

## The Differential Effects of Anti-Diabetic Thiazolidinedione on Prostate Cancer Progression Are Linked to the TR4 Nuclear Receptor Expression Status<sup>1,2</sup>

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### Abstract

The insulin sensitizers, thiazolidinediones (TZDs), have been used as anti-diabetic drugs since the discovery of their ability to alter insulin resistance through transactivation of peroxisome proliferator-activated receptors (PPARs). However, their side effects in hepatitis, cardiovascular diseases, and bladder cancer resulted in some selling restrictions in the USA and Europe. Here, we found that the potential impact of TZDs on the prostate cancer (PCa) progression might be linked to the TR4 nuclear receptor expression. Clinical surveys found that 9% of PCa patients had one allele TR4 deletion in their tumors. TZD increased cell growth and invasion in PCa cells when TR4 was knocked down. In contrast, TZD decreased PCa progression in PCa cells with wild type TR4. Mechanism dissection found that the Harvey Rat Sarcoma (HRAS) oncogene increased on TZD treatment of the TR4 knocked-down CWR22Rv1 and C4-2 cells, and interruption with HRAS inhibitor resulted in reversal of TZD-induced PCa progression. Together, these results suggest that TZD treatment may promote PCa progression depending on the TR4 expression status that may be clinically relevant since extra caution may be needed for those diabetic PCa patients receiving TZD treatment who have one allele TR4 deletion.

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### Introduction

Thiazolidinediones (TZDs), including rosiglitazone, pioglitazone, ciglitazone, and troglitazone, have been used extensively as successful anti-diabetic drugs after the discovery of their ability to alter the insulin sensitivity through activation of peroxisome proliferator-activated receptor gamma (PPARG, NR1C3) [1,2]. However, side effects

including hepatotoxicity, cardiovascular diseases, and bladder cancer have restricted some of their sales/usage in the USA and Europe [3–6]. Early studies indicated that TZDs might have protective effects in lung cancer but not in colorectal, prostate, or breast cancers [7,8]. Other reports suggested that TZDs could also reduce prostate cancer (PCa) cell growth [9]. However, an early report suggested that continued TZD

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treatment in diabetic patients might lead to increased bladder cancer risk in some patients [10], which raised a concern whether continued TZD treatment in diabetic patients might also have risks of promoting progression of other cancers.

Recent unexpected findings [11] showing TZDs could also function as ligands/activators to activate the TR4 nuclear receptor (TR4, NR2C2) [12–19] raised an interesting question about the potential impacts of this newly identified TZD-TR4 signaling on the classic TZD-PPAR signaling.

Here, we found 9% of PCa patients had lost one allele of *TR4* gene in their tumors compared to their adjacent benign tissues. *In vitro* cell lines and *in vivo* mice studies also found that the TR4 expression status in PCa might be able to alter the TZD's ability to induce PCa progression, which might raise a concern for those selective diabetic PCa patients receiving TZD treatment who had one allele TR4 deletion.

## Materials and Methods

### Prostate Tissue Microarray

A tissue microarray (TMA), consisting of representative lesions (three cores each) of benign appearing prostate and prostatic carcinoma from the tissue specimens obtained by radical prostatectomy, was constructed, as described previously [20]. Appropriate approval from the Institutional Review Board was obtained before construction and use of the TMA.

### Fluorescence In Situ Hybridization

Fluorescence *in situ* hybridization (FISH) was performed by the University of Rochester Medical Center Pathology Laboratory. Green 5-fluorescein dUTP-labeled human BAC clone RPCI-11 1104I16 probes for human TR4 were purchased from Empire Genomics (Buffalo, NY) and we followed their protocol for hybridization.

### Cell Culture

The PCa cell lines CWR22Rv1 and C4-2 were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS and 1% antibiotic-antimycotic (Invitrogen, Grand Island, NY). Stable CWR22Rv1 and C4-2 cell lines expressing scramble siRNA (scr) or siRNA against TR4 (siTR4) were established by transfecting pSuperior.retro.puro plasmids into the cells and selecting for stable cell lines by treatment with puromycin (1.2 µg/ml) for 2 weeks.

### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and 3 µg of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen). Quantitative real-time reverse transcription–polymerase chain reaction (PCR) was conducted using a Bio-Rad CFX96 System with SYBR Green to determine the level of mRNA expression of a gene of interest. Expression levels were normalized to the expression of β-actin RNA.

### Cell Proliferation Assay

Cells were treated with 4 µM TZD (all treatments used rosiglitazone) or DMSO vehicle control for 2 weeks before the assay. C4-2 and CWR22Rv1 cells were seeded in 24-well plates (10,000 cells per well) and cultured for 24, 48, 72, and 96 hours. Cells were harvested and cell numbers were calculated using 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) agent.

### Anchorage-Independent Colony Forming Assay

Cells were treated with 4 µM TZD or vehicle as control (DMSO) for 1 month before the assay. C4-2 and CWR22Rv1 cells were suspended at a density of  $2 \times 10^5$  cells/ml in 0.4% Noble agar (Sigma, St Louis, MO) containing media and were seeded on top of 0.8% agarose containing media in culture plates, and after agarose solidified, we added 2 ml of media on top. Media were changed every week for 1 month. Colonies were stained with *p*-iodonitroretrazolium violet (Sigma), photographed, and counted.

### Boyden Chamber Transwell Migration and Invasion Assays

Cells were treated with 4 µM TZD or DMSO for 2 months before the migration assay. Migration assay was performed using 24-well transwell inserts (8 µm pores) according to the manufacturer's instructions (Corning, #3422). C4-2 and CWR22Rv1 cells at  $10^5$  cells per well were seeded on the upper chambers of transwell plates and media containing 20% FBS (10% more than the upper chamber) were added to the lower chambers. For the invasion assay, the upper chambers with 8 µm pore polycarbonate membrane insert were pre-coated with Matrigel. Each sample was assayed in triplicate and incubated for 24 hours. The cells that migrated into the lower chambers were stained using 1% toluidine blue and counted under a microscope.

### Luciferase Reporter Assay

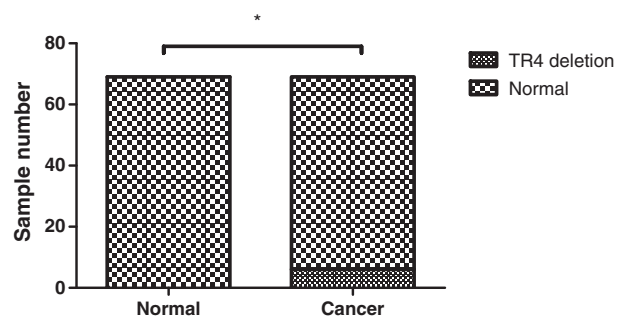
Harvey Rat Sarcoma (HRAS) promoter (–2053 to +144) was cloned to pGL3 luciferase plasmid. Cells were co-transfected with pGL3-HRAS promoter with pRL-TK as an internal control. Cells were lysed and detected for firefly luciferase activity by adding substrate LAR II. The internal *Renilla* luciferase control was detected by adding Stop & Glo Reagent (Promega, Madison, WIS).

### HRAS-Specific Inhibitor Treatment

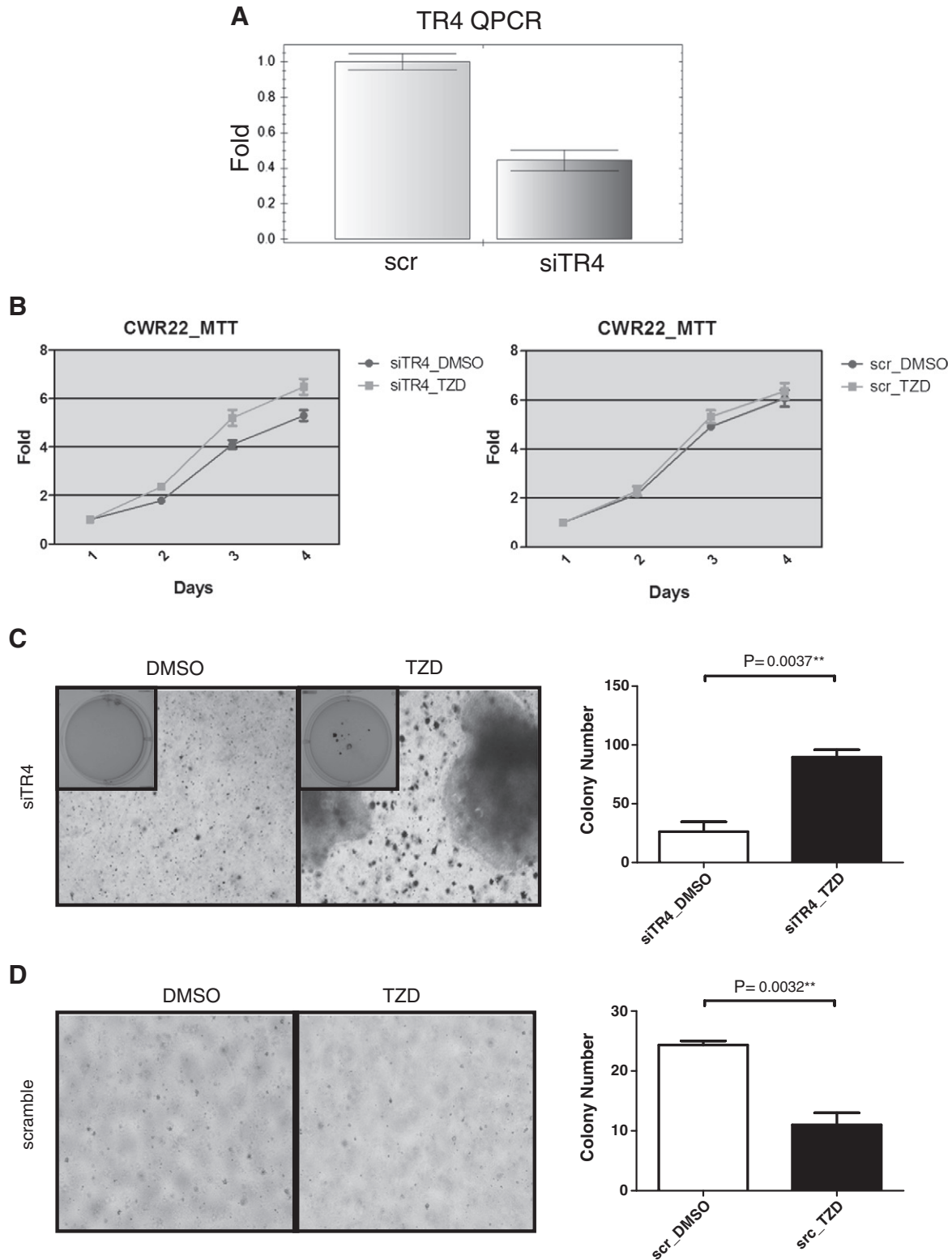
HRAS-specific inhibitor farnesyl thiosalicylic acid (FTS) was purchased from Cayman Chemical (Ann Arbor, MI). Cells were treated with 50 µM FTS for 2 weeks. The migration and invasion assays were performed after treatment.

### Orthotopic Xenograft Model

All animal procedures were approved by the Animal Care and Use Committee of the University of Rochester. Male 6- to 8-week-old nude mice were purchased from the National



**Figure 1.** Nine percent of PCa patients have TR4 deletion. Sixty-nine PCa specimens were tested by FISH. Six of 69 cancer samples showed one TR4 allele deletion, while none of the normal compartments of those 69 samples showed TR4 deletion. The statistical results were calculated using Fisher exact test (\* $P < 0.05$ ).

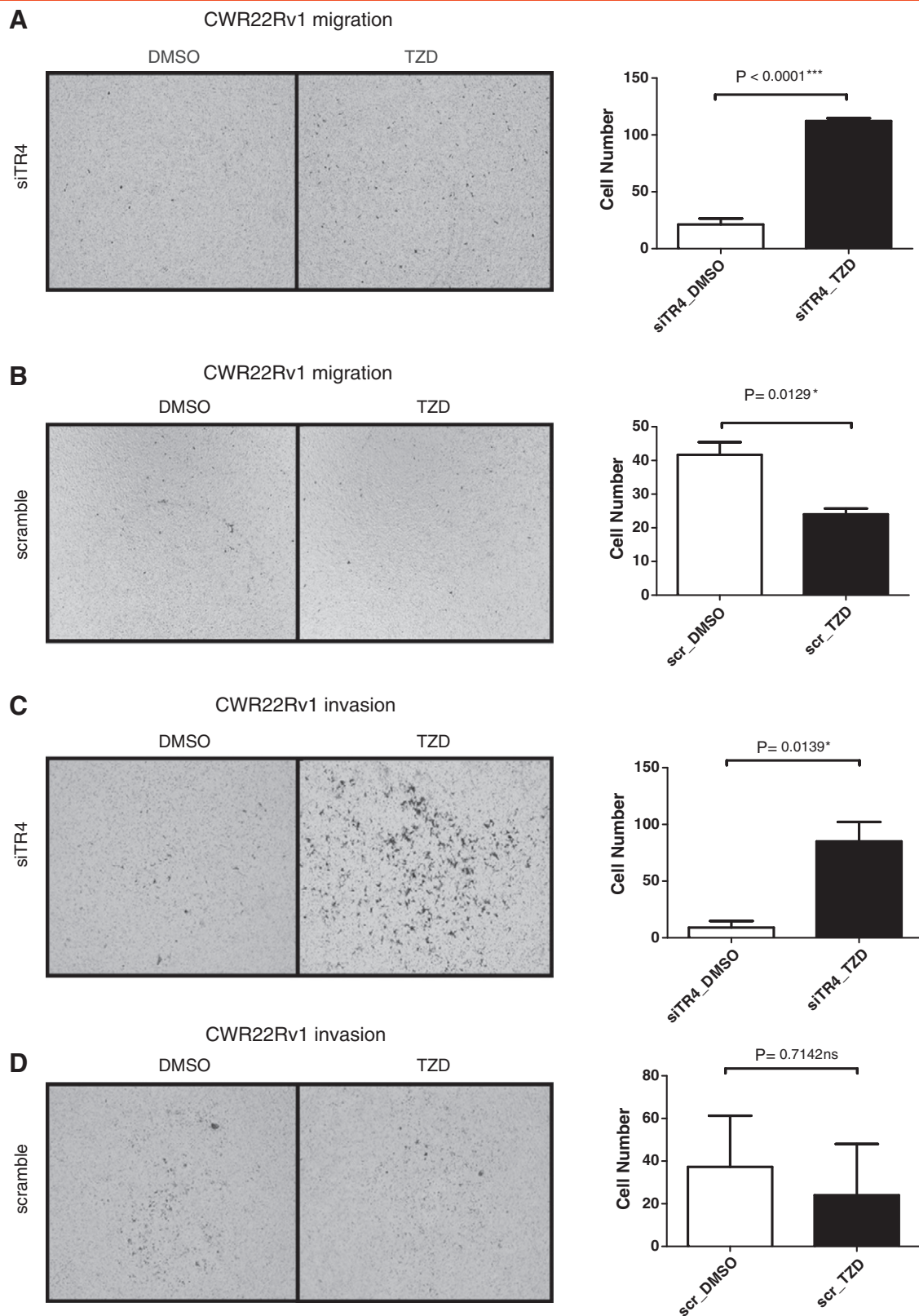


**Figure 2.** TZD treatment increases CWR22Rv1-siTR4 cell growth and colony formation. (A) Quantitative PCR showed TR4 mRNA level in CWR22Rv1-siTR4 and CWR22Rv1-scr cell lines. (B) TZD treatment increases CWR22Rv1-siTR4 (left panel) cell growth compared to no effect on CWR22Rv1-scr (right panel) using MTT assay. (C and D) TZD treatment increases CWR22Rv1-siTR4 (C) compared to decreases CWR22Rv1-scr (D) cell colony formation in soft agar gel. The numbers of colonies were counted, and the quantifications were shown in the right panels. All statistical analyses were performed using Student's *t* test (\*\**P* < 0.01).

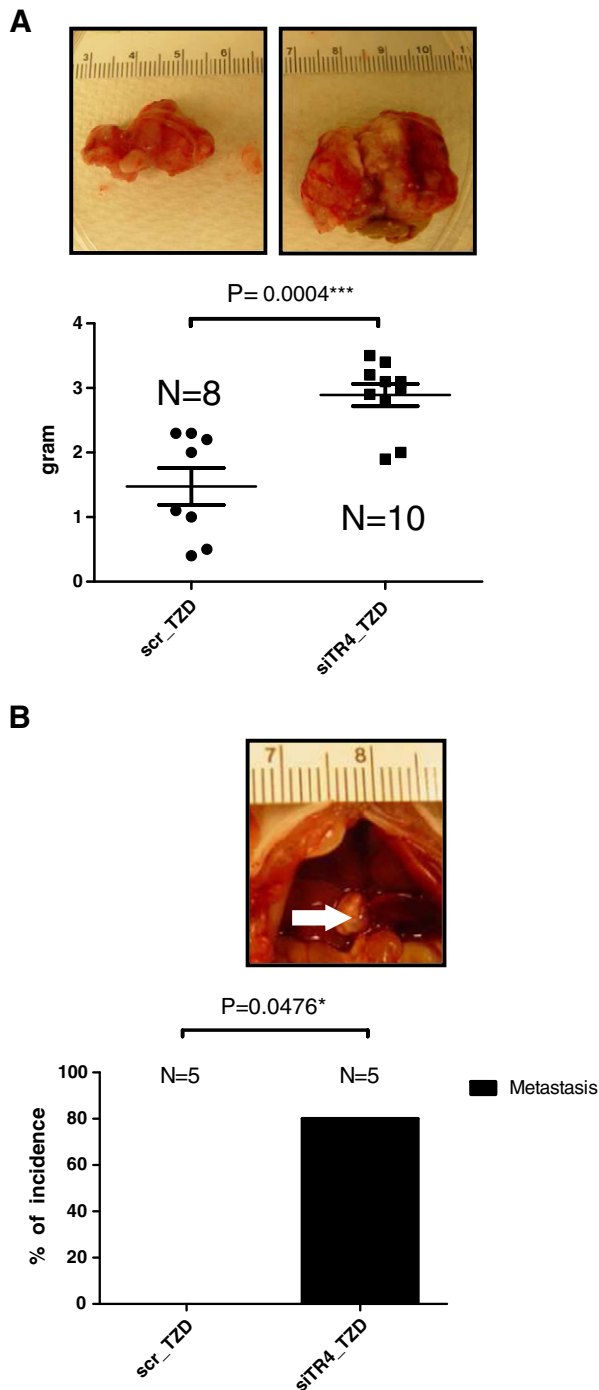
Cancer Institute (NCI). CWR22Rv1 ( $1 \times 10^6$ ) with Matrigel mixture was orthotopically injected into both anterior prostates in nude mice at 8 weeks of age. The primary and metastatic tumors were evaluated at 4 weeks endpoint.

#### Western Blot Analysis

CWR22Rv1 cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 1 mM okadaic acid and 1 mg/ml



**Figure 3.** TZD treatment increases CWR22Rv1-siTR4 cell migration and invasion. (A and B) TZD treatment increases CWR22Rv1-siTR4 (A) but decreases CWR22Rv1-scr (B) cell migration compared to vehicle control using Boyden chamber transwell migration assay. (C and D) TZD treatment increases CWR22Rv1-siTR4 (C) but has no effect on CWR22Rv1-scr (D) cell invasion compared to vehicle control using Boyden chamber transwell invasion assay coated with Matrigel. The numbers of colonies were counted, and the quantifications were shown in the right panels. All statistical analyses were performed using Student's *t* test (\* $P < 0.05$ , \*\*\* $P < 0.001$ , ns = not significant).



**Figure 4.** TZD treatment increases CWR22Rv1-siTR4 tumor formation and metastasis *in vivo*. CWR22Rv1 ( $1 \times 10^6$ ) with Matrigel mixture was orthotopically injected into both anterior prostates in five nude mice at 8 weeks of age. The primary and metastatic tumors were evaluated at 4 weeks endpoint. (A) TZD treatment increases CWR22Rv1-siTR4 (siTR4\_TZD) xenograft tumor ( $N = 10$ ) size when injected into nude mice prostate compared to CWR22Rv1-scr (scr\_TZD) control ( $N = 8$ ). The pictures (upper panel) represent the prostate tumor grown in the nude mice. The statistic results were calculated using Student's *t* test and shown in the lower panel (\*\*\* $P < 0.001$ ). (B) TZD treatment increases CWR22Rv1-siTR4 xenograft tumor metastasis when injected into nude mice prostate compared to CWR22Rv1-scr control. The incidence of metastasis was calculated as shown in the lower panel. The upper panel shows the large liver metastasis (arrow) in the CWR22Rv1-siTR4 TZD-treated mouse. The statistical results were calculated using Fisher exact test (\* $P < 0.05$ ).

aprotinin, leupeptin, and pepstatin]. Individual samples (50  $\mu$ g of proteins) were prepared for electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto poly(vinylidene fluoride) (PVDF) membranes (Millipore, Billerica, MA). After blocking the membranes with 5% fat-free milk in TBST [50 mM Tris (pH 7.5), containing 0.15 M NaCl and 0.05% Tween-20] for 1 hour at room temperature, the membranes were incubated with appropriate dilutions (diluted in 2.5% BSA) of specific Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After Tris buffered saline with Tween 20 (TBST) washing, the blots were incubated with anti-rabbit secondary antibodies with HRP and conjugated for 1 hour. After TBST washing, the blots were developed in ECL mixture (Thermo Fisher Scientific Inc., Rochester, NY).

#### Statistics

The data values were presented as the mean  $\pm$  SEM. *P* values were calculated by unpaired Student's *t* test or Fisher exact test.  $P < 0.05$  was considered statistically significant.

#### Results

##### *Nine Percent of PCa Patients Have One Allele TR4 Deletion*

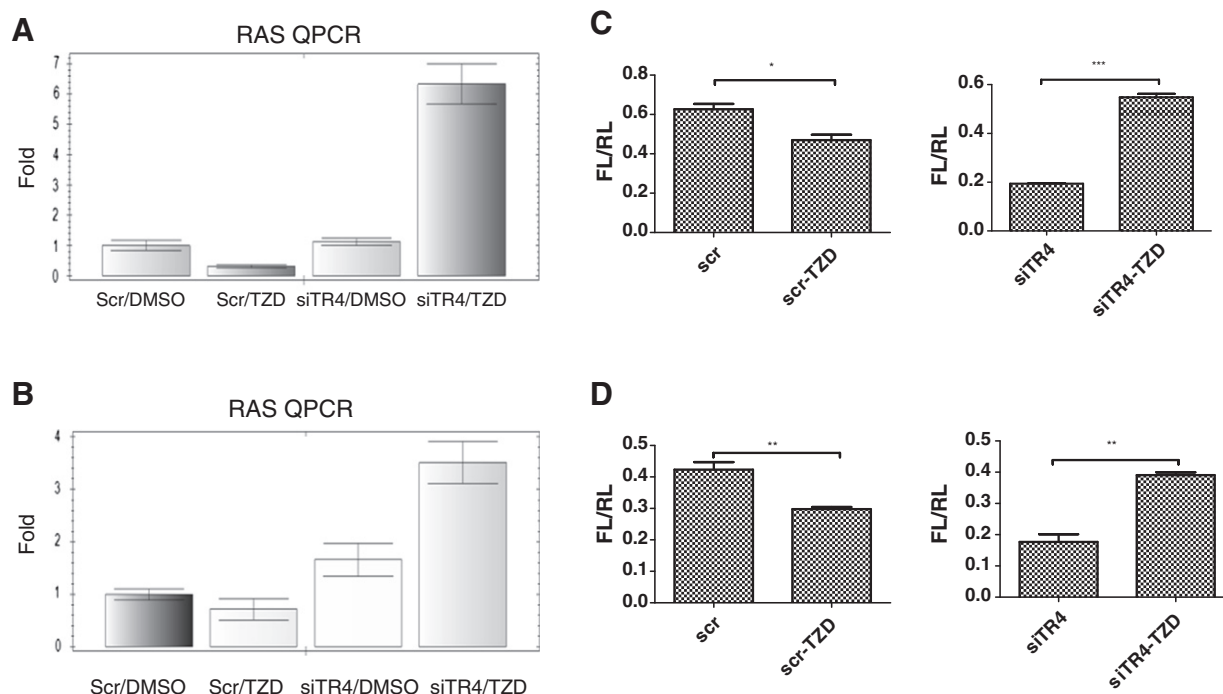
We tested TR4 deletion in a prostate TMA by FISH and found 9% (6 of 69) of PCa specimens had one allele TR4 deletion. In contrast, none of the control tissues/benign compartments from these 69 patients showed TR4 deletion ( $P < 0.0279$ ; Figure 1). There were no statistically significant differences in TR4 deletion among different tumor grades [1/25 Gleason score (GS)  $\leq 6$  vs 5/38 GS 7 vs 0/6 GS  $\geq 8$  tumors had the deletion] or stages (4/53 pT2 vs 2/16 pT3 tumors had the deletion). Additionally, no significant correlation between TR4 deletion and tumor recurrence was seen. These findings in clinical samples suggested a potential linkage between TR4 deletion and the development and progression of PCa.

##### *TZD Treatment–Altered PCa Cell Growth Is Linked to the TR4 Expression Status*

To test if TR4 deletion in PCa may be linked to PCa progression, we examined the effects of TZD treatment on PCa cells with altered TR4 expression to mimic the one allele TR4 deletion, since a recent report suggested that TZD treatment might be linked to the increased risk of bladder cancer [6], and importantly, TZD was also able to function as an agonist to transactivate TR4 [11]. We were interested to see if TZD treatment had any potential differential effects on the PCa progression in those PCa cells with or without TR4 deletion.

We first applied TR4-siRNA to knock down TR4 in PCa CWR22Rv1 cells (CWR22Rv1-siTR4) that led to nearly 50% suppression of TR4 mRNA (Figure 2A) and then treated the cells with TZD versus DMSO control. Interestingly, using MTT cell growth assay (Figure 2B), we found that TZD, compared to DMSO, treatment could promote PCa cell growth significantly in CWR22Rv1-siTR4 cells (left panel). In contrast, TZD treatment showed little effect on PCa cell growth compared to DMSO when the CWR22Rv1 cells were transfected with scramble-siRNA control (CWR22Rv1-scr; right panel).

We then applied the second growth assay, anchorage-independent growth assay, also known as colony formation assay, to confirm the above TZD differential effects. As expected, TZD, compared to DMSO, treatment could dramatically increase colony formation in CWR22Rv1-siRNA (Figure 2C). In contrast, TZD, compared to DMSO, treatment could decrease colony formation of CWR22Rv1-scr (Figure 2D).



**Figure 5.** TZD treatment has differential effect on HRAS expression. Quantitative PCR showed HRAS mRNA level in CWR22Rv1 (A) and C4-2 (B) cells. Each cell line was divided into four sublines with various treatments for comparisons (scramble siRNA with DMSO, scramble siRNA with TZD, TR4 siRNA with DMSO, and TR4 siRNA with TZD). In both cell lines, TZD decreases HRAS mRNA in the scramble siRNA subline but increases HRAS mRNA in the TR4 siRNA subline. (C and D) HRAS promoter (2.2 kb) was cloned into the luciferase reporter plasmid and then transfected into CWR22Rv1 or C4-2 with each cell line divided into the above four sublines. In both cell lines, TZD decreases HRAS-luciferase activity in the scramble siRNA subline but increases HRAS-luciferase activity in the TR4 siRNA subline (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). FL/RL: firefly luciferase/renilla luciferase.

Together, results from Figure 2, A to D, using different growth assays suggested that TZD treatment has differential effects on PCa progression that depends on the TR4 expression status.

#### TZD Treatment–Altered PCa Cell Migration/Invasion Is Linked to the TR4 Expression Status

To further study if TZD treatment might also have differential effects on PCa metastasis, we applied the Boyden chamber migration/invasion assays to examine the CWR22Rv1 metastatic ability. The results revealed that TZD, compared to DMSO, treatment enhanced CWR22Rv1-siTR4 cell migration significantly (Figure 3A). In contrast, TZD, compared to DMSO, treatment decreased CWR22Rv1-scr cell migration (Figure 3B).

For the invasion assay, we coated the membrane between the upper and lower wells with Matrigel to mimic the *in vivo* invasion of the cancer cells into the extracellular matrix. As shown in Figure 3C, TZD, compared to DMSO, treatment increased CWR22Rv1-siTR4 invasion significantly. In contrast, TZD, compared to DMSO, treatment slightly decreased CWR22Rv1-scr cell invasion (Figure 3D). Importantly, to ensure that such important findings are not just present in a single PCa cell line, we then repeated the above studies described in Figures 2 to 3 with the PCa C4-2 cell line and obtained similar results (Supplementary Figures 1 and 2).

Together, results from Figures 2, 3, Supplementary Figures 1 and 2 concluded that TZD treatment has differential effects on PCa progression that depends on TR4 expression status, which might suggest a potential risk to enhance the tumor progression in those PCa patients with one allele TR4 deletion receiving TZD treatment.

#### TZD Treatment Enhanced PCa Progression *In Vivo* When TR4 Is Knocked Down

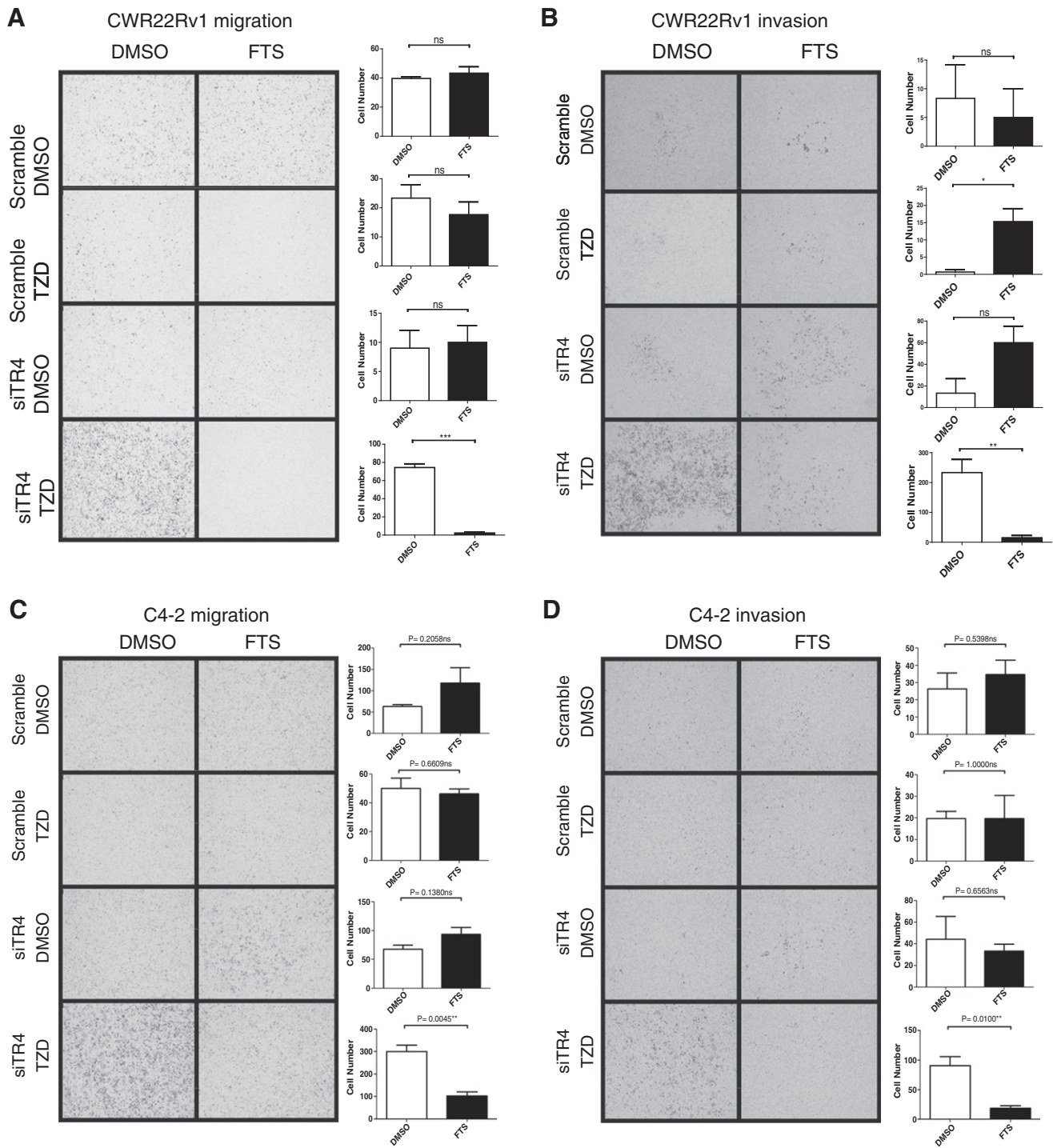
To confirm that the above *in vitro* findings may also occur in the *in vivo* mouse model, we xenografted TZD-treated CWR22Rv1-scr and CWR22Rv1-siTR4 cells into nude mouse anterior prostates and sacrificed the mice at 4 weeks after inoculation. The results showed that the CWR22Rv1-siTR4 mice grew larger tumors compared to the CWR22Rv1-scr mice (Figure 4A). As expected, CWR22Rv1-siTR4 mice also had more metastatic PCa tumors than the CWR22Rv1-scr mice (Figure 4B).

Together, the *in vivo* results from Figure 4, A and B, confirm the *in vitro* findings that TZD could increase the PCa growth and invasion depending on the TR4 expression level.

#### Mechanism Dissection and How TZD Treatment Has Differential Effect on PCa Progression

To dissect the potential mechanism(s) by which the TZD (rosiglitazone) has differential effects on PCa progression that may depend on the expression status of TR4, we first screened 35 metastasis-related genes (Supplementary Table 1) and found that *HRAS* oncogene mRNA increases in both PCa CWR22Rv1-siTR4 (Figure 5A) and C4-2-siTR4 (Figure 5B) treated with TZD but decreases in the scramble controls treated with TZD. Further mechanism dissection using luciferase reporter assay also proved that TZD was able to modulate *HRAS* expression at the transcriptional levels when TR4 was knocked down (Figure 5, C and D).

Importantly, we then applied the interruption approach by adding 50  $\mu$ M RAS-specific inhibitor FTS for 2 weeks in CWR22Rv1 and C4-2 stable sublines (scramble-siRNA-DMSO, scramble-siRNA-TZD,

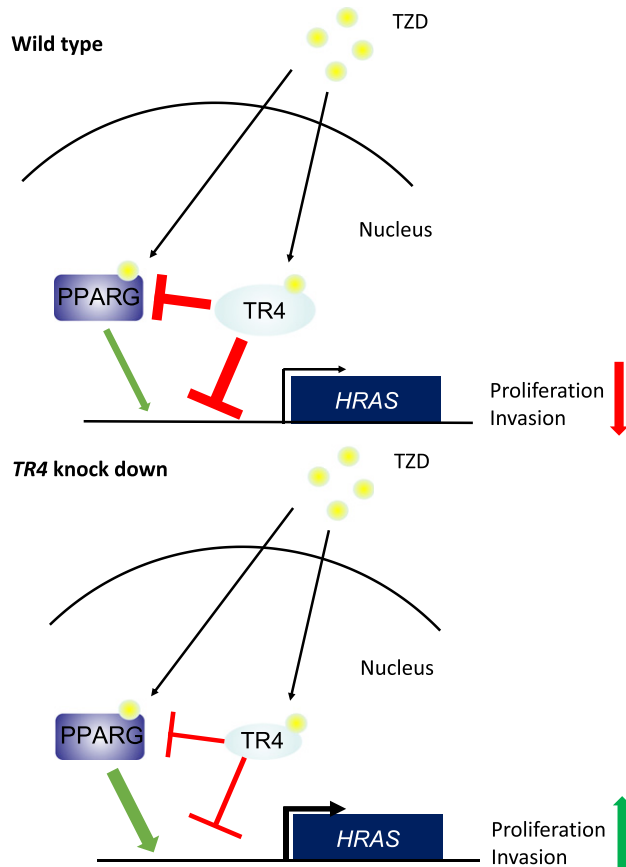


**Figure 6.** RAS-specific inhibitor FTS can interrupt the TZD-induced PCa migration and invasion. (A) Treatment with 50  $\mu$ M RAS inhibitor FTS successfully interrupted TZD-induced CWR22Rv1-siTR4 migration (bottom panel). FTS did not show any suppression effect in any of the other three controls. (B) Treatment with 50  $\mu$ M FTS successfully interrupted TZD-induced CWR22Rv1-siTR4 invasion (bottom panel). FTS did not show any suppression effect in the other three controls. (C) Treatment with 50  $\mu$ M FTS successfully interrupted TZD-induced C4-2 siTR4 migration (bottom panel). FTS did not show any suppression effect in any of the other three controls. (D) Treatment with 50  $\mu$ M FTS successfully interrupted TZD-induced C4-2-siTR4 invasion (bottom panel). FTS did not show any suppression effect in any of the other three controls. The statistical results were calculated using Fisher exact test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

TR4-siRNA-DMSO, and TR4-siRNA-TZD and found that FTS treatment could successfully interrupt TZD-induced PCa cell migration and invasion in both CWR22Rv1 and C4-2 TR4 knocked down cell lines (Figure 6, A–D), suggesting that TZD might need to function

through modulation of *HRAS* expression to have differential effects on PCa progression that depends on the expression of TR4 (Figure 7).

Together, results from Figures 5 and 6 concluded that those PCa patients with one allele TR4 deletion should be more cautious with



**Figure 7.** This schematic diagram depicts the TZD signaling through TR4. The upper panel shows that in the TR4 wild-type condition TZD cannot induce the *HRAS* expression and proliferation/invasion. The bottom panel shows that in the TR4 knockdown condition TZD can induce the *HRAS* expression and proliferation/invasion.

taking TZD or may consider additional treatment (for example, FTS or other drugs) to eliminate the potential side effects.

## Discussion

Among the clinical uses of many TZDs, troglitazone was the first TZD to be approved in 1997 for the treatment of patients with type 2 diabetes. However, the severe hepatotoxicity side effect resulted in drug withdrawal in 2000 [21]. Rosiglitazone was released in 1999 and was found to have the potential of increased heart attack risk [22]. Pioglitazone was approved in 1999 and found to have the potential side effect of increased bladder cancer risk and was withdrawn in France [10].

Here, we found that treatment with the TZD rosiglitazone had adverse effects when TR4 expression is knocked down. Given the fact that TZD can transactivate both TR4 and PPARG, it is very likely that TZD may have profound effects to activate PPARG in cells with TR4 deletion. Under this circumstance, TZD may become an enhancer to promote PCa progression. These results suggest that the expression levels of TR4 and PPARG in PCa cells may need a balance to maintain the proper and best TZD effects. Knocking down one of them (TR4 in this study) may lead to unbalanced TZD effects that may increase the risk for PCa progression (Figure 7).

Mechanism dissection revealed that the oncogene *HRAS* expression was increased when TR4 was knocked down in cells and then treated with TZD (Figure 5). *HRAS* plays important roles in regulating cell

proliferation, differentiation, and survival [23]. Activated *HRAS* could transduce the signal through the mitogen-activated protein kinase (MAPK) pathway and promote tumorigenesis [24]. Accumulating evidence supports that wild-type *RAS* can be chronically activated by the aberrant autocrine/paracrine growth factor signals in various tumors including PCa [25]. Importantly, the *RAS*/*RAF*/*MAPK* signals have also been linked to the progression of PCa into the castration-resistant stage [25]. Mice with transgenic *RAS* exhibited prostatic intraepithelial neoplasia and metaplastic changes [26], suggesting the potential roles for the promotion of PCa initiation. Furthermore, other reports also showed that *RAS* might promote angiogenesis by inducing vascular endothelial growth factor production that might be involved in the metastasis [27]. Early studies also indicated that the activated *RAS* might be able to promote metastasis in a non-metastatic rodent cell line [28,29], as well as in the xenografted mouse model [30]. Mulholland et al. also reported that *RAS*/*MAPK* activation together with *PTEN* loss could promote metastasis from PCa stem/progenitor cells [31]. Together, the results from *in vitro* cell lines, *in vivo* animals, and clinical studies all suggest that *RAS*/*MAPK* activation plays critical roles in cancer progression and metastasis.

Interestingly, in addition to the enhanced *HRAS* expression, we also found the expression of *RAF*, a *HRAS* downstream target, was enhanced when treating with TZD regardless of the TR4 level (Supplementary Figure 3). Nevertheless, targeting *HRAS* signals with the inhibitor FTS also successfully reversed the TZD-enhanced tumor cell migration and invasion (Figure 6). These results suggest that the combination of FTS with TZD may potentially eliminate the TZD side effects.

In summary, this study provides the first evidence showing that TZD may have differential roles to affect PCa progression under different TR4 expression levels. People that have the genetic defect of TR4 deletion caused by inheritance or mutation/deletion or any other molecules that can alter TR4 expression in the PCa should be more careful because of the potential side effects of being treated with TZD to battle diabetes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2015.02.005>.

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