TR4 Nuclear Receptor Alters the Prostate Cancer CD133\(^+\) Stem/Progenitor Cell Invasion via Modulating the EZH2-Related Metastasis Gene Expression

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Abstract

The testicular nuclear receptor 4 (TR4) is a member of the nuclear receptor superfamily that mediates various biologic functions with key impacts on metabolic disorders and tumor progression. Here, we demonstrate that TR4 may play a positive role in prostate cancer CD133\(^+\) stem/progenitor (S/P) cell invasion. Targeting TR4 with lentiviral silencing RNA significantly suppressed prostate cancer 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\(^{\prime}\) 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, TGFB1, SLUG, and MMP9. Rescue approaches via adding the EZH2 reversed the TR4-mediated prostate cancer S/P cell invasion. Together, these results suggest that the TR4–EZH2 signaling may play a critical role in the prostate cancer S/P cell invasion and may allow us to develop a better therapy to battle the prostate cancer metastasis.

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Introduction

Prostate cancer continues to be the most common malignant tumor in men in the United States. Androgen/androgen receptor (AR) signals play important roles in prostate cancer progression, and androgen deprivation therapy (ADT) is the standard therapy for the advanced prostate cancer. However, most ADT eventually fails and prostate cancer progresses into castration-resistant prostate cancer (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 prostate cancer 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 suppresses 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 prostate cancer (7), and its expression is positively linked with the progression and metastasis of prostate cancer (8).

Here, we demonstrate that targeting TR4 could suppress prostate cancer CD133\(^+\) S/P cell invasion via inhibiting of EZH2 expression and its downstream metastasis-related genes, including NOTCH1, SLUG, TGFB1, and MMP9.
Materials and Methods

Cell culture
C4-2 cells were a gift from Dr. Jer-Tsong Hsieh of University of Texas Southwestern Medical Center (Dallas, TX) in 2009 and were not authenticated by us. The CWR22Rv1 cell line was purchased from and authenticated by the ATCC in May 2010. Prostate cancer stem cells (PCSC) were purchased from Celprogen in 2011 and were not authenticated by us. C4-2 and CWR22Rv1 cell lines were maintained in RPMI-1640 media containing penicillin (25 U/mL), streptomycin (25 g/mL), 1% L-glutamate, and 10% FBS. Isolated CD133<sup>+</sup>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 apotransferrin, 0.03 mmol/L sodium selenite, 2 mmol/L progesterone, 5 mmol/L HEPES, 0.1% sodium bicarbonate, 10 ng/mL basic fibroblast growth factor, and 20 ng/mL EGF.

Magnetic beads isolation of CD133<sup>+</sup> cells
Isolation of CD133<sup>+</sup> prostate cancer cells was performed as previously described (9, 10). Briefly, magnetic beads (Invitrogen) were conjugated with biotinylated CD133 antibody (Miltenyi Biotec). Cells (2 x 10<sup>7</sup>) were detached with 5 mmol/L EDTA and incubated with beads, and separated by placing tubes in a magnetic field. The S/P markers of the sorted cells were confirmed by qPCR and immunofluorescence. Flow-cytometric analysis indicated that >95% of the sorted cells are CD133<sup>+</sup> cells (data not shown).

qRT-PCR
Total RNA was isolated by TRizol reagent (Invitrogen), and 1 μg of total RNA was then subjected to reverse transcription using Superscript III transcriptase (Invitrogen). 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). 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 Biotechnology, #sc-166574), TR4 (Perseus Proteomics, #PP-H0107B-00), EZH2 (Cell Signaling Technology, #5246P), NOTCH1 (Cell Signaling Technology, #3439), TGF<sub>B</sub>1 (Santa Cruz Biotechnology, #sc-146), SLUG (Cell Signaling Technology, #9585), or MMP9 (Santa Cruz Biotechnology, #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). Quantitations of blots are shown in Supplementary 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). The 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 (0.01 mol/L 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<sup>5</sup>) were resuspended with serum-free media and seeded in the upper chamber of Transwells. Media with 10% FBS were put in the lower chamber. After 24-hour 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.

Figure 1.
Higher expression of TR4 in prostate cancer stem/progenitor (S/P) cells. C4-2 (A) and CWR22Rv1 (B) prostate cancer 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 ± SD (error bars) are presented. Result shows the sorted CD133<sup>+</sup> S/P cells had significantly higher stem cell markers and TR4 level (C and D, left). Western blot data show that prostate cancer CD133<sup>+</sup> S/P cells had higher TR4 level (C and D, right) compared with parental cells.
3D invasion assay
The 3D invasion assay was as previously described (12). Briefly, 1 x 10^4 C104 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 weeks and then spheres and protrusions will be recorded under microscope.

ChIP assay
Chromatin immunoprecipitation (ChIP) experiments were performed following the Cold Spring Harbor ChIP protocol (13) with minor modifications. Cultured C4-2 prostate cancer cells (2 x 10^7) were harvested. The cells were treated with 1% formaldehyde at room temperature for 10 minutes to cross-link DNA. After sonication (10 x 10 seconds, 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) 24 hours after transfection, and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). pRL-TK served as internal control. The assays were run in triplicate.

Orthotopic-injected mouse model
Male 6- to 8-week-old nude mice were purchased from NCI (Bethesda, MD). Mice were divided into two groups, that is, the control group and the shRNA group. CW22Rv1 S/P cells (2 x 10^5) with shTR4 or Scramble (scr) control mixed with Matrigel (BD) were orthotopically injected into both anterior prostates. IVIS

Figure 2.
TR4 role in mediating prostate cancer metastasis. TR4 was successfully knocked down in both C4-2 and CWR22Rv1 CD133^+ S/P cells (A and 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 ± SD (error bars) are presented. The shTR4 groups had less invasion ability than control groups (B and E), C and F show 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 prostate cancer patients from the Oncomine database show that the patients with metastatic prostate cancer have a significantly higher TR4 level than the localized prostate cancer.
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 IHC.

IHC
Tumor tissues were fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. The primary antibodies of the TR4 (Perseus Proteomics, #PP-H0107B-00, 1:50), EZH2 (Cell Signaling Technology, #5246P, 1:100) were used for staining. The primary antibody was recognized by the biotinylated secondary antibody (Vector), 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±SD. The Student 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 prostate cancer 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 prostate cancer (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 prostate cancer progression (16). We were therefore interested in examining the potential linkage of TR4 in prostate cancer S/P cells-enhanced metastasis.

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

Together, results from Fig. 1A–D demonstrate the higher expression of TR4 in prostate cancer S/P cells.
TR4 promotes prostate cancer CD133⁺ S/P cell metastasis

To examine whether increased TR4 in CD133⁺ S/P cells may affect their influence on prostate cancer 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 and 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 prostate cancer C4-2 or CWR22Rv1 CD133⁺ S/P cell invasion (Fig. 2G–J).

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

Together, results from in vitro cell line studies (Fig. 2A–J) and human prostate cancer 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 prostate cancer CD133⁺ S/P cell invasion.

TR4 regulates EZH2 and its metastasis-related genes in prostate cancer S/P cells

To dissect the mechanism by which TR4 alters the S/P cell-mediated prostate cancer cell invasion in the C4-2 and CWR22Rv1 CD133⁺ S/P cells, we screened those genes that were reported to link to prostate cancer metastasis (Fig. 3A and Supplementary Table S1). The results revealed five overlapping potential key genes with KAI1 and TIMP2 increased and IGE, HIF2α, and EZH2 decreased upon knocking down of TR4 (Fig. 3B and 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 and CWR22Rv1 CD133⁺ S/P cells (Fig. 3D–F). We also obtained similar results when we replaced C4-2 CD133⁺ S/P cells with two other S/P cell lines (CWR22Rv1 CD133⁺ S/P and PCSC; Fig. 3D). PCSC cell line was originally obtained from a prostate cancer patient and immortalized by Celprogen.

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

Using NCBI GEO databases to analyze the human prostate cancer sample array, we also found the positive correlation of TR4 and EZH2 in 94 prostate cancer samples (GEO dataset accession GSE35988; ref. 27; Fig. 3F). Similarly, analysis of another dataset (GEO dataset accession GSE6919) revealed the positive correlation of TR4 and EZH2 in 89 human prostate cancer 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 three putative TR4REs within the \textit{EZH2} promoter region (\(\pm 1698\) to \(\pm 1683\); \(\pm 1055\) to \(\pm 1041\); \(\pm 149\) to \(\pm 163\)) using JASPAR database (Fig. 4B). Because it is commonly accepted that only binding sites in the conserved region are functional, we checked the sequence and found that all three sites are in the evolutionary conserved region. We then applied the ChIP assay and found that TR4 only bound to the first two putative TR4REs (Fig. 4C). We then linked the \textit{EZH2} promoter (\(\pm 2007\) to \(\pm 313\)) into pGL-3 basic luciferase reporter and examined TR4 influence on its activity. As shown in Fig. 4D, TR4 enhanced \textit{EZH2} promoter activity in a dose-dependent manner.

Because 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 the above results using a series of truncations of the \textit{EZH2} promoter reporter and results showed that truncation without the first TR4RE failed to induce \textit{EZH2} promoter activity (Fig. 4E), suggesting that this TR4RE may play essential roles to mediate the TR4 modulation of \textit{EZH2} expression. This conclusion is further confirmed via the ChIP assay, showing that knocking down TR4 with TR4-shRNA led to significantly suppressed the binding to the first TR4RE (Fig. 4F).

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

**TR4 promotes prostate cancer S/P cell invasion via inducing \textit{EZH2}**

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

Together, results from Fig. 5A–E suggest that targeting TR4 with TR4-shRNA may suppress prostate cancer CD133\(^+\) S/P cell invasion via modulation of \textit{EZH2} signaling.
Targeting TR4 suppresses prostate cancer S/P tumor metastasis in the in vivo mouse model

All the above in vitro data concluded that targeting TR4 could suppress prostate cancer CD133+ S/P cell invasion via alteration of EZH2. To further confirm this in vitro conclusion in vivo, we applied an orthotopically xenografted prostate cancer 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 RNA (scr). A total of 2 × 10^5 sorted CD133+ S/P cells were orthotopically xenografted into anterior prostates of nude mice, with 12 mice each group. There were 7 mice in the scr group and 6 mice in the shTR4 group that developed primary tumor. In the scr group, 4 out of 7 mice developed metastatic sites include liver, diaphragm, and lung. In the shTR4 group, only one mouse out of six developed lung metastasis. Both of the lung metastasis in the two groups can only be seen under IVIS scan. Tumor samples were collected for IHC staining (D) with quantitative data (E).

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 with vehicle control (Fig. 6D and 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 prostate cancer S/P cell invasion via regulation of EZH2 signals.

Discussion

Accumulating evidence shows that CD133 is an important S/P marker in prostate cancer (10, 17, 29). Vander and colleagues (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 days. More
importantly, the increased CD133⁺ cell population led to metastasis and poor prognosis in prostate cancer patients (21, 22). Here, we further proved that TR4 could alter the prostate cancer 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 prostate cancer CD133⁺ S/P cells have higher TR4 expression, and that may be important in mediating prostate cancer biologic progression. Yang and colleagues also found that TR4 was highly expressed in PCSC cells, the established PCSC line from Celprogen, as compared with C4-2 CD133⁻ cells (11). Upregulation 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 prostate cancer S/P cell invasion.

Recent studies indicated that EMT might play key roles in CSC function. Both EMT and CSCs play critical roles in tumor metastasis and they have biologic 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 and colleagues found that TR4 could promote prostate cancer metastasis via the CCL2/CCR2 signaling, the key player of EMT (16). In this study, we further found that TR4 could promote prostate cancer CD133⁺ S/P cell invasion via regulation of EZH2, which is important in both CSCs and EMT. These results therefore suggest that TR4 may promote prostate cancer metastasis through both the EMT-dependent pathway and CD133⁺ S/P cells.

In this study, we demonstrated that TR4 might promote prostate cancer S/P cell invasion. This is in contrast with an early study showing that TR4 might suppress prostate cancer initiation (6), suggesting that TR4 might play dual roles with a suppressor role in prostate cancer initiation and a promoter role in prostate cancer S/P cell invasion. Mechanism dissection suggested that TR4 might inhibit prostate cancer initiation via positively regulating DNA damage repair-related gene ATM, thus enhancing DNA repair to prevent tumorigenesis. In contrast, TR4 could also promote prostate cancer 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 versus tumor metastasis in prostate cancer (44). Furthermore, BRCA1 was reported as a tumor suppressor (45) in prostate cancer initiation but confers radiation resistance to prostate cancer (46).

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

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

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Zhu, D.-R. Yang, G. Li, Y. Shan, C. Chang Development of methodology: J. Zhu Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhu, X. Qiu Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhu, X. Qiu, C. Chang Writing, review, and/or revision of the manuscript: J. Zhu, H.-C. Chang, Y. Shan, C. Chang Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Shan, C. Chang Study supervision: D.-R. Yang, Y. Sun, X. Qiu, Y. Shan, C. Chang

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