RESEARCH ARTICLE

Triptolide Inhibits Histone Methyltransferase EZH2 and Modulates the Expression of Its Target Genes in Prostate Cancer Cells

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Abstract

The histone methyltransferase EZH2 (enhancer of zeste homolog 2) plays critical roles in prostate cancer (PCa) development and is a potential target for PCa treatment. Triptolide possesses anti-tumor activity, but it is unknown whether its therapeutic effect relates with EZH2 in PCa. Here we described EZH2 as a target for Triptolide in PCa cells. Our data showed that Triptolide suppressed PCa cell growth and reduced the expression of EZH2. Overexpression of EZH2 attenuated the Triptolide induced cell growth inhibition. Moreover, Triptolide treatment of PC-3 cells resulted in elevated mRNA levels of target genes (ADRB2, CDH1, CDKN2A and DAB2IP) negatively regulated by EZH2 as well as reduced mRNA levelsan of EZH2 positively regulated gene (cyclin D1). Our findings suggest the PCa cell growth inhibition mediated by Triptolide might be associated with downregulation of EZH2 expression and the subsequent modulation of target genes.

Keywords: EZH2 - EZH2 target genes - prostate cancer (PCa) - Triptolide

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Introduction

Prostate cancer (PCa) is the second most diagnosed type of cancer in males worldwide and its overall incidence shows an upward trend in China (Jemal et al., 2011; Ren et al., 2013). Androgen depletion has long been advocated for PCa treatment. However, the development into more aggressive hormone-independent PCa during the hormone therapy has raised concerns over its effectiveness, thus urging for the exploration of new strategies for the treatment of PCa (Feldman et al., 2001). Recent studies have found that deregulated epigenetic modifications including histone methylation play important roles in the onset and progression of numerous cancers (Ellis et al., 2009). The histone methyltransferase EZH2 (enhancer of zeste homolog 2) has become a novel target for research on cancer treatment (Schulz et al., 2006; Ellis et al., 2009). EZH2 performs its epigenetic role via regulation of histone methylation, and functions as the sole catalytic core subunit of the polycomb repressive complex 2 (PRC2), which catalyses the trimethylation of histone3 on lysine 27 (H3K27me3) and is implicated in gene repression. EZH2 serves in many fundamental biological processes, including embryonic development (O'Carroll et al., 2001), X chromosome inactivation (Plath et al., 2003), B cell development (Su et al., 2003), actin polymerization mediated T cell activation (Su et al., 2005), maintenance of circadian clock function (Etchegaray et al., 2006), cell differentiation (Gil et al., 2005), senescence(Kamminga et al., 2006) and transcriptional activation (Shi et al., 2007). Increasing evidences imply that deregulation of EZH2 closely correlates with tumors. Indeed, EZH2 is highly expressed in a variety of tumors (Chase et al., 2011). Knockdown of EZH2 expression shows cell cycle arrest and cell proliferation inhibition, while ectopic expression of EZH2 induces transcriptional repression and results in increased proliferation and advanced oncogenic capacity (Saramäki et al., 2006; Chang et al., 2012). Several tumor suppressor genes are described as direct targets of EZH2 (Yu et al., 2007; Min et al., 2010; Zhao et al., 2010). And it is demonstrated that overexpression of EZH2 is associated with multiple aspects of tumorigenesis, such as genomic aberrations, invasion, metastasis and angiogenesis (Saramäki et al., 2006; Sauvageau et al., 2010; Chase et al., 2011). Microarray studies have linked overexpression of EZH2 to PCa (Varambally et al., 2002). More than half of the hormone-refractory PCa possess increased copies of EZH2 gene, which correlates with high expression levels of EZH2 protein, whereas its amplification and overexpression is rare in early stage PCa, highlighting its potential function in PCa progression (Saramäki et al., 2006).

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The antitumor activity of Triptolide, one of the main active ingredients in the traditional Chinese medicine Tripterygium wilfordii Hook F, is well documented (Kiviharju et al., 2002; Phillips et al., 2007; Antonoff et al., 2009; Liu et al., 2009; Zhu et al., 2009; Wang et al., 2009; Zhao et al., 2010; Huang et al., 2012). Its antitumor activity involves a variety of cell signaling pathways and protein modification processes. Several targets of Triptolide have been described in a variety of tumors, such as the vascular endothelial growth factor (Zhu et al., 2009), NF-kappa B (Zhu et al., 2009), Hsp70 (Westerheide et al., 2006; Phillips et al., 2007; Antonoff et al., 2009). Triptolide also was proven to inhibit the expression of the SUMO-specific protease 1, the androgen receptor and c-Jun (Huang et al., 2012). As we have described above that EZH2 is a crucial mediator and play multiple roles in PCa, it is worth to consider that EZH2 might be one of the molecular targets by which Triptolide exerts its anti-PCa effect.

In this study, we showed that Triptolide suppressed the growth of PCa cells. Our data indicated that Triptolide inhibited the expression of EZH2 and modulated its downstream target genes in PCa cells, thus providing a novel insight into the anti-PCa effects of Triptolide.

Materials and Methods

Reagents

Triptolide was purchased from π-π Technologies (Shenzhen, China), dissolved in DMSO at a stock concentration of 50 mM and stored at 4°C. 10% Formaldehyde, 0.4% Trypan blue and 0.1% Crystal violet solutions were from Sigma (St. Louis, MO, USA). Thiazolyl Blue Tetrazolium Bromide (MTT) was from Invitrogen (Grand Island, NY, USA). RNAiso Plus Reagent, cDNA Reverse Transcription Kit and SYBR® PrimeScript™ RT-PCR Kit II were purchased from TaKaRa (Dalian, China). Antibody for EZH2 (#4905) was purchased from Cell Signaling Technology (Danvers, MA, USA), for β-actin (C4) was from Santa Cruz (Dallas, TX, USA). HRP conjugated goat anti-rabbit IgG or anti-mouse IgG were purchased from CoWin Biosciences (Beijing, China). ECL detection system was from Thermo scientific (Waltham, MA, USA).

Table 1. Oligonucleotide Sequences Used in This Study

Primer name	Primer sequence
EZH2 F1	GCCAGACTGGGAAGAAATCTG
EZH2 R1	TGTGTTGGAAAATCCAAGTCA
ß-actin F1	AATGTCGCGGAGGACTTTGAT
ß-actin R1	AGGATGGCAAGGGACTTCCTG
CDH1 F1	TGGAGAGACACTGCCAACTG
CDH1 R1	AGGCTGTGCCTTCCTACAGA
ADRB2 F1	CATGCGCCGGACCACGAC
ADRB2 R1	CATGCCCACCACCCACACCT
DAB2IP F1	CTCCCTCTCCAAGACAGCAG
DAB2IP R1	GGGAGGGGCAGATAAGCTAC
CDKN2A F1	CATAGATGCCGCGGAAGGT
CDKN2A R1	TCTCCAACTCAGGCTACCAG
cyclin D1 F1	CACGCGCAGACCTTCGTT
cyclin D1 R1	GCGGATTGGAAATGAACTTCA

Plasmid construction

EGFP-EZH2 construct was made by standard cloning method. Human EZH2 fragment was cloned in an XhoI and SmaI digested pEGFP-C1. The plasmid construct was confirmed by DNA sequencing.

Cell culture, transfection and drug treatment

PC-3 and LNCaP cells were cultured in RPMI1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Thermo Scientific) and penicillin/streptomycin at 37°C in a 5% CO_2 incubator. For transient transfection, cells were plated on a six well plate or 6-cm Petri dish to ~80% confluence and transfected with plasmids using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Cells were treated with Triptolide as described in individual experiments.

Cell proliferation assay

PC-3 and LNCaP cells were plated at 2×10^3 cells/well in 96-well plates and cultured until attachment, then treated with various doses of Triptolide for 24 h and 48 h, and DMSO was used as negative control. Cells proliferation inhibition was determined by MTT assay. The following formula was used: cell inhibition rate (%) =[1-(OD of the experimental samples/OD of the control)]×100%. All experiments were performed in triplicate.

Clonogenic assay

PC-3 cells were initially plated in 6-cm plates at different densities and after treatment with 0.1 μM Triptolide (DMSO as negative control) for 7 days, the colonies were fixed with a 10% formaldehyde solution and stained with a 0.1% crystal violet solution. The plates were photographed and colony formation was analyzed.

Quantitative real-time PCR

PC-3 or LNCaP cells were treated with various doses of Triptolide for 24h or with 0.1μM Triptolide for desired time. RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. 500ng total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (TaKaRa). The real-time PCR were performed on the Bio-Rad CFX 96 Real-time PCR system using SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa) and specific primers (Table 1). The mRNA level of each gene was normalized to β-actin with the ΔΔCT method using Bio-Rad CFX Manager V1.1.308.1111 software. The relative mRNA level for each gene was calculated by dividing its normalized expression in treated samples by that in untreated control sample.

Cell lysate and Western blot analysis

PC-3 cells were treated with various doses of Triptolide for 24h. Cells were collected and lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1% NP-40, 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride and 1× Roche protease inhibitor cocktail solution). After incubation on ice for 30 min, the lysates were cleared by centrifugation at 10,000 g for 10 min at 4°C and the protein concentrations were determined using Bradford

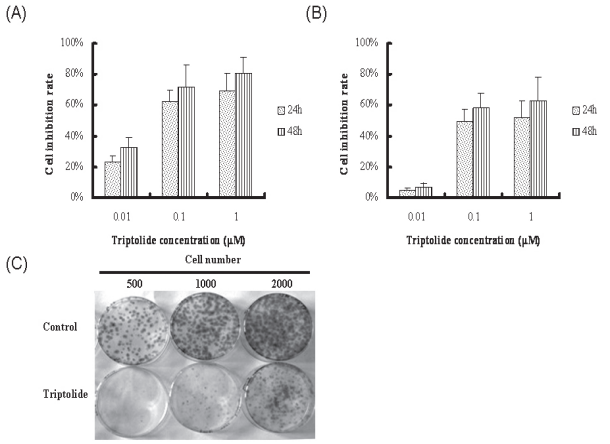

Figure 1. Triptolide Inhibits Cell Growth in PCa Cell Lines. LNCaP (A) or PC-3 cells (B) were treated with the indicated doses of Triptolide for 24 and 48h, respectively. The inhibition on cell growth was observed by MTT assay. All assays were performed in triplicate, and values present as the mean ± SD of three independent experiments. (C) Effect of Triptolide on PC-3 colony formation. PC-3 cells were plated at different densities and treated with 0.1μM Triptolide for 7 days. The clone formation was assessed with the Crystal violet staining

assay (Bio-Rad, Hercules, CA, USA). Proteins were denatured by boiling in 6×SDS sample buffer for 5min at 95°C and subjected to SDS-PAGE followed by Western blotting. Protein blots were probed with the indicated primary antibodies and appropriate secondary antibodies and protein bands were visualized using the ECL system.

Statistical analysis

Results were expressed as the mean ± SD. t test was applied to evaluate the differences between groups. For all the tests, the level of significance was set at $P < 0.05$.

Results

Triptolide inhibits the growth of PCa cells

The inhibitory activity of Triptolide on various cancer cells growth has been studied (Kiviharju et al., 2002; Phillips et al., 2007; Antonoff et al., 2009; Liu et al., 2009; Zhu et al., 2009; Wang et al., 2009; Zhao et al., 2010). Here we determined the effect of Triptolide on the growth of PCa cells. LNCaP cells or PC-3 cells were treated with different doses of Triptolide (0.01 μM, 0.1 μM and 1 μM) for 24h and 48h. As depicted on Figure 1, a dose-dependent inhibition of PCa cell growth was achieved following the treatment of Triptolide in both LNCaP cells (Figure 1A) and PC-3 cells (Figure 1B); and the inhibition rate of cell growth increased with the extension of incubation time as well. It also showed that Triptolide had stronger inhibitory effect on LNCaP cells relative to PC-3 cells.

To further investigate the inhibitory effect of Triptolide on PCa growth, we performed clonogenic assay with PC-3 cells. As shown on Figure 1C, 0.1 μM Triptolide treatment for 7 days resulted in an extremely reduction of colony formation in comparison with the control group, and the colonies formed in the presence of Triptolide were also much smaller, thus showing Triptolide inhibited the growth of PC-3 cells.

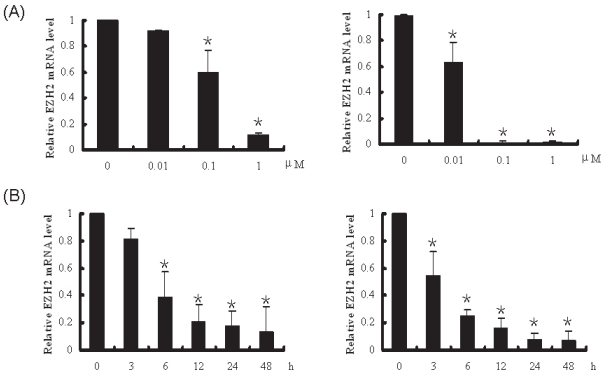

Figure 2. Effects on the EZH2 mRNA of PCa Cells Treated with Triptolide. (A) Triptolide decreased EZH2 mRNA level in a dose-dependent manner in both PC-3 (left panel) and LNCaP cells (right panel). Cells were treated with the indicated doses of Triptolide for 24 h. Total RNA was isolated and EZH2 and ß-actin (used as an internal control) mRNA levels were analyzed by qRT-PCR. (B) Triptolide decreased EZH2 mRNA level in a time-dependent manner in both PC-3 (left panel) and LNCaP cells (right panel). Cells were treated with 0.1μM Triptolide for the desired times. EZH2 and ß-actin mRNA levels were detected by qRT-PCR. Quantification results of EZH2 mRNA level were shown as folds of control and expressed as the mean ± SD (n=3). *$P < 0.05$

Taken together, our data suggest that Triptolide efficiently inhibits the growth of PCa cells.

Triptolide decreases EZH2 mRNA level in PCa cells in a dose- and time-dependent manner

As EZH2 is overexpressed and plays crucial roles in PCa (Varambally et al., 2002; Saramäki et al., 2006), we checked whether Triptolide could inhibit its expression on mRNA level in PCa cells. After treatment with different doses of Triptolide for 24h, total RNA from PC-3 or LNCaP cells were extracted and EZH2 mRNA level was analyzed by quantitative real-time PCR. As showed on Figure 2A (left panel), the treatment of PC-3 cells with 0.01 μM Triptolide had no obvious effect on the EZH2 mRNA level, meanwhile, a dose-dependent inhibition was observed in the 0.1 μM and 1 μM treatment groups, which significantly decreased the mRNA level to 59.7±17.3% and 11.6±1.8% compared with the control group ($P < 0.05$), respectively. Similarly, increasing doses of Triptolide resulted in enhanced inhibitory effect on the EZH2 mRNA levels in LNCaP cells, which decreased to 63.6±14.9%, 1.9±0.7% and 2.2±0.5% in the 0.01μM, 0.1μM and 1μM treatment groups relatively to their corresponding control group (Figure 2A, right panel).

To further confirm that the Triptolide-induced downregulation of EZH2 mRNA level was a time-dependent process, PC-3 or LNCaP cells were incubated with 0.1 μM Triptolide for desired time. Quantitative real-time PCR results were shown in Figure 2B. Although the treatment with 0.1 μM Triptolide for 3h resulted in no significant reduction on the EZH2 mRNA level in PC-3 cells, the inhibition rate of EZH2 mRNA levels increased with the prolonging of the treatment. After 6h or more, Triptolide pronouncedly decreased the levels of EZH2 mRNA compared with the control group (Figure 2B, left panel) ($P < 0.05$). In addition, similar results were obtained

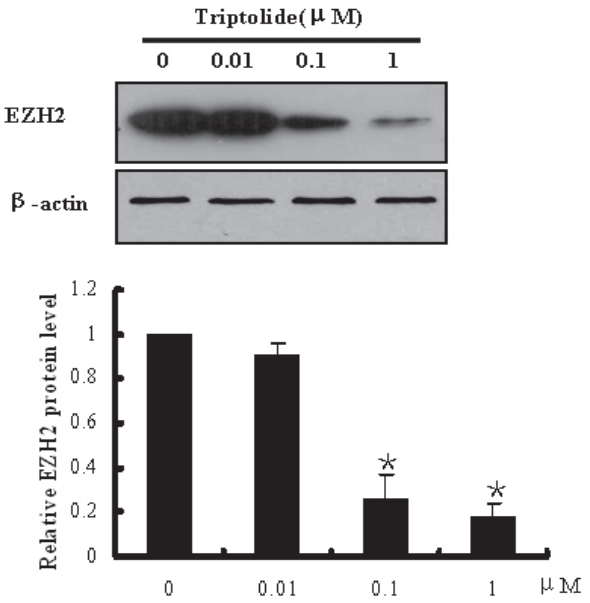

Figure 3. Triptolide Suppresses EZH2 Protein Expression in PCa Cells. PC-3 cells were treated with the indicated doses of Triptolide for 24h. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western blot with the indicated antibodies. β-actin was used as a loading control. The signal intensity of the indicated bands was evaluated and the data were shown as folds of control and expressed as the mean ± SD (n=3). *$P < 0.05$

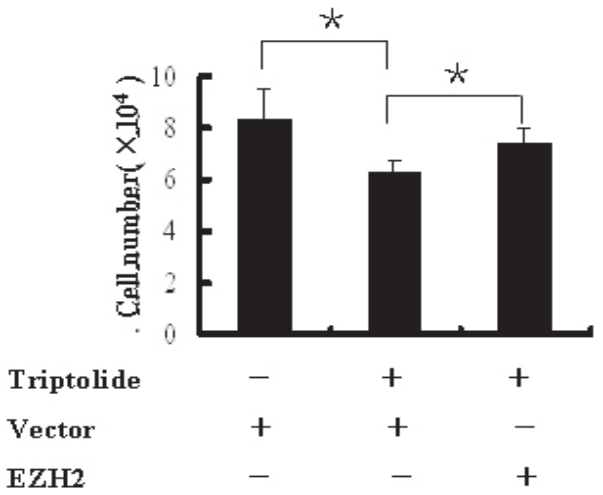

Figure 4. Overexpression of EZH2 Attenuates the Inhibitory Effect on PC-3 Cell Growth of Triptolide. PC-3 cells were transfected with EGFP-EZH2 or vector. Forty-eight hours after transfection, cells were incubated with or without 0.1 μM Triptolide for another 24 h and cell number was counted. Cell number values present as the mean ± SD of three independent experiments. *$P < 0.05$

with Triptolide treated LNCaP cells (Figure 2B, right panel), although unlike for PC-3 cells, 0.1 μM Triptolide treatment for 3h significantly reduced the EZH2 mRNA level ($P < 0.05$).

All together, these results show that Triptolide can downregulate EZH2 expression at the transcription level in both PCa cell lines in a dose- and time-dependent manner.

Triptolide decreases EZH2 protein level in PC-3 cells

As we have showed that Triptolide decreased the expression of EZH2 at mRNA level, we therefore investigated whether Triptolide could inhibit the expression of EZH2 at the protein level. PC-3 cells were treated with various doses of Triptolide for 24 h, and cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting analysis. As depicted by Figure 3, Triptolide significantly decreased the EZH2 protein level in a dose-dependent manner, confirming the inhibitory effect of Triptolide on EZH2 protein expression in PC-3 cells.

EZH2 is involved in Triptolide mediated growth inhibition of PC-3 cells

Since we observed that Triptolide inhibited PCa cell growth and suppressed EZH2 expression at both transcriptional and translational levels, to address if the inhibitory effect on PCa cell growth induced by Triptolide relies on EZH2, we analyzed the cell growth after Triptolide treatment in PC-3 cells with overexpression of EGFP tagged EZH2. PC-3 cells were transfected with EGFP-EZH2 or vector. Forty-eight hours later, cells were incubated with or without 0.1μM Triptolide for another 24h and cell number was counted. As shown in Figure 4, Triptolide treatment led to significantly decreased cell number compared with control group ($P < 0.05$); EZH2 overexpression rescued the decline in cell growth and significantly reversed Triptolide-induced cell growth inhibition ($P < 0.05$). These results suggest that EZH2 may play a functional role in growth inhibition of PC-3 cells.

Triptolide modulates EZH2 downstream target genes mRNA expression in PC-3 cells

EZH2 has been previously reported to silence a number of target genes, and therefore plays a driving role in many aspects of the cancer development (Yu et al., 2007; Min et al., 2010; Yu et al., 2010; Zhao et al., 2010). For example, EZH2 can bind and silence the CDKN2A (cyclin-dependent kinase inhibitor 2A) tumor suppressor locus (Wu et al., 2011). EZH2 also inhibits the expression of CDH1 (E-cadherin), to promote tumor progression and metastasis (Cao et al., 2008). Through silencing of DAB2IP gene, EZH2 triggers prostate tumorigenesis and metastasis (Min et al., 2010). EZH2 can suppress the expression of the β-2 adrenergic receptor (ADRB2) to induce cell invasion and prostate epithelial cells transformation (Yu et al., 2007). We postulated that EZH2 downregulation by Triptolide would lead to increased mRNA levels of these genes which were negatively regulated by EZH2. To explore the consequences of Triptolide treatment on the expression of EZH2 downstream target genes, PC-3 cells were treated with 0.1μM Triptolide for 24h, total RNA were extracted and analyzed by quantitative real-time PCR to monitor the changes in the expression level of the indicated genes. As shown in Figure 5A, compared with the control group, Triptolide treatment led to a significant increase on the mRNA levels of these four target genes negatively regulated by EZH2 ($P < 0.05$), supporting that Triptolide induced the expression of these EZH2 target genes. On the other hand, EZH2 also functions as a transcription activator (Shi et al., 2007). To further evaluate the effect of Triptolide on EZH2 function, cyclin

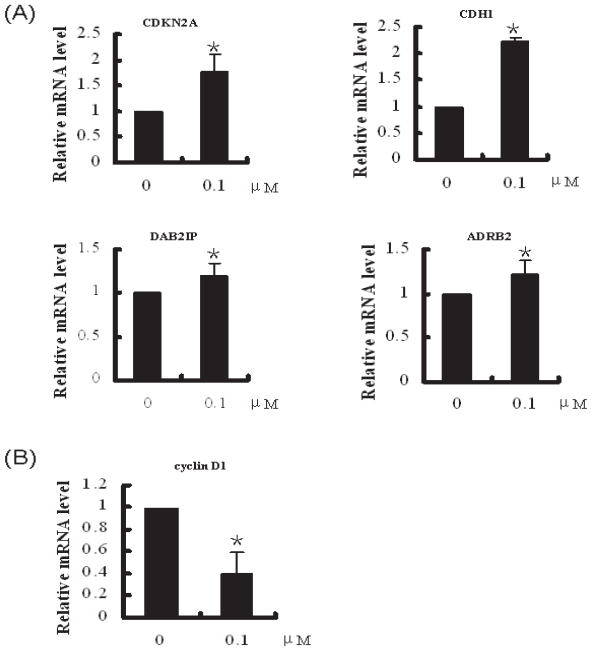

Figure 5. Triptolide Modulates the Expression of EZH2 Target Genes. PC-3 cells were treated with 0.1μM Triptolide for 24h. Total RNA was isolated and qRT-PCR was performed with specific primers for the indicated target genes that are either transcriptionally repressed (A) or activated (B) by EZH2. Quantification results were shown as folds of control and expressed as the mean ± SD (n=3). *$P < 0.05$

D1, the known transactivated gene by EZH2 (Shi et al., 2007), was investigated. Contrary to what was observed with EZH2-negatively regulated genes, the treatment of PCa cells with Triptolide led to a drastic reduction on the cyclin D1 mRNA level ($P < 0.05$) (Figure 5B). Together, these results indicate that EZH2 target genes are modulated by Triptolide, suggesting Triptolide alters the function of EZH2.

Discussion

Chinese medicinal herbs have been an invaluable source for the discovery and development of therapeutic agents. As one major active component of Tripterygium wilfordii Hook F, Triptolide possesses various biological functions (Wang et al., 2011). Consistent with previous studies that Triptolide showed antitumor properties by suppressing cell growth (Zhu et al., 2009; Zhao et al., 2010), our data demonstrate Triptolide exerted an inhibitory effect on PCa cell growth (Figure 1). Interestingly, compared with PC-3 cells, LNCaP cells showed more sensitivity to Triptolide since higher cell inhibition rate was achieved, which might be due to its intrinsic characteristics. Although several targets involved in the antitumor activity of Triptolide have been described (Kiviharju et al., 2002; Phillips et al., 2007; Antonoff et al., 2009; Liu et al., 2009; Zhu et al., 2009; Wang et al., 2009; Zhao et al., 2010; Huang et al., 2012), its mechanism of action is still not fully elucidated. EZH2 has offered a potential target for Triptolide in anti-PCa treatment. Overexpression of EZH2 occurs in PCa (Varambally et al., 2002; Saramäki et al., 2006). EZH2 plays crucial roles in the epigenetic control of tumorgensis (Chase et al., 2011); changed epigenetic modifications of genes might play as important roles as genetic mutations in tumorigenesis and are reversible and preventable (Ellis et al., 2009). In the present study, Triptolide displayed potent inhibitory effect on EZH2 expression at both mRNA and protein levels in PCa cells (Figure 2 and 3). Furthermore, EZH2 overexpression rescued the significant inhibition on cell growth mediated by Triptolide (Figure 4). These data revealed that Triptolide exerted its cell growth inhibitory effect via EZH2. Besides blocking cell growth, EZH2 depletion inhibits metastasis and suppresses angiogenesis (Fussbroich et al., 2011). So Triptolide mediated EZH2 downregulation represents an attractive strategy for therapeutic interventions in PCa. Numerous studies suggested that the expression of EZH2 was modulated at the transcriptional, posttranscriptional and post-translational levels (Chang et al., 2012). Recent works reported that Triptolide bound to a subunit of the transcription factor TFIIH (XPB) and induced degradation of the subunit of RNA polymeraseII (Rpb1) (Titov et al., 2011; Wang et al., 2011), resulting in transcriptional inhibition. Future work is needed to elucidate whether these novel mechanisms contribute to the effect of Triptolide on EZH2 and to provide more details on how Triptolide downregulates the expression of EZH2.

EZH2 is generally related with transcriptional repression (Chase et al., 2011). Through trimethylating histone in the promoter regions of tumor suppressor genes, EZH2 involves in transcriptional repression of these genes. While EZH2 is highly expressed in a wide range of cancer types (Chase et al., 2011), it is believed that inappropriately silencing tumor suppressor genes by overexpression of EZH2 contributes to tumorigenesis. So, we investigated if Triptolide treatment could affect these EZH2 regulated downstream genes and involve in epigenetic regulation in PC-3 cells. Indeed, EZH2 target genes were obviously transcriptionally derepressed by Triptolide treatment (Figure 5A), indicating that Triptolide restored the EZH2-mediated epigenetic silencing of target genes expression and lower EZH2 level induced by Triptolide was probably responsible for it. In addition, in contrary to its established role as a transcription repressor, EZH2 can transactivate cyclin D1 gene (Shi et al., 2007). Our data revealed that Triptolide markedly reduced cyclin D1 expression (Figure 5B), and further confirmed that Triptolide could alter the functions of EZH2. Since these indicated genes function variously in cancer, their modulation would thus be consistent with anti-PCa effects of Triptolide. However, additional studies are needed to address the exact mechanisms how EZH2 downregulation induced by Tripolide alters the expression of these genes as well as the following consequences of modulating these target genes.

Based on what we observed, we suppose that the inhibition on PCa cell growth by Triptolide is associated with the downregulated expression of EZH2 which subsequently modulates its target genes expression. In view of previous studies (Liu et al., 2009; Titov et al., 2011; Huang et al., 2012), we can not state certainly that EZH2 inhibition is the sole mechanism of action that results in cell growth inhibition; however, considering

the importance of EZH2 in PCa (Varambally et al., 2002; Saramäki et al., 2006), downregulation of EZH2 is probably a novel mechanism for Triptolide. To date, few EZH2 pharmacological inhibitors have been developed. Our results indicate Triptolide acts as an EZH2-targeting inhibitor in PCa cell lines. Recent work has reported that 3-deazaneplanocin (DZNep), a widely used but not specific EZH2 inhibitor that reduces EZH2 protein level but not mRNA level, was shown to inhibit PCa cell growth and reduce tumor formation (Chase et al., 2011; Crea et al., 2011). In order to further assess the specificity of EZH2 inhibition by Triptolide, it will be of interest to make a comparison with DZNep.

In summary, our results demonstrate that Triptolide suppressed PCa cell growth accompanied by potent inhibition of EZH2 expression and modulation of its downstream target genes. This study expands a possible mode of action of Triptolide in anti-PCa treatment.

Acknowledgements

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