TR4 nuclear receptor alters the prostate cancer CD133+ stem/progenitor cell invasion via modulating the EZH2-related metastasis gene expression

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Running Title: TR4 Promotes Metastasis in PCa Stem Cells

Keywords: TR4, EZH2, prostate cancer, Stem/Progenitor cells, metastasis

Abbreviations: PCa, prostate cancer; ADT, androgen deprivation therapy; S/P cell, stem/progenitor cell; CRPC, castration resistant prostate cancer; TR4RE, TR4 response element;

Acknowledgements: This study was supported by NIH grants (CA122840 and CA156700) for C Chang, George Whipple Professorship Endowment for C Chang, the Taiwan Department of Health Clinical Trial for C Chang, Research Center of Excellence (DOH99-TD-B-111-004 to China Medical University, Taichung, Taiwan) for C Chang, NSFC (Grant No. 81472776) for D Yang.

Conflict of interest: The authors declare no competing interests.

Word count: 3600 words.

Figures: 6

Supplementary Figures: 2

Supplementary Tables: 1
Abstract

The testicular nuclear receptor 4 (TR4) is a member of the nuclear receptor superfamily that mediates various biological functions with key impacts on metabolic disorders and tumor progression. Here we demonstrate that TR4 may play a positive role in prostate cancer (PCa) CD133+ stem/progenitor (S/P) cell invasion. Targeting TR4 with lentiviral silencing RNA significantly suppressed PCa CD133+ S/P cell invasion both in vitro and in vivo. Mechanism dissection found that TR4 transcriptionally regulates the oncogene EZH2 via binding to its 5' promoter region. The consequences of targeting TR4 to suppress EZH2 expression may then suppress the expression of its downstream key metastasis-related genes including NOTCH1, TGFβ1, SLUG and MMP9. Rescue approaches via adding the EZH2 reversed the TR4-mediated PCa S/P cell invasion. Together, these results suggest that the TR4→EZH2 signaling may play a critical role in the PCa S/P cell invasion and may allow us to develop a better therapy to battle the PCa metastasis.

Introduction

Prostate cancer (PCa) continues to be the most common malignant tumor in men in the United States. Androgen/androgen receptor (AR) signals play important roles in PCa progression, and androgen deprivation therapy (ADT) is the standard therapy for the advanced PCa. However, most ADT eventually fails and PCa progresses into castration resistant PCa (CRPC) that is often accompanied with metastasis. The detailed mechanisms, however, remain unclear.

One key factor that may contribute to the metastasis development, despite the ongoing ADT treatment, is the increasing population of stem/progenitor (S/P) cells, and targeting cancer S/P cells has been suggested as a potential novel therapy to suppress the PCa metastasis (1).

Testicular nuclear receptor 4 (TR4) may modulate many signals by interacting with other nuclear receptors including AR and estrogen receptor. Results from TR4 knockout (TR4KO) mice studies have shown that TR4 may play key roles to influence embryonic development, stem cell pluripotency and progression of several diseases including metabolic disorders and...
various tumors (2-6).

Enhancer of zeste homolog 2 (EZH2) is a member of the polycomb repressive complex 2 (PRC2), which includes suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). EZH2 may serve as a histone methyltransferase to methylate the histone H3 lysine 27 (H3K27) at target gene promoters that lead to epigenetic silencing. EZH2 interacts with SNAI1 and suppress E-cadherin to promote epithelial–mesenchymal transition (EMT) to influence the cancer metastasis. EZH2 is essential for embryonic development and stem cell pluripotency. Increased expression of EZH2 was found in a variety of human cancers including PCa (7) and its expression is positively linked with the progression and metastasis of PCa (8).

Here we demonstrate that targeting TR4 could suppress PCa CD133+ S/P cell invasion via inhibiting of EZH2 expression and its downstream metastasis-related genes including NOTCH1, SLUG, TGFβ1 and MMP9.

Materials and Methods

Cell Culture

C4-2 cells were a gift from Dr. Jer-Tsong Hsieh of University of Southwestern Medical Center in 2009 and were not authenticated by us. The CWR22Rv1 cell line was purchased from and authenticated by the American Type Culture Collection (ATCC, Manassas, VA) in May, 2010. Prostate cancer stem cells (PCSCs) were purchased from Celprogen (San Pedro, CA) in 2011 and were not authenticated by us. C4-2 and CWR22Rv1 cell lines were maintained in RPMI 1640 media containing penicillin (25 units/ml), streptomycin (25 g/ml), 1% L-glutamate, and 10% fetal bovine serum (FBS). Isolated CD133+ S/P cells and PCSC cells were incubated in DMEM/F12 media with 0.6% glucose, 10 mg/ml putrescine, 50 mg/ml insulin, 100 mg/ml apo-transferin, 0.03 mM sodium selenite, 2 mM progesterone, 5 mM HEPES, 0.1% sodium bicarbonate, 10 ng/ml bFGF and 20 ng/ml EGF.

Magnetic beads isolation of CD133+ cells

Isolation of CD133+ PCa cells was performed as previously described (9, 10). Briefly, magnetic beads (Invitrogen, Grand Island, NY) were conjugated with biotinylated CD133
antibody (Miltenyi Biotec, Cambridge, MA). Cells \((2 \times 10^7)\) were detached with 5 mM EDTA and incubated with beads, and separated by placing tubes in a magnetic field. The stem/progenitor markers of the sorted cells were confirmed by qPCR and immunofluorescence. Flow cytometric analysis indicated that >95% of the sorted cells are CD133+ cells (data not shown).

**Quantitative real-time PCR**

Total RNA was isolated by Trizol reagent (Invitrogen, Grand Island, NY), and 1 µg of total RNA was then subjected to reverse transcription using Superscript III transcriptase (Invitrogen, Grand Island, NY). Quantitative real-time PCR (qRT-PCR) was conducted using a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression of specific genes. Expression levels were normalized to the expression of GAPDH and all reactions were run at least in triplicate.

**Western Blot Analysis**

Cells were washed with PBS and lysed in RIPA buffer. Proteins (30 µg) were separated on 10% or 15% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat milk in PBST for 1 hour at room temperature, and then incubated with diluted primary antibodies against GAPDH (Santa Cruz, #sc-166574), TR4 (Perseus Proteomics, #PP-H0107B-00), EZH2 (Cell Signaling, #5246P), NOTCH1 (Cell Signaling, #3439), TGFβ1 (Santa Cruz, #sc-146), SLUG (Cell Signaling, #9585), or MMP9 (Santa Cruz, #sc-10737) overnight at 4°C. Blots were incubated with HRP conjugated secondary antibody for 1 hour at room temperature, washed, and developed in the ECL system (Bio-Rad, Hercules, CA, USA). Quantitations of blots are shown in Fig. S1.

**Plasmids and lentivirus**

TR4 shRNA sequence was cloned into pLKO.1 puro plasmid. To overexpress EZH2, EZH2 cDNA was cloned into PWPI vector. Lentivirus packing and production was the same as previously described(11). Human EZH2 full-length promoter (-2007+-313), along with truncated promoter (-1083+-313, -458+-313, -187+-313,), were amplified and cloned into pGL3-basic vector, respectively.

**Invasion Assay**

Growth factor reduced Matrigel (BD, #356234) was diluted with coating buffer (0.01M Tris,
0.7% NaCl, pH 8.0) at the ratio of 1:15, coated onto the 8 μm transwell plates (Corning, #3422), and incubated at 37°C for 2 hours. Cells (10^5) were re-suspended with serum-free media and seeded in the upper chamber of transwells. Media with 10% FBS was put in the lower chamber. After 24 hours incubation, cells invaded to the lower part of the membrane were harvested, fixed with methanol, and stained with 0.1% Toluidine Blue. Invaded cells were counted under microscope. Triplicate experiments were performed.

3-D invasion assay

The 3-D invasion assay was as previously described (12). Briefly, 1x10^4 cells were suspended in 200 μl media containing 1% Matrigel (Trivigen, #3500-096-K) and then plated into the previously prepared collagen/Matrigel mixture coated plates. Media will be replenished every 3 days for 2 wks and then spheres and protrusions will be recorded under microscope.

ChIP Assay

ChIP experiments were performed following the Cold Spring Harbor ChIP protocol (13) with minor modifications. Cultured C4-2 PCa cells (2 × 10^7) were harvested. The cells were treated with 1% formaldehyde at room temperature for 10 min to cross-link DNA. After sonication (10 × 10 sec, 10% amplitude on a Branson Sonifier 450), cross-linked chromatin was precleared with protein A/G-agarose beads and then immunoprecipitated using anti-TR4-specific antibody or IgG overnight at 4°C. Supernatants from the no-antibody-added samples were used to measure total input chromatin. The chromatin was then incubated overnight at 65°C to reverse cross-links. DNA was then treated with RNase A, purified using gel extraction columns (OMEGA) and resuspended in 50 μL TE buffer. Then TR4 occupancy on chromatin was assessed by PCR with locus-specific primers.

Luciferase reporter gene assays

C4-2 cells were seeded in 24-well plates and were transfected with PGL3 reporter, pCMX/pCMX-TR4, and 1 ng pRL-TK using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared with Passive Lysis Buffer (Promega, Madison, WI) 24 hrs after transfection, and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI). pRL-TK served as internal control. The assays were run in triplicate.

Orthotopic-injected mice model

Male 6- to 8-week-old nude mice were purchased from NCI (Bethesda, MD, USA). Mice were
divided into 2 groups include the control group and the shRNA group. CW22Rv1 S/P cells (2 × 10^5) with shTR4 or Scramble (scr) control mixed with Matrigel (BD) were orthotopically injected into both anterior prostates. IVIS system was used to monitor the tumor size and metastasis 4 weeks after injection. Mice were then sacrificed; tumor tissue samples were fixed, processed into paraffin tissue sections and used for immunohistochemistry.

**Immunohistochemistry**

Tumor tissues were fixed in 4% neutral buffered para-formaldehyde and embedded in paraffin. The primary antibodies of the TR4 (Perseus Proteomics, #PP-H0107B-00, 1:50), EZH2 (Cell Signaling, #5246P, 1:100) were used for staining. The primary antibody was recognized by the biotinylated secondary antibody (Vector, Burlingame, CA), and visualized by VECTASTAIN ABC peroxidase system and peroxidase substrate DAB kit (Vector). The positive staining signals were semiquantitated by ImageJ software.

**Statistical analysis**

Values were expressed as mean ± S.D. The Student’s t and ANOVA tests were used to calculate P values. P values were two-sided, and considered statistically significant when <0.05.

**Results**

**Higher expression of TR4 in PCa CD133+ S/P cells**

Recent studies found that higher S/P cell population in tumors may play important roles in tumor initiation, invasion and recurrence, and targeting S/P cells may represent another powerful therapy to suppress PCa (14). Importantly, recent studies also suggested that CRPC might function through increased S/P cell population to promote the CRPC metastasis (15), and TR4 has also been reported to play important roles in PCa progression (16). We were therefore interested in examining the potential linkage of TR4 in PCa S/P cells-enhanced metastasis.

We used the CD133+ as the marker of PCa S/P cells since increasing evidence indicated that PCa S/P cells are characterized with high expression of CD133 (10, 17, 18), and CD133+ cancer stem cells are responsible for cellular migration and metastasis in cancer (19, 20),
including PCa(21, 22). We first sorted out PCa S/P cells from PCa C4-2 and CWR22Rv1 cells via CD133+ antibody(9, 10), and results revealed CD133+ sorted S/P cells have distinct morphologies (Fig. 1A, B) with higher expression of the S/P markers of CD133 and Nanog (Fig. 1C, D, left panels) as compared to parental cells. Importantly, we also found higher expression of TR4 in CD133+ S/P cells at protein and mRNA level (Fig. 1C, D), and knocked down TR4 in CD133+ S/P cells led to morphology change to more differentiated PCa cells (Fig. S2).

Together, results from Fig. 1A-D demonstrate the higher expression of TR4 in PCa S/P cells.

**TR4 promotes PCa CD133+ S/P cells metastasis**

To examine whether increased TR4 in CD133+ S/P cells may impact their influence on PCa metastasis, we knocked-down TR4 and assayed the influence on cell invasion using chamber transwell invasion assays. The results revealed that knocked-down TR4 (Fig. 2A) led to suppressing the C4-2 CD133+ S/P cell invasion (Fig. 2B, C). Similar results were also obtained when we replaced CD133+ S/P cells derived from C4-2 cells with those from CWR22Rv1 cells (Fig. 2D-F).

We then used another 3D invasion assay to further confirm the previous results, and results again revealed that knocking-down TR4 with TR4-shRNA suppressed PCa C4-2 or CWR22Rv1 CD133+ S/P cell invasion (Fig. 2 G-J).

Using NCBI GEO databases(GEO dataset accession GSE6919)(23) to analyze the human PCa sample array with TR4 expression also indirectly suggested that PCa tissues with metastasis have a higher TR4 expression than those localized PCa tissues (Fig. 2K).

Together, results from in vitro cell lines studies (Fig. 2A-J) and human PCa sample survey (Fig. 2K) all suggest that higher TR4 expression in S/P cells may link to the S/P cell ability to metastasize and targeting TR4 leads to suppressed PCa CD133+ S/P cell invasion.

**TR4 regulates EZH2 and its related metastasis genes in PCa S/P cells**

To dissect the mechanism by which TR4 alters the S/P cells-mediated PCa cell invasion in the C4-2 and CWR22Rv1 CD133+ S/P cells, we screened those genes that were reported
to link to PCa metastasis (Fig. 3A, supplementary table 1). The results revealed 5 overlapping potential key genes with KAI1 and TIMP2 increased and IGF, HIF2α, and EZH2 decreased upon knocking down of TR4(Fig. 3B, C). We decided to focus on EZH2 as it plays important roles in cancer S/P cells and its expression was significantly decreased in both C4-2 and CWR22Rv1 CD133+ S/P cells.

Early studies suggested that EZH2 might function through modulation of several key metastasis-associated genes including NOTCH1, SLUG, TGFβ1 and MMP9 to promote cancer cell invasion (24-26). We first demonstrated that knocking-down TR4 suppressed the expression of both EZH2 and these metastasis-related genes in C4-2 CD133+ S/P cells (Fig. 3D). Similar results were also obtained when we replaced C4-2 CD133+ S/P cells with other two S/P cell lines (CWR22Rv1 CD133+ S/P and PCSC) (Fig. 3D). PCSC is a PCa stem cell line originally obtained from a PCa patient and immortalized by Celprogen (San Pedro, CA).

Together, results from Fig. 3A-D suggest that TR4 may function through modulation EZH2-related signal (see cartoon, Fig. 3E) to influence the PCa S/P cell invasion.

Using NCBI GEO databases to analyze the human PCa sample array, we also found the positive correlation of TR4 and EZH2 in 94 PCa samples (GEO dataset accession GSE35988) (27) (Fig. 3F). Similarly, analysis of another dataset (GEO dataset accession GSE6919) revealed the positive correlation of TR4 and EZH2 in 89 human PCa samples (Fig. 3G).

TR4 regulates EZH2 expression at the transcriptional level

TR4 is a transcription factor and can modulate its downstream target genes via binding to the TR4 response element (TR4RE) of its downstream genes (28). To further dissect the molecular mechanism by which TR4 affects EZH2 expression, we first searched for potential TR4REs (Fig. 4A) and found 3 putative TR4REs within the EZH2 promoter region (-1698~-1683; -1055~-1041; +149~+163) using JASPAR database (Fig. 4B). Since it is commonly accepted that only binding sites in the conserved region are functional, we checked the sequence and found all three sites are in the evolutionary conserved region. We then applied the ChIP assay and found that TR4 only bound to the first 2 putative TR4REs (Fig. 4C). We then linked the EZH2 promoter (-2007~+313) into pGL-3 basic luciferase reporter and examined TR4 influence on its activity. As shown in Fig. 4D, TR4 enhanced EZH2
promoter activity in a dose-dependent manner.

Since TR4RE1 and 2 are only about 600 nucleotides apart, the limitation of ChIP technology makes it very difficult to determine whether TR4 is recruited to both sites or only one of these two sites. We further confirmed above results using a series of truncation of the EZH2 promoter reporter and results showed that truncation without the first TR4RE led TR4 failed to induce EZH2 promoter activity (Fig. 4E), suggesting this TR4RE may play essential roles to mediate the TR4 modulation on EZH2 expression. This conclusion is further confirmed via ChIP assay showing knocking-down TR4 with TR4-shRNA led to suppress significantly the binding to the first TR4RE (Fig. 4F)

Taken together, results from both ChIP and luciferase reporter assays all conclude that TR4 may induce EZH2 expression by binding directly to the EZH2 promoter region.

**TR4 promotes PCa S/P cell invasion via inducing EZH2**

To link TR4 induced EZH2 expression to TR4 capacity to promote PCa S/P cell invasion, we applied the interruption approach via overexpression of EZH2 using lentivirus system to see if this could reverse the TR4-shRNA suppression effect. The Western blot results revealed that adding EZH2 could reverse (rescue) the suppression effect of TR4-shRNA on EZH2 and its downstream metastasis-related genes (Fig. 5A). Importantly, adding EZH2 also reversed partially the TR4 ability to influence the CD133+ S/P cell invasion both in C4-2 (Fig. 5B, C) and CWR22Rv1 cells (Fig. 5D, E).

Together, results from Fig. 5A-E suggest that targeting TR4 with TR4-shRNA may suppress PCa CD133+ S/P cell invasion via modulation of EZH2 signaling.

**Targeting TR4 suppresses PCa S/P tumor metastasis in *in vivo* mouse model**

All the above *in vitro* data concluded that targeting TR4 could suppress PCa CD133+ S/P cell invasion via alteration of EZH2. To further confirm this *in vitro* conclusion *in vivo*, we applied an orthotopically xenografted PCa S/P cells mouse model. CWR22Rv1 cells stably transfected with firefly luciferase reporter gene were sorted by MACS with CD133 antibody and infected with either TR4-shRNA or scramble. $2 \times 10^5$ sorted CD133+ S/P cells were orthotopically xenografted into anterior prostates of nude mice, with 12 mice each group.
There were seven mice in the scr group and six in the shTR4 group developed primary tumor. In the scr group, four out of seven mice developed metastatic sites include liver, diaphragm, and lung. In the shTR4 group, only one mouse developed lung metastasis. Both of the lung metastasis in the two groups can only be seen under IVIS scan. The results revealed that mice with knocked-down TR4 in S/P cells had much fewer metastatic tumors detected (Fig. 6A, B, C), suggesting TR4 indeed is the key player to promote PCa S/P cell invasion.

We then analyzed the expression of TR4 and EZH2 using IHC staining in the in vivo mouse model and found that EZH2 expression is much lower in the knocked-down TR4 tumors as compared to vehicle control (Fig. 6D, E).

Together, results from Fig. 6A-E in vivo mouse model studies are in agreement with the above in vitro cell lines studies and demonstrated that targeting TR4 could suppress PCa S/P cell invasion via regulation of EZH2 signals.

Discussion

Accumulating evidence show that CD133 is important stem/progenitor marker in PCa(10, 17, 29). Vander et al.(18) reported that clonogenic ability of sorted CD133+ CWR22Rv1 cells is 2.4 times greater than the parental cells and average colony size was two times larger by 10 day. More importantly, the increased CD133+ cell population led to metastasis and poor prognosis in PCa patients(21, 22). Here we further proved that TR4 could alter the PCa CD133+ S/P cell invasion via modulating the EZH2-related metastasis gene expression.

Nuclear receptors have been reported to act as regulators of cancer S/P cell metabolism (30). Genome-wide gene expression data suggest that TR4 is expressed at a higher level in S/P cells (30), which is consistent with our data showing that PCa CD133+ S/P cells have higher TR4 expression, and that may be important in mediating PCa biological progression. Yang et al. also found TR4 was highly expressed in PCSC, the established PCa stem cell line from Celprogen, as compared to C4-2 CD133- cells(11). Up-regulation of EZH2 has been reported to increase the population of cancer S/P cells in other tumors(31-35), while targeting EZH2 using antagonist or silencing RNA decreased the self-renewal ability and tumorigenesis (36, 37). These findings also fit well with our findings here showing that TR4 may function
through modulation of EZH2 signaling to mediate PCa S/P cell invasion.

Recent studies indicated that EMT might play key roles in the cancer stem cells (CSCs) function. Both EMT and CSCs play critical role in tumor metastasis and they have biological similarities (38-40). These EMT-phenotype cells share molecular characteristics with CSCs(41). On the other hand, signaling pathways involved in the regulation of stem cell function also can trigger EMT programs(42), and EMT inducers can also play key roles in the maintenance of CSCs(43). Ding et al. found that TR4 could promote PCa metastasis via the CCL2/CCR2 signaling, the key player of EMT(16). In this study we further found that TR4 could promote PCa CD133+ S/P cells invasion via regulation of EZH2, which is important in both CSCs and EMT. These results therefore suggest that TR4 may promote PCa metastasis through both EMT-dependent pathway and CD133+ S/P cells.

In this study we demonstrated that TR4 might promote PCa S/P cell invasion. This is in contrast to an early study showing TR4 might suppress PCa initiation (6), suggesting that TR4 might play dual roles with a suppressor role in PCa initiation and a promoter role in PCa S/P cells invasion. Mechanism dissection suggested that TR4 might inhibit PCa initiation via positively regulating DNA damage-repair related gene ATM, thus enhancing DNA repair to prevent tumorigenesis. In contrast, TR4 could also promote PCa S/P cell invasion via modulation of EZH2 and its downstream metastasis-related genes. Other nuclear receptors, including the AR, may also play dual yet opposite roles in tumor initiation vs. tumor metastasis in PCa (44). Furthermore, BRCA1 was reported as a tumor suppressor (45) in PCa initiation but confers radiation resistance to PCa (46).

Early studies indicated that TR4 might be regulated by certain molecules including metformin (47), thiazolidinedione-rosiglitazone, and polyunsaturated fatty acids (PUFAs) (28). Since metformin has been linked to suppress the PCa progression (48-50), it will be interesting to see if metformin can also suppress PCa S/P cell invasion via modulation TR4 activity.

In summary, the studies presented herein demonstrate that TR4 might be able to function through modulation EZH2 expression to alter its downstream signals to enhance the PCa S/P cell mediated invasion. Targeting TR4 may becoming a new potential therapeutic approach to better suppress PCa S/P cell invasion.
Acknowledgements

This study were supported by NIH grants (CA122840 and CA156700) for C Chang, George Whipple Professorship Endowment for C Chang, the Taiwan Department of Health Clinical Trial for C Chang, Research Center of Excellence (DOH99-TD-B-111-004 to China Medical University, Taichung, Taiwan) for C Chang. NSFC (Grant No. 81472776) for D Yang.

Conflict of interest

The authors declare no competing interests.

References


50. Ben Sahra I, Tanti JF, Bost F. The combination of metformin and 2-deoxyglucose inhibits autophagy and
Figure legends

Fig. 1. Higher expression of TR4 in PCa stem/progenitor cells
C4-2 (A) and CWR22Rv1 (B) PCa S/P cells were sorted by MACS with CD133 antibody. Sorted stem cells were examined by qPCR with CD133 and Nanog stem markers and TR4 primers. Parental cells were used as control. Triplicate experiments were performed, and mean values ± S.D. (error bars) are presented. Result shows the sorted CD133+ S/P cells had significant higher stem markers and TR4 level (C, D, left panels). Western blot data show that PCa CD133+ S/P cells had higher TR4 level (C, D, right panels) compared to parental cells.

Fig. 2. TR4 role in mediating PCa metastasis
TR4 was successfully knocked down in both C4-2 and CWR22Rv1 CD133+ S/P cells (A, D). Then both TR4 knocked-down (shTR4) and control S/P cells (scr) were used for invasion assays. Triplicate experiments were performed, and mean values ± S.D. (error bars) are presented. The shTR4 groups had less invasion ability than control groups (B, E). C-F shows the quantitative data. Then the invasion ability change was measured by the 3D invasion assay that shows the similar results (G-J). K. gene array data of 88 PCa patients from the Oncomine database shows that the patients with metastatic PCa have a significantly higher TR4 level than the localized PCa.

Fig. 3 TR4 regulates EZH2 and its downstream
A. 30 PCa metastasis-related genes were screened by qPCR assay. B-C. Five out of the 30 metastasis related genes changed dramatically in both the C4-2 and CWR22Rv1 CD133+ S/P cells with EZH2 expression decreased most significantly. D. Western blots were performed to check EZH2 and it downstream genes change after knocking-down TR4. EZH2, NOTCH1, SLUG, TGFβ1 and MMP9 were significantly decreased in the three different cell lines include C4-2 and CWR22Rv1 CD133+ S/P cells and PCa stem cell line PCSC. Triplicate experiments were performed. E. Shows the integrated signaling pathways. F-G. Show the positive correlation between TR4 and EZH2 in clinical human PCa samples.
Fig. 4 TR4 transcriptionally regulates EZH2
A. TR4RE motif sequences. B. Three putative TR4REs predicted by JASPAR from EZH2 promoter. C. ChiP assay showed TR4 protein binding to the chromatin at the first and second putative TR4RE in EZH2 promoter. D. Promoter luciferase reporter assay showed that TR4 could promote EZH2 promoter activity in a dose dependent pattern. E. Luciferase reporter assay of series truncation of EZH2 promoter. At the loss of the first TR4RE, TR4 could no longer induce EZH2 promoter activity. F. ChiP assay of the first TR4RE of EZH2 promoter were performed in TR4 knocking-down cells. Results showed that the signal was significantly decreased. Luc assays were run in triplicate.

Fig. 5. TR4 promotes invasion in PCa CD133+ S/P cells via inducing of EZH2
A. Western blot shows EZH2 and its downstream genes decreased with knocking-down TR4, which can be partially reversed by overexpression of EZH2. B. C4-2 CD133+ S/P cells invasion assay showed shTR4 decreased invasion ability. Effect of decreased invasion ability via knocking down of TR4 was partially reversed by overexpressing EZH2. C. The quantitative data. D-E. the similar results in CWR22Rv1 CD133+ S/P cells. Triplicate experiments were performed.

Fig 6. Targeting TR4 suppresses PCa S/P tumor metastasis in vivo
A. Luc-CWR22Rv1 CD133+ cells with shTR4 or control were implanted into nude mice with 12 mice in each group. The tumor growth and metastasis were detected by IVIS system. B. Quantitative data of tumor metastases detected by IVIS system and autopsy. In the scr group, 4/7 mice developed metastatic sites include liver, diaphragm, and lung. In the shTR4 group, only one mouse out of six developed lung metastasis. C. Gross appearance of metastases in control group, white arrows point out metastasis. Both of the lung metastasis in the two groups can only be seen under IVIS scan. D. Tumor samples were collected for IHC staining with E. quantitative data.
Fig 1

A. C4-2 CD133+ C4-2 parental

B. CWR22Rv1 CD133+ CWR22Rv1 parental

C. mRNA fold change

D. mRNA fold change

TR4
GAPDH

TR4
GAPDH
Fig 2

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

P = 0.001

K. 

P = 0.0001

I. 

J. 

P = 0.011

P = 0.015

P = 0.009
Fig 3

A. 3D Metastasis related genes

B. C4-2 S/P shTR4/scr

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C. CWR22Rv1 S/P shTR4/scr

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D. TR4, EZH2, Notch1, TGFβ1, SLUG, MMP9, GAPDH

E. TR4 → EZH2 → direct or indirect

F. Scatter plot of TR4 vs. EZH2

G. Scatter plot of TR4 vs. NOTCH1

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Fig 4

A.

B.

C.

D.

E.

F.
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Mol Cancer Ther Published OnlineFirst April 1, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0971

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