresponding enzalutamide resistant counterparts, PC3-EnzR and DU145-EnzR, were co-cultured with NFs or CAFs to evaluate the effects of TME in driving enzalutamide resistance. Cell viability of prostate cancer cells was examined by MTT assay. The study also utilized recombinant human neuregulin-1 (NRG1) protein and siRNA to modulate NRG1 expression in CAFs. RT-qPCR, Western blot, and ELISA were employed to assess gene and protein expressions related to the NRG1-HER3 signaling pathway and its association with enzalutamide resistance. CAFs significantly promoted cell growth and enzalutamide resistance of PC3-EnzR and DU145-EnzR cells through substantial increased secretion of NRG1 by CAFs. Co-culturing enzalutamide-resistant prostate cancer cells (PC3-EnzR and DU145-EnzR) with CAFs further enhanced enzalutamide resistance, as evidenced by elevated IC50 values. Inhibition of NRG1 in CAFs attenuated their impact on enzalutamide resistance, providing insight into the role of NRG1 in mediating the crosstalk between CAFs and prostate cancer in the context of enzalutamide resistance. This study elucidates the pivotal role of CAF-secreted NRG1 in promoting enzalutamide resistance in prostate cancer, providing valuable insights for developing targeted therapeutic strategies to overcome resistance in advanced prostate cancer.

Keywords: Prostate cancer, enzalutamide resistance, tumor microenvironment, cancer-associated fibroblasts, NRG1

Introduction

Prostate cancer is the second most frequent malignancy diagnosed in men and the fifth leading cause of death worldwide [1, 2]. The escalating incidence, particularly in economically developed regions, suggests a looming threat to male health, positioning prostate cancer as a potential front-runner in male cancer incidence in China [3]. Clinical interventions for prostate cancer encompass androgen deprivation therapy (ADT), surgery, radiotherapy, and chemotherapy [4, 5]. While ADT remains a cornerstone across various stages, the emergence of castration-resistant prostate cancer (CRPC) post-treatment poses a substantial challenge, driving prostate cancer metastasis and lethality [5-8].

Within the intricate landscape of the prostate tumor microenvironment (TME), cancer-associated fibroblasts (CAFs) emerge as integral components [9, 10]. Originating from the activation of normal fibroblasts by factors such as transforming growth factor-β (TGF-β), CAFs play a pivotal role in reshaping the TME, influencing the invasive, metastatic, and drug-resistant characteristics of prostate cancer [11]. This dynamic interplay between tumor cells and the microenvironment underscores the need for a nuanced understanding of the molecular crosstalk driv-

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ing prostate cancer progression and therapy resistance.

Enzalutamide, a second-generation androgen-deprivation drug, has demonstrated efficacy in extending the survival time of prostate cancer patients [12]. However, the inevitable development of resistance poses a significant hurdle in long-term treatment outcomes [13, 14]. Recent studies indicate a noteworthy phenomenon: post-ADT, CAFs secrete substantial amounts of neuregulin-1 (NRG1). NRG1, a membrane glycoprotein belonging to the epidermal growth factor ligand family, binds to HER3 receptors on prostate cancer cells, activating downstream signaling pathways [15, 16]. This intricate molecular cascade emerges as a key contributor to enzalutamide resistance, marking NRG1 as a potential target in circumventing treatment resistance.

In this study, we investigate the intricate interplay between CAF-secreted NRG1 and the development of enzalutamide resistance in prostate cancer cells. By elucidating the molecular mechanisms underpinning this phenomenon, we aim to provide crucial insights into the adaptive strategies adopted by prostate cancer cells in the face of androgen deprivation therapy. Our findings not only contribute to understanding the resistance mechanisms but also unveil potential therapeutic avenues for sensitizing prostate cancer cells to enzalutamide, offering a paradigm shift in addressing ADT resistance.

Materials and methods

Cell culture

Two human prostate cancer cell lines, DU 145 and PC3, were ordered from IBCB cell bank (Shanghai, China). DU145 cells were maintained in DMEM medium (Gibco, Carlsbad, USA), which supplemented with 10% fetal bovine serum (FBS); PC3 cells were cultured in Ham's F12 medium (Gibco, Carlsbad, USA) supplemented with 10% FBS. Enzalutamide-resistant PC3 and DU145 cell lines, PC3-EnzR and DU145-EnzR, were established in our laboratory by exposing monolayer-cultured cells to incremental concentrations of enzalutamide, was purchased from Selleck Chemicals (MDV-3100, Houston, USA).

Fibroblasts isolation and culture

NFs and CAFs were isolated from adjacent normal tissues and prostate cancer specimens, respectively. The adjacent normal tissues and prostate cancer specimens utilized in our research were collected from five pairs of samples. A summary of the clinicopathological features is provided in the Table S1. All enrolled patients did not receive androgen deprivation therapy prior to surgery. Briefly, tissue samples were minced into pieces after rinsing with PBS. Samples were digested in collagenase A at 37°C, for 8 hours. After removing tissue debris, cells were collected and maintained in DMEM medium supplemented with 10% FBS. Passage 2-6 fibroblasts were used in this study following established research protocols [17].

The co-culture system of NFs or CAFs with prostate cancers cells was performed, and the co-culture protocol detailed in our previous study [18].

Cell transfection

The siRNA targeting NRG1 (siNRG1) and negative control (NC), closed into pcDNA3 overexpression plasmid, were procured from Sangon Biotech (Shanghai, China). siNRG1#1, Sense 5'-GGUCUGAACGAAACAAUAUTT-3'; Antisense 5'-AUAUUGUUUCGUUCAGACCTT-3'. siNRG1#2, Sense 5'-GCCACUCUGUAAUCGUGAUTT-3'; Antisense 5'-AUCACGAUUACAGAGUGGCTT-3'. CAFs were seeded in 6-well plates and cultured overnight, the NC or siNRG1 were transfected into cells using Lipofectamine™ RNAiMAX (ThermoFisher, Waltham, USA) following the manufacturer's guidelines, for 48 hours. Cells were then collected for subsequent experiments.

qRT-PCR

Total RNA from indicated cells was extracted using Takara Trizol (Tokyo, Japan). The cDNA was synthesized by using the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). RT-qPCR was conducted using Taqman PCR master mix on the StepOnePlus real-time PCR System (ThermoFisher, Waltham, USA) for NRG1 expression analysis. The primer sequences are listed below: NRG1: Forward 5'-CGG TGT CCA TGC CTT CCA T-3', Reverse 5'-GTG TCA CGA GAA GTA GAG GTC T-3'; GAPDH: Forward P 5'-TGT GGC ATC AAT GGA TTT GG-3', Reverse P 5'-ACA CCT

GTA TTC CGG GTC AAT-3'. The relative mRNA levels were determined using the $2^{\Delta\Delta CT}$ method, with GAPDH serving as an internal control.

Western blot

Total proteins from indicated cells were extracted using RIPA lysis buffer (ThermoFisher, Waltham, USA). Equal amounts of cell lysates were electrophoresed on SDS-polyacrylamide gels, and the proteins were transferred to PVDF membranes. Primary antibodies included NRG1 (Abcam #ab53104, 1:1000 dilution), HER3 (CST #12708, 1:1000 dilution), pHER3 (CST #12708, 1:2000 dilution), MMP1 (LSBio #LS-C352518, 1:500 dilution), MMP9 (LSBio #LS-C31757, 1:1000 dilution), and GAPDH (CST #2118, 1:1000 dilution). Protein signals were visualized using ECL reagent (ThermoFisher, Waltham, USA), and quantified by Image J software.

ELISA

The concentration of NRG1 protein in cell culture medium was determined by using ELISA kit (R&D #DY377, Minneapolis, USA), following the manufacturer instructions.

Cell viability assay

Cell viability of PC3 and DU145, the enzalutamide resistant counterpart, PC3-EnzR and DU145-EnzR, was measured by using MTT assay Kit (Abcam, Shanghai, China). In brief, cells in the culture or co-culture system exposed with NFs or CAFs were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined.

Animal study

To determine the physiological significance of the cell treatment, male BALB/c nude mice (18-22 g, 6 weeks old) were used for the *in vivo* study. Four groups of cells were used to establish an in vivo model: (1) PC3-EnzR cells cocultured with CAFs; (2) PC3-EnzR cells cocultured with CAFs transfected with siNRG1; (3) PC3-EnzR cells cocultured with CAFs transfected with siNRG1 and treated with recombinant human NRG1 (10 ng/mL); (4) Untreated PC3-EnzR cells. Each group of 1×10^7 cells suspended in 200 µL PBS was injected subcutaneously into the left flank of each mouse. Tumor growth was monitored once the tumor volume reached 200 mm^3. Tumor volume was calculated using the formula (length × $width^2$)/2. On day 21, animals were sacrificed, and the tumors were isolated and weighed. The study was approved by LONGHUA Hospital Shanghai University of Traditional Chinese Medicine.

Statistical analysis

All the statistical analyses in this study were performed by using Prism 9.0. Data are presented as mean ± SEM. The two-tailed unpaired Student's t-test, one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, and two-way ANOVA with Sidak's multiple comparisons test were used to evaluate the statistical differences in the treatment.

Results

CAFs promote cell growth of prostate cancer cells

We investigated the impact of CAFs on the growth of prostate cancer cells. To elucidate the potential molecular underpinnings of this observed effect, we conducted gene and protein analyses of NRG1 in both normal fibroblasts (NFs) and CAFs. Intriguingly, compared to NFs, CAFs exhibited a significant upregulation in the expression of NRG1 (**Figure 1A-C**). Building upon our prior work [19], we established a co-culture system involving fibroblasts and PC3/DU145 prostate cancer cells (**Figure 1D**). Notably, the co-culture of normal fibroblasts with PC3 and DU145 cells did not significantly impact cell growth. However, the introduction of CAFs into the co-culture system resulted in a substantial promotion of PC3 and DU145 cell growth (**Figure 1E** and **1F**). This finding underscores the potential role of NRG1 in mediating the growth-promoting influence of CAFs on prostate cancer cells within the TME.

CAFs promote enzalutamide resistance in prostate cancer cells

Next, we examined the impact of CAFs on enzalutamide resistance in prostate cancer cells. As shown in **Figure 2A**, PC3 cells were exposed to varying concentrations of enzalutamide for 48 hours, both in standard culture conditions and within a co-culture system

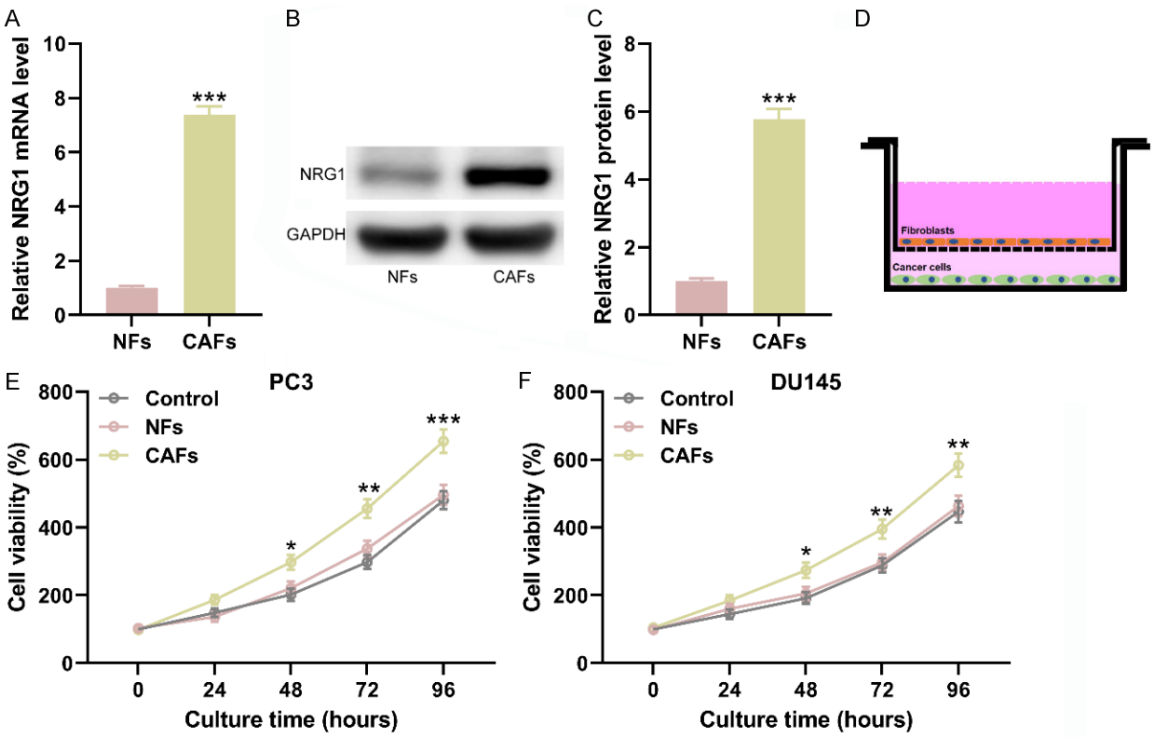

Figure 1. Cancer associated fibroblasts promoted cell growth of prostate cancer cells. RT-qPCR and Western blotting were used to measure the mRNA and protein expressions of NRG1 from normal fibroblasts (NFs) and cancer associated fibroblasts (CAFs) (A-C). (D) Schema for co-culture system of prostate cancer cells and fibroblasts. (E and F) MTT assay showed cell viability of PC3 and DU145 cells harvested from the co-culture system exposed with NFs or CAFs for different times. Results are shown as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to NFs.

alongside either NFs or CAFs. Utilizing the MTT assay, we measured cell viability and subsequently determined the IC50 value of enzalutamide (**Figure 2C**). Similarly, **Figure 2B** depicts the analogous experiment conducted with DU145 cells, presenting a comprehensive evaluation of enzalutamide response under different conditions (**Figure 2B** and **2D**). The results revealed that PC3 (DU145) cells co-cultured with NFs exhibited no significant change in enzalutamide IC50 compared to directly cultured PC3 (DU145) cells (**Figure 2C** and **2D**). Intriguingly, PC3 (DU145) cells co-cultured with CAFs displayed a significant increase in enzalutamide IC50, indicating that the presence of CAFs induces enzalutamide resistance in PC3 (DU145) cells (**Figure 2C** and **2D**). These findings underscore the role of the tumor microenvironment, specifically CAFs, in modulating the response of prostate cancer cells to enzalutamide.

CAFs further promote enzalutamide resistance in enzalutamide resistant prostate cancer cells

We extended our analysis to investigate the capacity of CAFs to further enhance enzalutamide resistance in prostate cancer cells, particularly focusing on enzalutamide-resistant counterparts (PC3-EnzR and DU145-EnzR). PC3 cells and PC3-EnzR cells, under the influence of NFs or CAFs, were exposed to varying concentrations of enzalutamide for 48 hours (**Figure 3A**). Employing the MTT assay, we assessed cell viability and determined the IC50 value of enzalutamide (**Figure 3C**). Similarly, **Figure 3B** portrays the parallel experiment conducted with DU145 cells and DU145-EnzR cells, providing a comprehensive evaluation of enzalutamide response under different conditions (**Figure 3B** and **3D**). The results demonstrated a noteworthy trend: the presence of CAFs significantly heightened enzalutamide resistance in enzalutamide-resistant prostate cancer cells. PC3-EnzR and DU145-EnzR cells co-cultured with CAFs exhibited a substantial increase in enzalutamide IC50 compared to their counterparts co-cultured with NFs or directly cultured alone (**Figure 3C** and **3D**). These findings underscore the crucial role of CAFs in exacerbating the resistance phenotype in enzalutamide-resistant prostate cancer cells, further emphasizing the impact of the

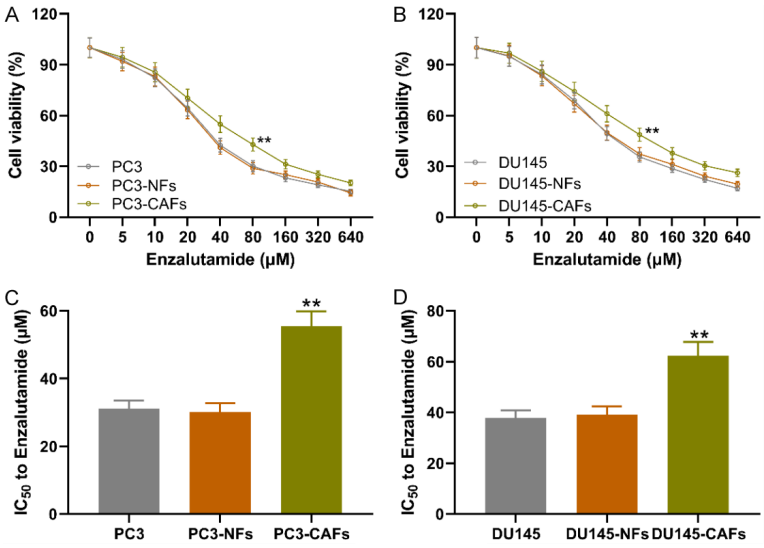

Figure 2. Cancer associated fibroblasts promoted enzalutamide resistance in prostate cancer cells. (A) PC3 cells, PC3 cells in the co-culture system exposed with NFs, PC3 cells in the co-culture system exposed with CAFs were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (C). (B) DU145 cells, DU145 cells in the co-culture system exposed with NFs, DU145 cells in the co-culture system exposed with CAFs were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (D). Results are shown as means ± SEM. **P < 0.01 compared to NFs.

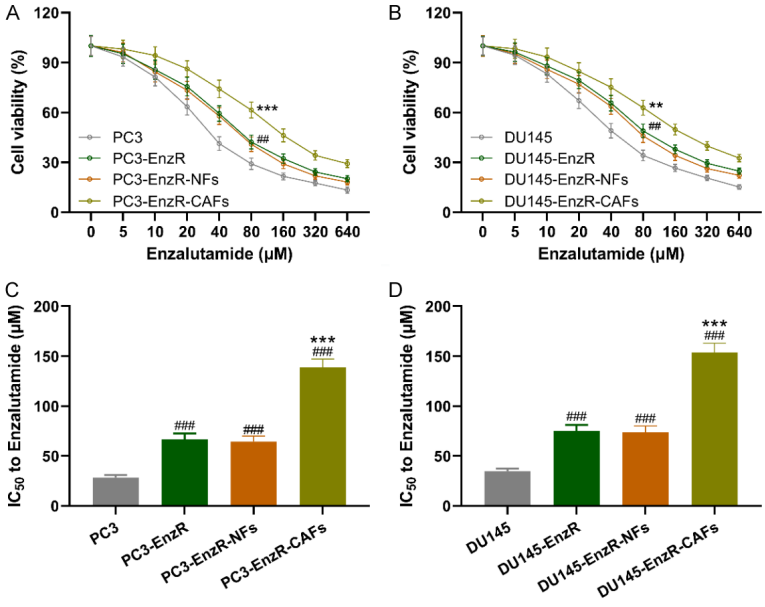

Figure 3. Cancer associated fibroblasts further promoted enzalutamide resistance in enzalutamide resistant prostate cancer cells. (A) PC3 cells, the enzalutamide resistant counterpart (PC3-EnzR), PC3-EnzR cells in the co-culture system exposed with NFs, and PC3-EnzR cells in the co-culture system exposed with CAFs were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (C). (B) DU145 cells, the enzalutamide resistant counterpart (DU145-EnzR), DU145-EnzR cells in the co-culture system exposed with NFs, and DU145-EnzR cells in the co-culture system exposed with CAFs were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (D). Results are shown as means ± SEM. **P < 0.01, ***P < 0.001 compared to NFs. ##P < 0.01, ###P < 0.001 compared to negative controls (PC3 or DU145).

tumor microenvironment on therapeutic outcomes.

CAFs induce NRG1 secretion and activate HER3 signaling in enzalutamide-resistant prostate cancer cells

Our investigation focused on elucidating the mechanisms underlying enzalutamide resistance in PC3-EnzR cells when co-cultured with CAFs. Notably, co-culturing PC3-EnzR cells with CAFs led to the secretion of NRG1, a crucial ligand in the ErbB signaling pathway. ELISA analysis demonstrated elevated levels of NRG1 in the culture medium from PC3-EnzR cells co-cultured with CAFs compared to PC3-EnzR cells alone (**Figure 4A**). Furthermore, Western blot was performed to assess the downstream effects of NRG1 secretion (**Figure 4B** and **4C**). PC3-EnzR cells in the co-culture system with CAFs exhibited enhanced phosphorylation of HER3, indicative of increased ErbB pathway activation. Additionally, the protein expressions of MMP1 and MMP9, known downstream targets of HER3 signaling associated with cancer progression, were upregulated in PC3-EnzR cells co-cultured with CAFs (**Figure 4D-F**). To validate the role of NRG1 in mediating the effects obser-

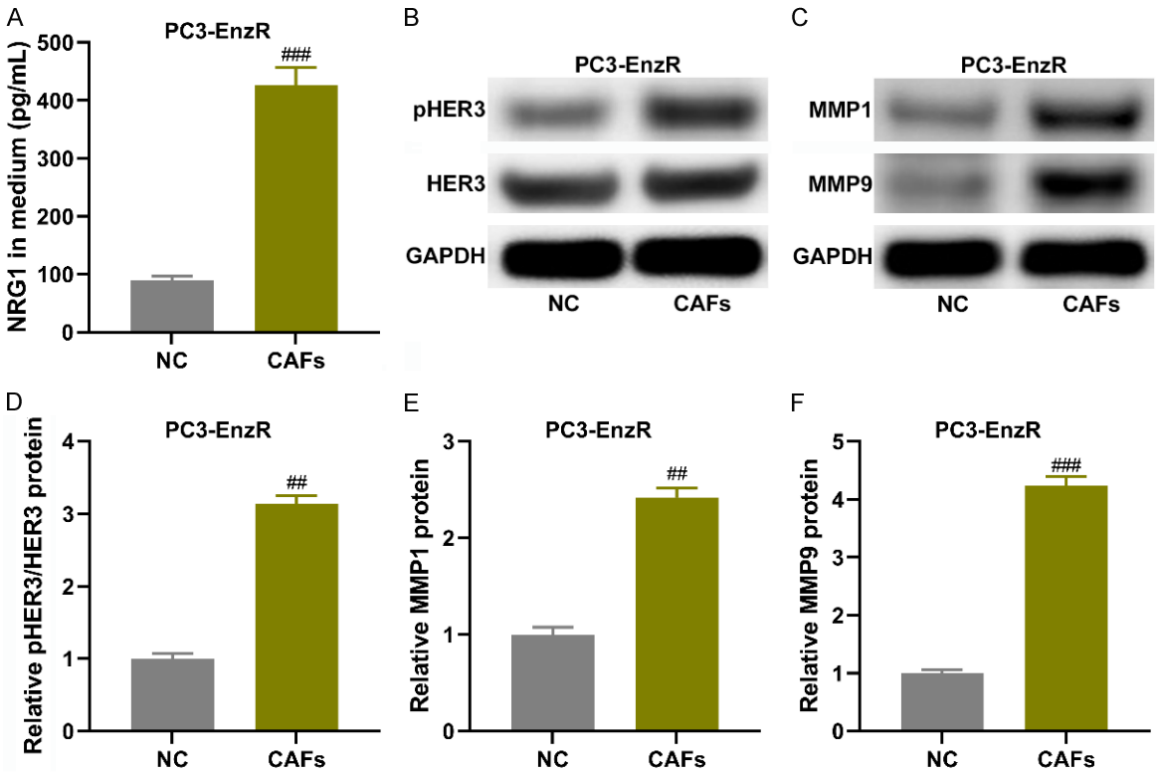

Figure 4. Cancer associated fibroblasts secreted NRG1 in the in the co-culture system exposed with CAFs and promoted phosphorylation of HER3 in enzalutamide resistant PC3 cells (PC3-EnzR). Levels of NRG1 in the culture medium from PC3-EnzR or PC3-EnzR cells in the co-culture system exposed with CAFs were measured by ELISA (A). PC3-EnzR or PC3-EnzR cells in the co-culture system exposed with CAFs were harvested and western blotting was used to measure the protein expressions of pHER3, HER3, MMP1 and MMP9 (B-F). GAPDH was used as a loading control and the expressions were normalized to NC (PC3-EnzR culture). Results are shown as means ± SEM. ##P < 0.01, ###P < 0.001 compared to NC.

ved on HER3 phosphorylation, we knocked down NRG1 in CAFs. It was found that knockdown of NRG1 in CAFs leads to a decrease in HER3 phosphorylation in PC3-EnzR cells (Figure S1). Moreover, the addition of recombinant NRG1 restores the phosphorylation levels, indicating that NRG1 is indeed a critical mediator in this process.

A parallel investigation was conducted on enzalutamide-resistant DU145 cells (DU145-EnzR) co-cultured with CAFs. Similar to PC3-EnzR cells, co-culture with CAFs resulted in the secretion of NRG1, as evidenced by increased NRG1 levels in the culture medium compared to DU145-EnzR cells alone (**Figure 5A**). Subsequent Western blot analysis revealed that CAF-induced NRG1 secretion led to enhanced phosphorylation of HER3 in DU145-EnzR cells co-cultured with CAFs (**Figure 5B** and **5C**). Additionally, the expressions of MMP1 and MMP9 were elevated in DU145-EnzR cells co-cultured with CAFs, suggesting a parallel activation of HER3 signaling and the downstream effector proteins (**Figure 5D-F**). These findings suggest a potential molecular link between CAF-induced NRG1 secretion and the activation of HER3 signaling in enzalutamide-resistant prostate cancer cells.

Inhibition of NRG1 in CAFs attenuated the enzalutamide resistant of prostate cancer cells

To further confirm the involvement of NRG1 secretion in CAFs-mediated enzalutamide resistance, we employed two experimental approaches inspired by previous study [20]. First, the expression of NRG1 in CAFs was inhibited using siRNA to discern the impact of CAF-derived NRG1 on enzalutamide resistance in PC3 and DU145 cells. Second, recombinant human NRG1 protein was utilized to stimulate prostate cancer cells, mimicking the effect of CAF-secreted NRG1.

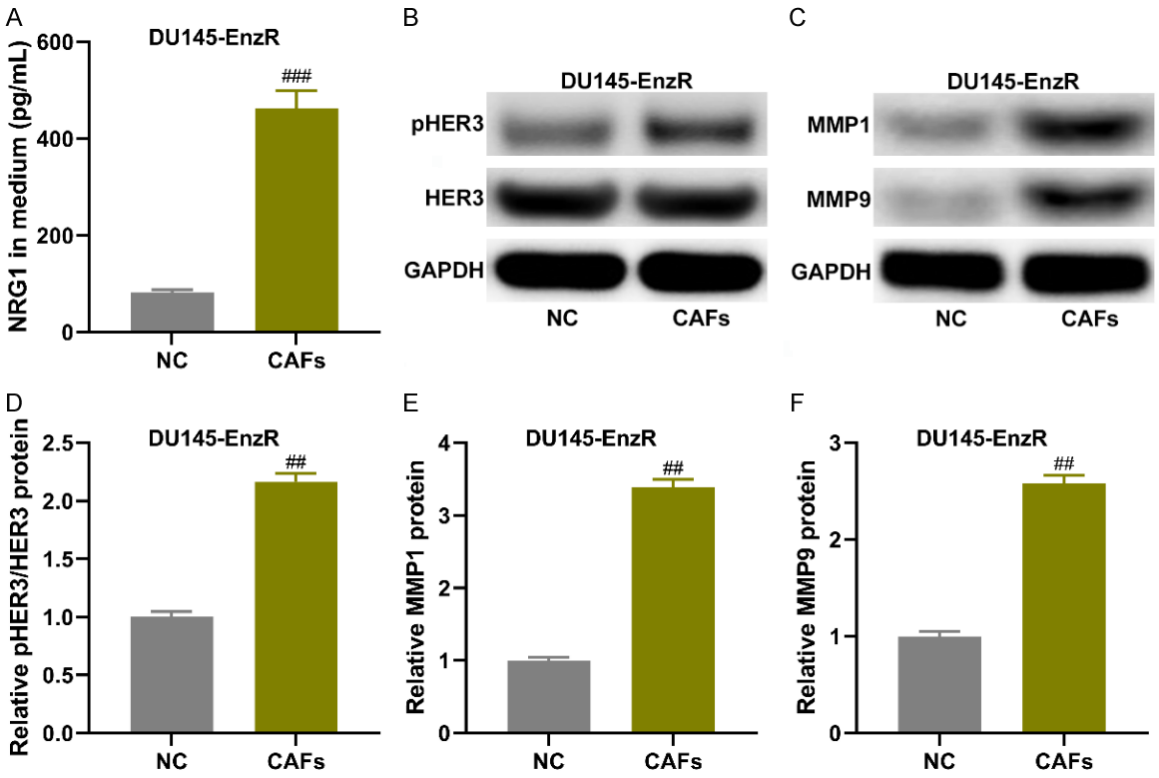

Figure 5. Cancer associated fibroblasts secreted NRG1 in the in the co-culture system exposed with CAFs and promoted phosphorylation of HER3 in enzalutamide resistant DU145 cells (DU145-EnzR). Levels of NRG1 in the culture medium from DU145-EnzR or DU145-EnzR cells in the co-culture system exposed with CAFs were measured by ELISA (A). DU145-EnzR or DU145-EnzR cells in the co-culture system exposed with CAFs were harvested and western blotting was used to measure the protein expressions of pHER3, HER3, MMP1 and MMP9 (B-F). GAPDH was used as a loading control and the expressions were normalized to NC (DU145-EnzR culture). Results are shown as means ± SEM. ##P < 0.01, ###P < 0.001 compared to NC.

Upon transfection of CAFs with siNRG1, both RT-qPCR and Western blot analyses confirmed a significant reduction in the mRNA and protein expressions of NRG1 compared to non-transfected CAFs (**Figure 6A-C**). Subsequently, the culture medium from PC3-EnzR cells in the co-culture system exposed to CAFs or CAFs transfected with siNRG1 was subjected to ELISA to measure NRG1 levels. As expected, siNRG1 transfection led to a marked decrease in NRG1 secretion by CAFs (**Figure 6D**). PC3-EnzR and DU145-EnzR cells were subjected to enzalutamide treatment under different conditions. In PC3-EnzR cells, inhibition of NRG1 expression in CAFs resulted in a weakened impact on enzalutamide resistance, as evidenced by reduced cell viability and a lower IC50 value compared to the co-culture system with non-transfected CAFs (**Figure 6E** and **6G**). Conversely, when recombinant human NRG1 was introduced into the culture medium of siNRG1-transfected CAFs, the enzalutamide resistance-promoting effect was restored (**Figure 6E** and **6G**). Similarly, in DU145-EnzR cells, the inhibition of NRG1 in CAFs attenuated enzalutamide resistance, leading to decreased cell viability and a lower IC50 value compared to the co-culture system with non-transfected CAFs (**Figure 6F** and **6H**). The addition of recombinant human NRG1 to siNRG1-transfected CAFs reinstated enzalutamide resistance in DU145-EnzR cells (**Figure 6F** and **6H**). Similar results were observed using an additional siRNA against NRG1 (siNRG1#2, Figure S2). Moreover, *in vivo* animal tumor model study further confirmed the *in vitro* findings that CAF secreted NRG1 contributes to enzalutamide resistance in prostate cancer (Figure S3). These results provide direct evidence that the promotion of enzalutamide resistance by CAFs is, at least in part, mediated through the secretion of NRG1, emphasizing the therapeutic potential of tar-

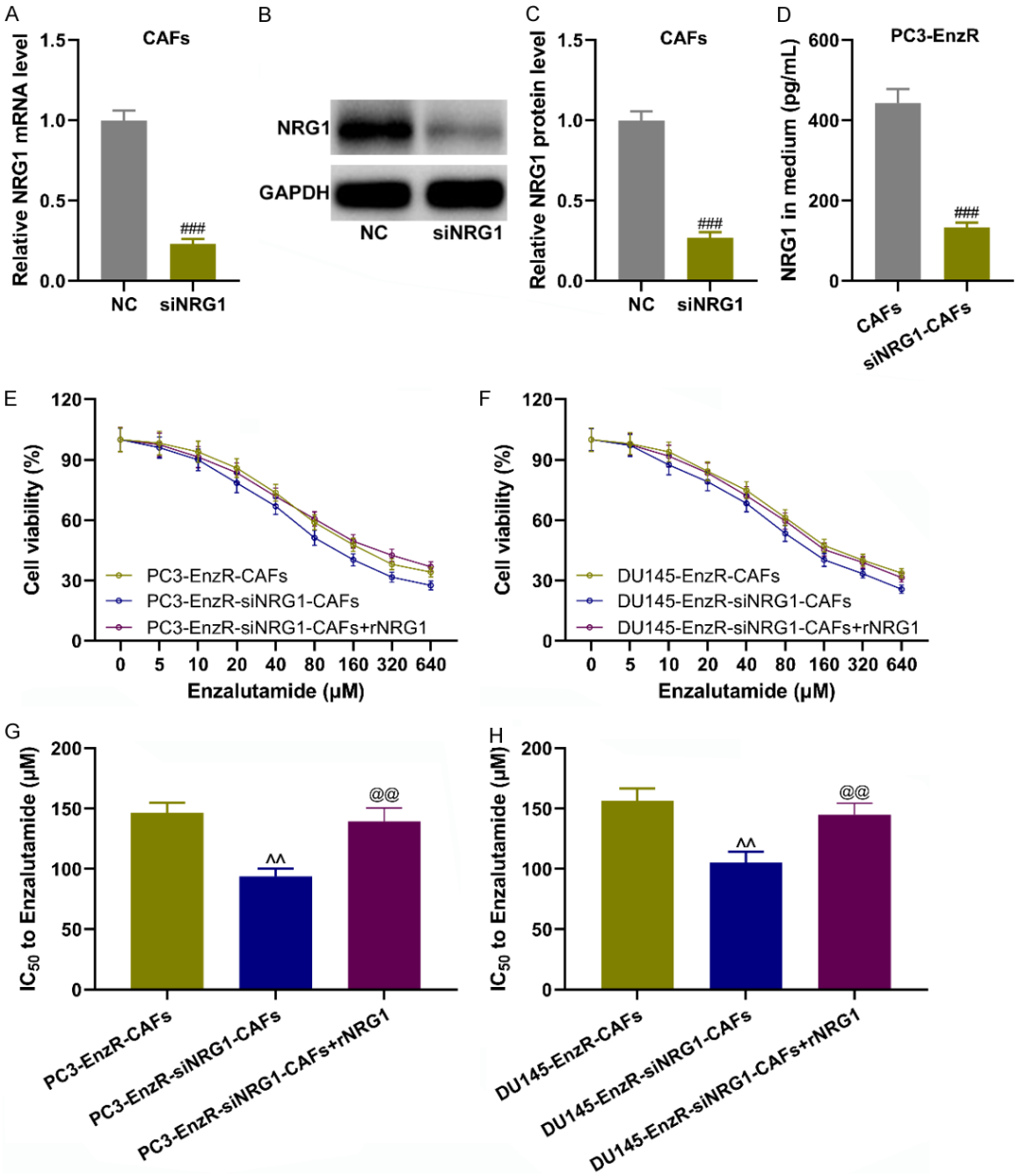

Figure 6. Inhibition of NRG1 in cancer associated fibroblasts (CAFs) attenuated the effects of CAFs on enzalutamide resistance in enzalutamide resistant prostate cancer cells. CAFs were transfected with siNRG1 for 48 hours, RT-qPCR and Western blotting were used to measure the mRNA and protein expressions of NRG1 (A-C). (D) Levels of NRG1 in the culture medium from PC3-EnzR in the co-culture system exposed with CAFs or PC3-EnzR cells in the co-culture system exposed with CAFs transfected with siNRG1 were measured by ELISA. (E) PC3-EnzR in the co-culture system exposed with CAFs, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1 and also recombinant human NRG1 (10 ng/mL) was added into the culture medium were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (G). (F) DU145-EnzR in the co-culture system exposed with CAFs, DU145-EnzR in the co-culture system exposed with CAFs transfected with siNRG1, DU145-EnzR in the co-culture system exposed with CAFs transfected with siNRG1 and also recombinant human NRG1 (10 ng/mL) was added into the culture medium were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (H). Results are shown as means ± SEM. ###P < 0.001 compared to NC. ^^P < 0.01 compared to co-culture system exposed with CAFs, @@P < 0.01 compared to co-culture system exposed with CAFs transfected with siNRG1.

geting NRG1 in the tumor microenvironment to overcome enzalutamide resistance in prostate cancer.

Discussion

Prostate cancer, characterized by its high prevalence and substantial mortality rates, remains a formidable challenge in men's health [2]. Our investigation into the intricacies of CRPC reveals the critical role played by TME, particularly CAFs, in influencing the response to enzalutamide, a second-generation anti-androgen.

Our findings align with previous studies demonstrating the active involvement of CAFs in shaping the TME and promoting prostate cancer aggressiveness. These fibroblasts, activated by factors such as TGF-β secreted by tumor cells, undergo transformation into CAFs, contributing to tumor invasion, metastasis, and drug resistance [21-24]. Our findings reveal a compelling association between CAFs and enzalutamide resistance in prostate cancer cells. Co-culture experiments demonstrated that CAFs significantly enhance the proliferation of enzalutamide resistant PC3 and DU145 cells. This observation is consistent with previous studies that highlight the active participation of CAFs in promoting various aspects of prostate cancer aggressiveness [25-28]. In our study, we have developed a Transwell co-culture system, which allows us to precisely assess the impact of fibroblast secretions on tumor cell behavior without the direct cellular contact seen in the study. This methodological refinement enables a more accurate dissection of the paracrine signaling mechanisms involved. Additionally, our investigation utilizes enzalutamide-resistant PC3 and DU145 cell lines (designated as PC3-EnzR and DU145-EnzR), which were specifically engineered by our laboratory. These cell lines serve as a robust model to elucidate how CAFs secreting NRG1 influence the antiandrogen resistance observed in PC3-EnzR and DU145-EnzR cells. By doing so, we aim to provide a deeper understanding of the mechanisms underlying antiandrogen resistance in prostate cancer, potentially leading to novel therapeutic strategies targeting these interactions.

Intriguingly, our study delves into the molecular underpinnings of CAF-mediated enzalutamide resistance, focusing on NRG1. NRG1, a member of the epidermal growth factor (EGF) family, has been implicated in various cancers, including prostate cancer [20, 29]. The upregulation of NRG1 in CAFs compared to normal fibroblasts (NFs) underscores its specific role in modulating the NRG1-HER3 axis. This finding is in line with research by Zhang et al. [20], which demonstrated the involvement of NRG1-HER3 signaling in promoting resistance to anti-androgen therapy. And an *in vivo* animal tumor model was used to further confirm the physiological significance of our findings.

Our study extends the understanding of the NRG1-HER3 axis by showcasing that inhibition of NRG1 in CAFs attenuates their impact on enzalutamide resistance. This aligns with recent findings by Gil et al. [30], emphasizing the therapeutic potential of targeting NRG1 in overcoming drug resistance. Moreover, our results provide direct evidence of NRG1 secretion by CAFs and its subsequent effects on HER3 phosphorylation, as well as the expression of MMP1 and MMP9. These molecular changes have been associated with enhanced invasiveness and metastasis in prostate cancer [30-32]. The present study contributes to the growing body of evidence supporting the pivotal role of NRG1-HER3 signaling in enzalutamide resistance, shedding light on a potential target for overcoming therapeutic challenges in CRPC.

While our study adds valuable insights into the interplay between CAFs, NRG1, and enzalutamide resistance, acknowledging its limitations is crucial. The reliance on in vitro co-culture systems and cell lines necessitates validation through in vivo models. Additionally, the potential heterogeneity in CAF populations and their dynamic nature in the TME warrant further exploration. Future studies should aim to translate these findings into clinical relevance, potentially paving the way for combinatorial therapeutic approaches targeting the NRG1-HER3 axis.

Conclusion

Our work sheds light on the intricate interactions within the prostate cancer TME, emphasizing the role of CAFs and NRG1 in driving enzalutamide resistance. By understanding the molecular nuances of CAF-mediated drug resis-

tance, we open avenues for targeted therapies that could redefine the treatment landscape for CRPC patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Lei Chen, Yigeng Feng and Renjie Gao, Surgical Department I (Urology Department), LONGHUA Hospital Shanghai University of Traditional Chinese Medicine, No. 725 Wanping Road South, Xuhui District, Shanghai 200032, China. Tel: +86-18964832103; E-mail: chenlei2114@shutcm.edu.cn (LC); fengyigeng2410@shutcm.edu.cn (YGF); gaorenjie02254@shutcm.edu.cn (RJG)

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Table S1. The clinicopathologic features of 5 patients

Features	Number of patients
Age	
< 60	2
≥ 60	3
Tumor stage	
pT1-2	1
pT3-4	3
N1	1
Gleason score	
≤ 7	1
> 7	4
PSA (ng/mL)	
≤ 10	1
> 10	4

PSA: prostate-specific antigen.

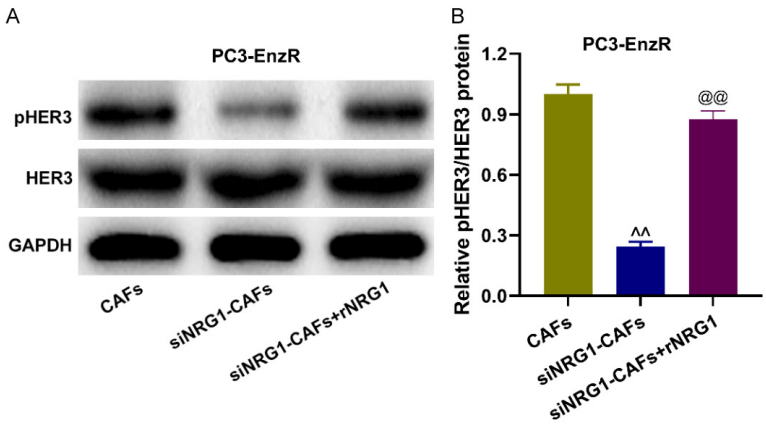

Figure S1. Inhibition of NRG1 in cancer associated fibroblasts (CAFs) attenuated the effects of CAFs on phosphorylation of HER3 in enzalutamide resistant PC3 cells (PC3-EnzR). PC3-EnzR in the co-culture system exposed with CAFs, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1 and also recombinant human NRG1 (10 ng/mL) were harvested and western blotting was used to measure the protein expressions of pHER3, HER3 (A). GAPDH was used as a loading control and the expressions were normalized to CAFs (B). Results are shown as means ± SEM. ^^P < 0.01 compared to co-culture system exposed with CAFs, @@P < 0.01 compared to co-culture system exposed with CAFs transfected with siNRG1.

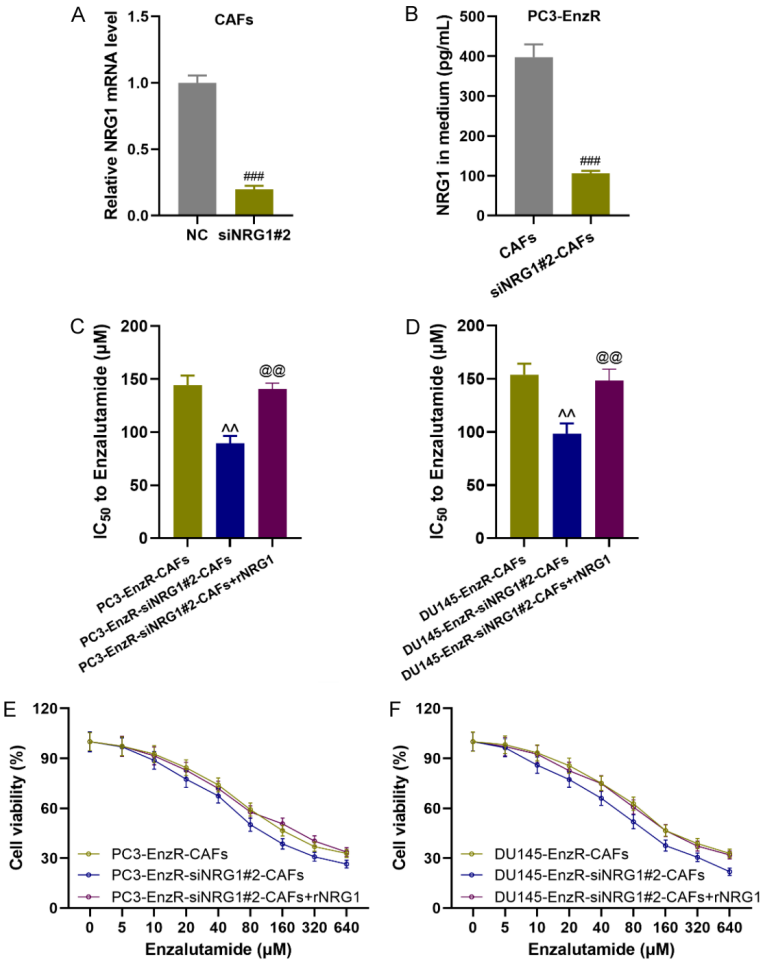

Figure S2. Inhibition of NRG1 in cancer associated fibroblasts (CAFs) attenuated the effects of CAFs on enzalutamide resistance in enzalutamide resistant prostate cancer cells. CAFs were transfected with siNRG1#2 for 48 hours, RT-qPCR was used to measure the mRNA expressions of NRG1 (A). (B) Levels of NRG1 in the culture medium from PC3-EnzR in the co-culture system exposed with CAFs or PC3-EnzR cells in the co-culture system exposed with CAFs transfected with siNRG1#2 were measured by ELISA. (E) PC3-EnzR in the co-culture system exposed with CAFs, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1#2, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1#2 and also recombinant human NRG1 (10 ng/mL) was added into the culture medium were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (C). (F) DU145-EnzR in the co-culture system exposed with CAFs, DU145-EnzR in the co-culture system exposed with CAFs transfected with siNRG1#2, DU145-EnzR in the co-culture system exposed with CAFs transfected with siNRG1#2 and also recombinant human NRG1 (10 ng/mL) was added into the culture medium were subjected to indicated concentrations of enzalutamide for 48 h, MTT was used to measure the cell viability and IC50 value of enzalutamide were then determined (D). Results are shown as means ± SEM. ###P < 0.001 compared to NC. ^^P < 0.01 compared to co-culture system exposed with CAFs, @@P < 0.01 compared to co-culture system exposed with CAFs transfected with siNRG1.

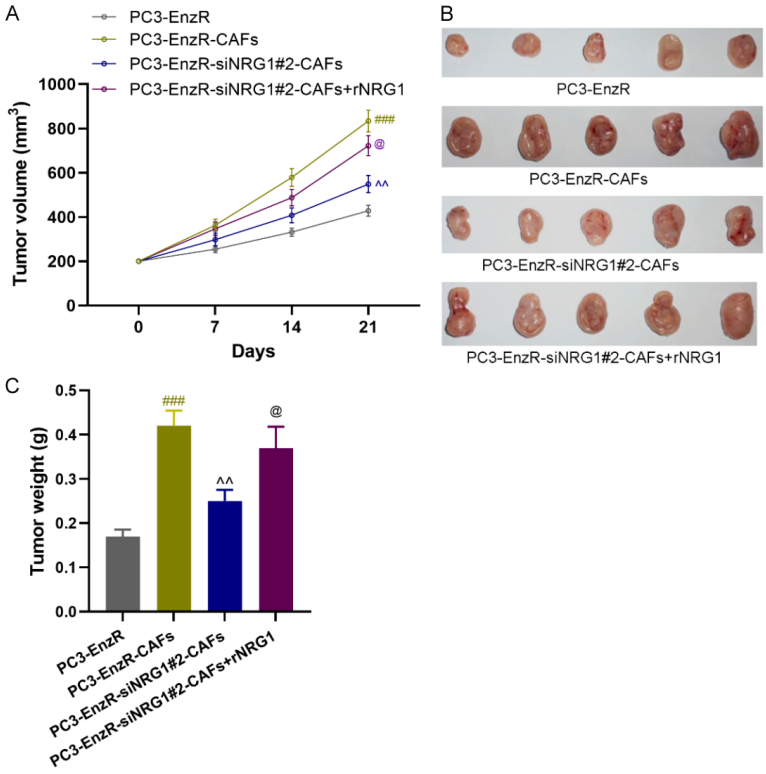

Figure S3. PC3-EnzR, PC3-EnzR in the co-culture system exposed with CAFs, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1, PC3-EnzR in the co-culture system exposed with CAFs transfected with siNRG1 and also recombinant human NRG1 (10 ng/mL) were harvested and used to build xenograft tumors. The tumor volume (A), tumor images (B) and tumor weight (C) were monitored. 5 mice in each group. Results are shown as means ± SEM. ###P < 0.001 compared to PC3-EnzR, ^^P < 0.01 compared to co-culture system exposed with CAFs, @@P < 0.01 compared to co-culture system exposed with CAFs transfected with siNRG1.