RESEARCH ARTICLE

Triptolide Promotes Senescence of Prostate Cancer Cells Through Histone Methylation and Heterochromatin Formation

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Abstract

Background: Triptolide is a medicinal herb-derived diterpene triepoxide with potent anti-tumor activity, mainly ,correlated with its ability to inhibit and inactivate subunits of RNA polymerase II, thereby suppressing global gene transcription. Epigenetic imbalance including histone methylation are well known to play important roles in prostate cancer (PCa) onset and progression. The goal of this study was to investigate whether triptolide might exert anti-PCa influence by reshaping the histone methylation landscape. **Methods:** Triptolide-treated PCa cell lines were analyzed by RT-qPCR and western blotting for expression of histone demethylases and associated markers. Detection of senescence was achieved using senescence associated β-galactosidase staining and analyses of apoptosis and cell cycle were performed by flow cytometry. Senescence–associated heterochromatin foci were detected by immunofluorescence while chromatin immunoprecipitation associated with qPCR (CHIP-qPCR) was applied to assess accumulation of histone markers on promoters of target genes. Cell viability was determined using the CCK-8 assay. **Results:** We found triptolide to enhance H3K27me3 levels by down-regulating JMJD3 and UTX and also H3K9me3 through up-regulation of SUV39H1. Furthermore, it up-regulated expression of HP1α. Thereby, heterochromatin formation and deposition on promoters of E2F1-target genes was promoted, correlating with suppression of gene transcription, decreased cell viability and induction of a senescence-like phenotype in PCa cells. **Conclusions:** Our results indicate that triptolide exerts anti-tumor effects including PCa cell senescence at least partially through increasing the levels of repressive histone H3 methylation and formation of a repressive chromatin state in PCa cells. Further studies of its potential as an epigenetic anti-PCa drug appear warranted.

Keywords: Triptolide- prostate cancer- senescence- Histone- Methylation- Heterochromatin

Asian Pac J Cancer Prev, **18 (9)**, 2519-2526

Introduction

Post-translational modification of histones are reversible and inheritable changes that affect gene expression in a sequence-independent manner (Feinberg, 2004). Histone methylation is one of the best characterized histone post-translational modifications and is reversely carried-out by histone methyltransferases (HMT) and histone demethylases (HDM) with high substrate specificity (Lennartsson and Ekwall, 2009). For instance, trimethylation of histone H3 on Lysine 27 (H3K27me3) is carried-out by the HMT Enhancer of zeste homolog 2 (EZH2) and reversed by the HDMs jumonji domain containing 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (UTX). Similarly, trimethylation of histone H3 on Lysine 9 (H3K9me3) is carried-out by the (suppressor of variegation 3-9 homolog 1) SUV39H1 amongst other HMTs and reversed by the JMJD2 family of HDMs. Histone methylation has multiple physiological functions, including gene transcription regulation, maintenance of genome integrity and epigenetic phenomena, which are dependent on the residue and the degree of methylation (Jenuwein and Allis, 2001; Kouzarides, 2007; Lennartsson and Ekwall, 2009; Mellor, 2005). Tri-methylation of H3 lysine 4 (H3K4me3) and H3 lysine 36 (H3K36me3) are associated with transcription activation; conversely, H3K27me3 H3K9me2/3 are particularly associated with transcription suppression. H3K9me2/3 also acts as a marker of heterochromatin. It serves as a binding site for heterochromatin protein 1 (HP1) and mediates chromatin condensation and heterochromatin formation, leading to transcription suppression. De-regulated H3K27me3 levels have been reported in pancreatic, ovarian cancer, breast cancer (Gao et al., 2014) and PCa (Kim and Roberts, 2016). Imbalanced histone methylation results from mutation or abnormal expression of HMTs and HDMs (Bhaumik et al., 2007; Waldmann and Schneider, 2013) including EZH2, JMJD3 and UTX (Hubner and Spector, 2010). These findings suggest abnormal histone

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methylation may be a potential target for tumor therapy.

Triptolide is an active diterpene triepoxide extracted from the medicinal herb Tripterygium wilfordii Hook F (Kupchan et al., 1972) and has shown potent anti-tumor effect against several kinds of cancer including PCa (Huang et al., 2012; Titov et al., 2011). To date, the molecular mechanisms underlying the anti-tumor activities of Triptolide include (a) inhibition of NF-κB pathway in anaplastic thyroid carcinoma (ATC) cells (Zhu et al., 2009); (b) inhibition of Heat shock protein 70 (HSP70) in Pancreatic cancer cells (Phillips et al., 2007); (c) global suppression of transcription through inhibition of the transcription factor TFIIH ATPase activity in human cervical cancer hela cell lines (Titov et al., 2011) and proteasome-mediated degradation of RPB1, the catalytic subunit of RNA polymerase II (Pol II) in human non–small cell lung cancer A549, leukemia KG1, colon cancer DLD1 (Vispe et al., 2009), human cervical cancer hela (Wang et al., 2011) human ovarian cancer SK-OV-3 and prostate cancer PC-3 cell lines (Manzo et al., 2012). These findings suggest that Triptolide perform its anti-tumor effect in multiple ways. In this paper we report a novel anti-tumor mechanism by which Triptolide induces H3K9me3 and H3K27me3 and promotes heterochromatin formation, leading to suppression of gene transcription and induction of a senescence-like phenotype in PCa cells.

Materials and Methods

Cell culture

PC-3 and LNCaP cells were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Cells were cultured in RPMI1640 (GIBCO) supplemented with 10% fetal bovine serum (Thermo scientific) and 100 units/ml penicillin and streptomycin, and maintained in an incubator at 37 °C with 5% CO_2.

Cell transfection

PC-3 cells were plated in 96-well plates (3×10^3 cells/well for cell viability assay), 6-well plates (2×10^6 cells/well for PCR, WB, Senescence) or 10 cm petri dishes (12×10^6 cells/dish for CHIP) until attached. 2 μg PCMV-HA-JMJD3 Plasmid (Addgene plasmid # 24167) or 50 pmol siRNAs for SUV39H1 and HP1α (GenePharma Co) (Table 1) were transiently transfected into cells using Lipofectamine 2000 reagent (Life technologies) according to the manufacturer's instructions. 48 h after transfection, cells were treated with various concentrations of Triptolide (π-π technologies) for another 24 h, then collected for downstream experiments.

Senescence Associated β-Galactosidase Staining

PC3 cells were seeded directly on coverslips in 6 wells plates at a density of 1×10^5cells/well and allowed to grow until 70-80% confluence. Cells were then treated with 100 nM Triptolide or DMSO for 24h and SA-β-Galactosidase activity was using the SA-β-Galactosidase staining kit (Beyotime) according to the manufacturer instructions. Senescent cells stain in blue. Image was recovered using a CKX41 inverted microscope fitted with a DP22 microscope digital camera (Olympus).

Quantitative real-time PCR

PC-3 cells were grown in 6-well plates overnight and treated with various doses of Triptolide for 24 h. Total RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. 500 ng total mRNA was reverse transcribed to cDNA using PrimeScript™ RT Master Mix (Perfect Real Time, Takara RR036A). Real-time PCR were performed on the Bio-Rad CFX 96 Real-time PCR system using SYBR® Premix Ex TaqTM II (Tli RnaseH Plus, TaKaRa DRR820) and specific primers (Table 1). Each analysis was performed in triplicate. 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 the untreated control sample.

Western blotting

PC-3 and LNCaP cells were treated with various doses of Triptolide for 24 h. Cells were collected and lysed with RIPA lysis buffer (Beyotime, P0013B), supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 1 × Roche protease inhibitor cocktail (4693116001). The protein concentration was determined using the BCA Protein Assay Kit (CWBIO, CW0014). Cell lysates containing equal amounts of protein were separated on SDS-PAGE gels, electro-transferred onto NC membranes and incubated with appropriate primary and secondary antibodies. Protein blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific 34080) and visualized with a ChemiDoc XRS+ imaging system (Bio-Rad, USA). β-actin or α-Tubulin antibody (AT819-1) was used as a loading control. HA tags antibody (CW0260) from CWBIO. Antibodies for EZH2 (#4905), H3 (#4499), JMJD3 (#3457), H3K27m3 (#9756), HRP-conjugated goat anti-mouse IgG (#7076) and anti-rabbit IgG (#7074) were from Cell Signaling Technology. Antibody for H3K9m3 (ab71999) was from abcam. Antibodies for Ki67 (sc15402), Rb1a (sc-73598) and β-actin (C4) were from Santa Cruz. Antibodies for SUV39H1 (10574-1-AP), HP1a (11831-1-AP), P16-INK4A (10883-1-AP), Cyclin A2 (18202-1-AP) were purchased from Proteintech.

Apoptosis and cell cycle analysis

PC-3 cells were cultured in 6-well plate and treated with Triptolide or DMSO for 24 h. For apoptosis analysis, cells were co-labeled with Annexin V (AV) and propidium iodide (PI) using the Annexin V-FITC Apoptosis Assay Kit (Beyotime , C1063), according to the manufacturer's instructions. For cell cycle analysis, cells were fixed with cold 70% ethanol and stained with PI using the DNA content Quantitation kit (KeyGEN, KGA512) following the manufacturer's protocol. The stained cells were analyzed using a Cyflow® Cube flow cytometer (PARTEC, Germany). Data were analyzed using FlowJO 7.6.5 software.

Immunofluorescence (IF)

PC-3 cells were grown on sterilized glass coverslips overnight and treated with 100 nM Triptolide or DMSO for 24 h. Cells on coverslips were fixed with 4% paraformaldehyde solution, blocked with 1% BSA in PBS, incubated with appropriate primary antibody overnight and Alexa Flour 555-conjugated goat anti-rabbit secondary antibody (IgG) for 1 h, then stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime , C1005), and transferred onto a slide. Images were captured using a BX51+DP70 fluorescence microscope (Olympus, Japan).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using a ChIP Assay Kit (Beyotime P2078), according to the manufacturer's instruction. In brief, PC-3 cells were transfected with SUV39H1 or non-specific control shRNAs for 48 h, then treated with Triptolide for another 24 h. Immunoprecipitation was performed as per the manufacturer's instruction. Immunoprecipitated DNA was further analyzed by real-time PCR using a SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa) and specific primers (Table 1). Each analysis was performed in triplicate.

Cell viability assay

PC-3 cells were plated in 96-well plates at a density of 3×10^3 per well until attached. Cells were transfected as described above, then treated with 100nM Triptolide or DMSO (negative control) for 24h. 10 μl cell counting kit-8 (CCK-8) solution (Beyotime, C0038), was added per well and the plate was incubated for 1 h at 37°C. The absorbance of each well was measured on an M200pro Multimode Plate Reader (Tecan) at 450 nm and 630 nm. Each treatment was performed in triplicate and experiments were repeated 3 times.

Statistical analysis

SigmaPlot - scientific data analysis and graphing software version 9 was used for data analysis and results were expressed as the mean ± SD. Student's 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 enhances H3K27me3 level by down-regulating the histone demethylases JMJD3 and UTX

In order to investigate the effect of Triptolide on histone methylation in PCa cells, PC-3 cells were treated with different doses of Triptolide and total protein was analyzed by western blotting. We found that Triptolide treatment decreased EZH2 protein level but unexpectedly, increased the levels of H3K27me3 and histone H3 in PC-3 cells in a dose-dependent manner (Figure 1A). We also found that Triptolide significantly reduces UTX and JMJD3 mRNA (Figure 1B) in a dose-dependent manner and protein levels in a dose- and time-dependent manners in LNCaP and PC-3 cells (Figure 1C and 1D). We next transfected PC-3 cells with an HA-tagged JMJD3 expression plasmid for 48 h, then treated with 100 nM Triptolide for another 24 h

Table 1. Sequences of Primers and Small Interfering RNAs Used in This Study

Genes	Forward 5'- 3'	Reverse 5'- 3'
Primers for real-time PCR		
ß-actin	AATGTCGCGGAGGACTTTGAT	AGGATGGCAAGGGACTTCCTG
RB1	TCACCTTGAATCTGCTTGTCC	TGGAGATCTTACAGGAGAAAGATACA
CCNA2	TGAAGAGGCAACCAGACATCAC	AGCCAAATGCAGGGTCTCAT
CDKN2A	CATAGATGCCGCGGAAGGT	TCTCCAACTCAGGCTACCAG
CDK2	GCGAATTCCCCAGCCCTAATCTCA	GCCTCGAGAACCCTCTTCAGCAATAA
JMJD3	GGAGGCCACACGCTGCTAC	GCCAGTATGAAAGTTCCAGAGCTG
UTX	TTTGTCAATTAGGTCACTTCAACCTC	AAAAAGGCAGCATTCTTCCAGTAGTC
SUV39H1	GCAGCATACTCAATGAGCAGA	GTGGGATCCCCGTTGGAAAT
HP1a	ACTGGGGCGATCCGGTAGGT	AGGCCACCAGGTCC CTTGGA
BCL2	ACCGGGAGATGTCGCCCCTGG	TCCCACCAGGGCCAA ACTGAGC
Cyclin E1	ACTTGGCTCTGCCTACGGGGG	CACACTGGTCCCTCGCCGTCC
NFKB1	CGGACTCGCCACCCGGCTTC	TGGGCCATCTGCTGTTGGCAGT
Primers for ChIP		
GAPDH	TACTAGCGGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA
Cyclin E1	TTTTGCCGCTCCAGCGCCGCTCG	GCAGGGACGGGGAATCAGGGGCGG
BCL2	CGGCCTCTGTTTGATTTCTCCTGGC	TGTGGCCCAGATAGGCACCCAG
SENP1	GCCAGAGTGCAAATGGCCAATGGAG	TGGGGCTCAAAAGACTTCGACGACA
NFKB1	CCTCCGTGCTGCCTGCGT	CGAGAGAGCATACAGACAGACGGACAC
Small interfering RNAs		
Non-specific control	UUCUCCGAACGUGUCACGUTT	
SUV39H1	ACCUCUUUGACCUGGACUATT	
HP1α	GGCUUUUCUGAGGAGCACATT	

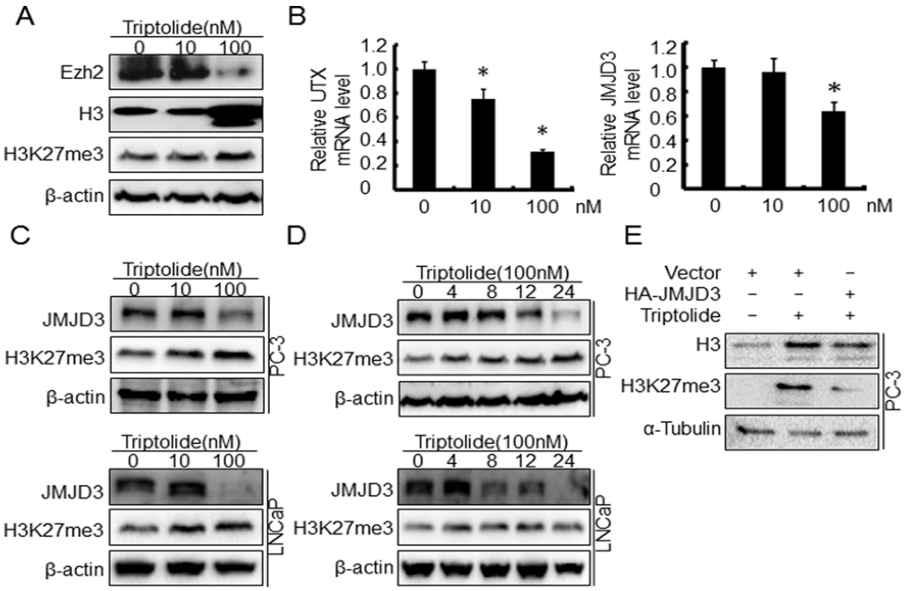

Figure 1. Triptolide Enhances H3K27me3 by Down-Regulating JMJD3 and UTX in PCa Cells. (A) Triptolide down-regulates EZH2 expression yet enhances H3K27me3 level. PC-3 and LNCap cells were treated with the indicated doses of Triptolide and analyzed by western blotting. β-actin was used as a loading control. (B) Triptolide decreases JMJD3 and UTX mRNA levels. PC-3 cells were treated with the indicated doses of Triptolide for 24 h, then JMJD3 and UTX mRNA levels were determined by real-time PCR with specific primers. * indicates $P < 0.05$. (C) and (D) Triptolide decreases the JMJD3 protein level in dose-dependent (C) and time-dependent (D) manners, and enhances the H3K27me3 level. PC-3 cells were treated with the indicated doses of Triptolide and analyzed by western blotting. β-actin was used as a loading control. (F) Ectopic expression of JMJD3 attenuates the Triptolide-induced accumulation of H3K27me3. PC-3 cells were transfected with an HA-JMJD3 expression plasmid or blank vector for 24 h, then treated with 100 nM Triptolide for an additional 24 h. Cells were collected and analyzed by western blotting. α-Tubulin was used as a loading control.

and analyzed the level of H3K27me3. Ectopic expression of JMJD3 partially attenuates the Triptolide-induced enhancement of H3K27me3 (Figure 1E), suggesting that Triptolide induces H3K27me3 accumulation through down-regulation of JMJD3 and UTX.

Triptolide induces heterochromatin formation in PCa cells

We next examined the effect of Triptolide on H3k9me3, another histone repressive mark. LNCaP and PC-3 cells were treated with various concentrations of Triptolide for 24 h and total RNA and protein were analyzed by real-time PCR and western blotting respectively. Triptolide significantly increased the protein level of SUV39H1 and associated H3K9me3 in LNCaP and PC-3 cells in both dose- and time-dependent manners (Figure 2A and 2B).Triptolide also increases SUV39H1 mRNA level in PC-3 cells (Figure 2C). We next knocked down SUV39H1 in PC-3 cells using a specific siRNA, then treated the cells with Triptolide. Western blot analysis show that knock-down of SUV39H1 attenuates Triptolide-induced enhancement of H3K9me3. Thus, Triptolide enhances H3K9me3 level through up-regulation of SUV39H1. Triptolide also increased significantly HP1α mRNA level (Figure 2C) and protein level in a dose- and time-dependent manner (Figure 2A and 2B). These results suggest that Triptolide induces heterochromatin formation in PCa cells.

Triptolide induces a senescence-like phenotype in PC-3 cells

Formation of heterochromatin involves in oncogene- and drug-induced cell senescence. Therefore, we investigated the effect of Triptolide on senescence markers. PC-3 cells were treated with 100 nM Triptolide for 24h and the activity of senescence-associated-β-galactosidase (SA-β-gal) was assessed. As shown on Figure 3A, Triptolide increased the number of SA-β-gal positive PC-3 cells (stained blue) when compared to the DMSO-treated group. Further, Triptolide induced the formation of nuclear structures resembling heterochromatic foci enriched with H3K9me3 in PC-3 cells (yellow arrows, Figure 3B) as revealed by immunofluorescent staining. We also performed flow cytometric analysis of cellular DNA in PC-3 cells treated with 100 nM Triptolide for 24h. Triptolide significantly increased the accumulation of cells in S phase (Figure 3C). We then used real-time PCR and immunoblotting to examine changes in expression of some molecular markers of senescence in cells treated with Triptolide for 24 h. The p53 pathway and the pRB1 pathway are two major effector pathways through which cell-cycle regulatory proteins induce cellular senescence. Since PC-3 is a p53-null cell line, we mainly investigated the pRB1 pathway. As shown in Figure 3D, the mRNA levels of RB1 and CDKN2A (p16) were significantly up-regulated by Triptolide, and the mRNA levels of CCNA2 and CDK2 were significantly down-regulated. Furthermore, the protein levels of RB1 and p16 were up-regulated by Triptolide, and the protein levels of Cyclin A2 and the cell growth marker Ki67 were down-regulated (Figure

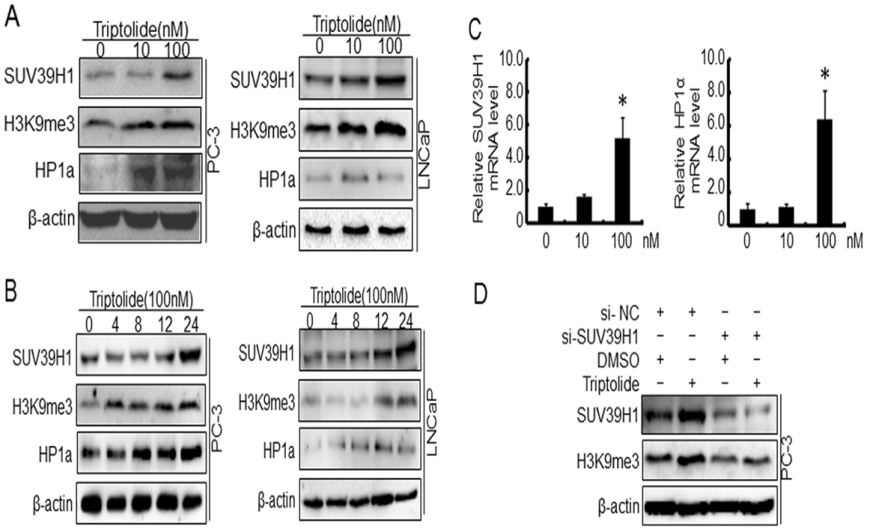

Figure 2. Triptolide Induces Heterochromatin Formation in PCa Cells. (A) and (B) Triptolide up-regulates SUV39H1 and HP1α expression in dose-dependent (A) and time-dependent (B) manners, and enhances the H3K9me3 level. PC-3 and LNCap cells were treated with the indicated doses of Triptolide and analyzed by western blotting. β-actin was used as a loading control. (C) Triptolide increases SUV39H1 and HP1-alpha mRNA levels. PC-3 cells were treated with the indicated doses of Triptolide, and analyzed by real-time PCR with specific primers. * indicates P <0.05. (D) Knock-down of SUV9H1 attenuates the Triptolide-induced accumulation of H3K9me3. PC-3 cells were transfected with SUV9H1 siRNA or non-specific control for 24 h then treated of 100 nM Triptolide for an additional 24 h. Cells were collected and analyzed by western blotting. β-actin was used as a loading control.

3E). Together, these data indicate that Triptolide induces a senescence-like phenotype in PC-3 cells through activation of the pRB1 pathway.

Triptolide-induced cellular effects is mediated through formation of a repressive chromatin state

We examined whether heterochromatin formation contributes to other cellular effect of Triptolide. E2F1-responsive genes are regulated by accumulation of H3K9me3. Therefore, we performed chromatin immunoprecipitation assays and found that Triptolide significantly induces deposition of H3K9me3 on the promoters of E2F1 target genes as compared to the DMSO treated group (Figure 4A). Meanwhile, siRNA

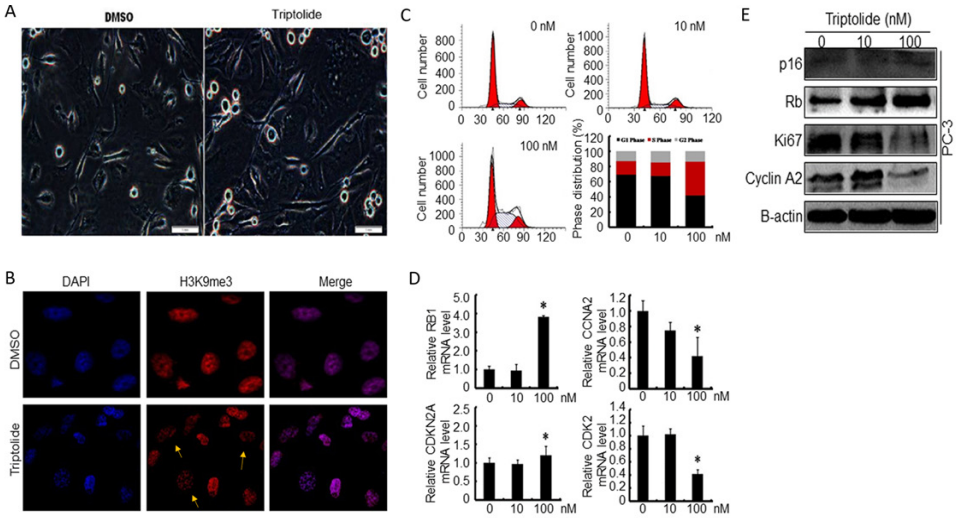

Figure 3. Triptolide Induces a Senescence-Like Phenotype in PC-3 Cells. (A) Effect of triptolide on senescence-associated-β-galactosidase production by PC-3 cells. PC-3 cells were treated with 100 nM Triptolide or DMSO for 24h and then stained for SA-β-galactosidase activity. Senescent cells stain in blue. Image was recovered using an inverse phase light microscope. (B) Immunofluorescent images of H3K9me3 and senescence-associated heterochromatic foci (SAHF) in PC-3 cells treated with Triptolide or DMSO. Cells were treated with 100 nM Triptolide for 24 h and analyzed by immunofluorescence with anti-H3K9me3 primary antibody (Red). DNA was stained with DAPI (blue). Cell images were captured with a fluorescence microscope. SAHF are depicted with yellow arrows (C) Cell cycle analysis of PC-3 cells by flow cytometry. After treatment with various doses of Triptolide for 24 h, PC-3 cells were fixed and stained with propidium iodide (PI). DNA content was detected by flow cytometry. (D) Effect of Triptolide on the mRNA level of senescence-related genes. Cells were treated with the indicated doses of Triptolide and analyzed by real-time PCR with specific primers. * indicates P <0.05. (E) Effect of Triptolide on the level of senescence-related proteins. Cells were treated with the indicated doses of Triptolide and analyzed by western blotting. β-actin was used as a loading control.

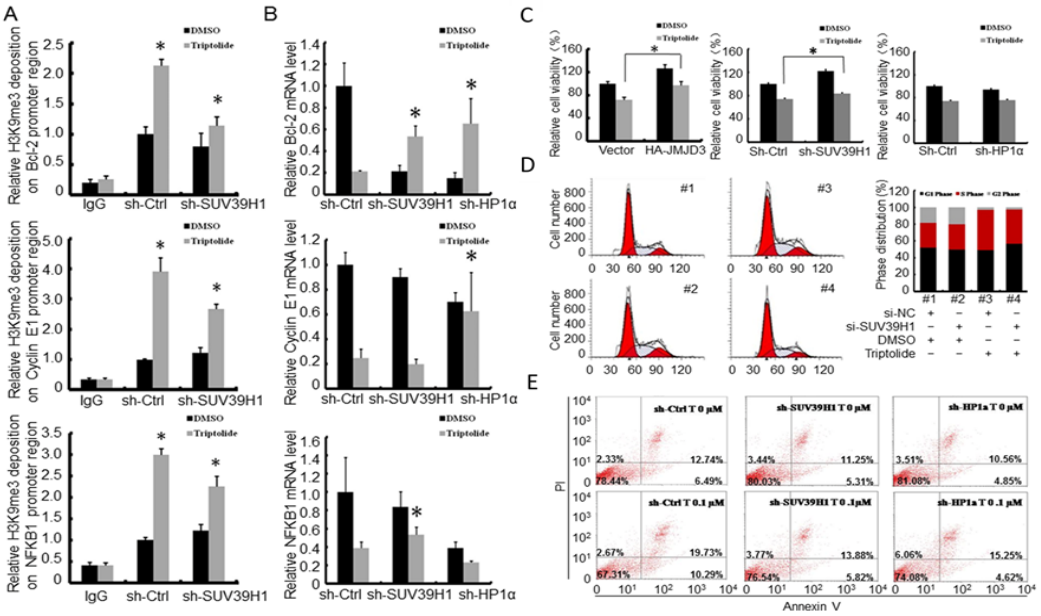

Figure 4. Triptolide-Induced Cellular Effects is Mediated Through Formation of a Repressive Chromatin State. (A) Triptolide induces H3K9me3 deposition on the promoter regions of E2F1 target genes. PC-3 cells were transfected with SUV39H1 siRNA or non-specific control for 48 h then treated with 100 nM Triptolide or DMSO for an additional 24 h. Chromatin was extracted and immunoprecipitated (CHIP) with specific anti-H3K9me3 antibody or control IgG. The target DNA level was analyzed by real-time PCR using GAPDH as an internal control. Results are presented as relative values compared to the negative control. "*" means $P < 0.05$. (B) Triptolide inhibits the expression of E2F1 target genes. PC-3 cells were transfected with siRNAs specific to SUV39H1 and HP1α for 48 h then treated with 100 nM Triptolide or DMSO for an additional 24 h. The mRNA levels of several E2F1 target genes were analyzed by real-time PCR. Results are presented as relative values compared to the negative control. * means $P < 0.05$. (C) Ectopic expression of JMJD3 or knock-down of SUV39H1 and HP1α attenuates the growth inhibition effect of Triptolide. PC-3 cells were transfected with JMJD3 expression plasmids or SUV39H1 and HP1α siRNAs for 48 h, then treated with 100 nM Triptolide or DMSO for an additional 24 h, Cell viability was assessed by CCK-8 assay. (D) Knock-down of SUV39H1 attenuates the S-phase arrest induced by Triptolide. PC-3 cells were transfected with SUV39H1 or control siRNAs for 48 h, then treated with 100 nM Triptolide or DMSO for an additional 24 h. Cells were fixed and stained with PI, and DNA content was determined by flow cytometry. (E) Knock-down of SUV39H1 and HP1α attenuates the apoptosis induction effect of Triptolide. PC-3 cells were transfected with SUV39H1, HP1α or control siRNAs for 48 h then treated with 100 nM Triptolide or DMSO for an additional 24 h. After cells were stained with AV-FITC (green) and PI (red), they were analyzed by flow cytometry. The flow cytometry traces show the percentage of cells that are intact (AV−/PI−) or at different stages of apoptosis (AV+/PI−, AV+/PI+ and AV−/PI+).

knock-down of SUV39H1 attenuates H3K9me3 deposition on those promoters (Figure 4A), suggesting that Triptolide-induced deposition of H3K9me3 on target gene promoters is highly dependent on SUV39H1. We also found that Triptolide significantly decreases the mRNA level of E2F1 target genes and knock down of SUV39H1 or HP1α partially attenuated the inhibitory effect of Triptolide in PC-3 cells (Figure 4B), indicating that Triptolide suppresses gene expression partially through enhancing the deposition of H3K9me3 on gene promoters and inducing heterochromatin formation.

We next transfected PC-3 cells with either a plasmid expressing HA-tagged JMJD3 or siRNAs specific for SUV39H1 and HP1α for 48 h, then treated with 100 nM Triptolide for an additional 24 h. Cell viability assays showed that over-expression of JMJD3 or knock-down of SUV39H1 significantly reduces the cytotoxicity of Triptolide to PC-3 cells (Figure 4C). Cell cycle analysis showed that knock-down of SUV39H1 significantly suppresses the S-phase arrest of Triptolide-treated PC-3 cells (Figure 4D). Similar results were also observed with knock-down of HP1α (data no shown). Using flow cytometry analysis, we found that knock-down of SUV39H1 or HP1α significantly inhibited the apoptotic activity of Triptolide (Figure 4E).

Discussion

The small molecule Triptolide has demonstrated potent anti-tumor effect against several kinds of cancer through a wide range of molecular mechanisms (Huang et al., 2012; Manzo et al., 2012; Phillips et al., 2007; Titov et al., 2011; Vispe et al., 2009; Wang et al., 2011; Zhu et al., 2009). However, there is a knowledge gap about the effect of Triptolide on histone methylation in PCa. We previously showed that Triptolide significantly inhibits the growth of PCa cells through downregulation of EZH2, a key subunit of the Polycomb repressive complex 2 (Tamgue et al., 2013). In light of that finding, we hypothesized that Triptolide may induce changes in the histone modification pattern in PCa cells. In this paper, we found that Triptolide enhanced H3K27me3 levels despite down-regulating EZH2 in PC-3 cells (Figure 1A). This result was unexpected since downregulation of EZH2 by Triptolide decreased H3K27me3 level in the multiple myeloma cell line U266 (Zhao et al., 2010). Since H3K27me3 is a marker

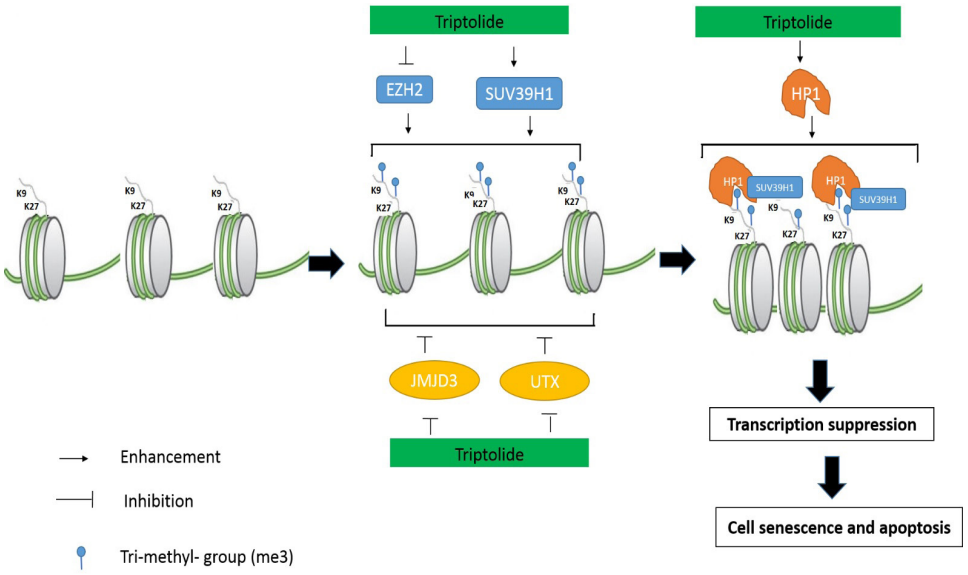

Figure 5. Proposed Model of the Epigenetic-Mediated Transcription Suppression Mechanisms and Anti-PCa Effect of Triptolide. Triptolide enhances the levels of H3K27me3 and H3K9me3 by down-regulating JMJD3/UTX and up-regulating SUV39H1 respectively. Triptolide also upregulates HP1α which then promotes heterochromatin formation, suppression of genomic transcription, and induction of PCa cell senescence.

of gene repression we reasoned that Triptolide would induce the accumulation of repressive marks, consistently with its reported transcription suppression effect. We found that Triptolide indeed induces heterochromatin formation in PCa cells through different mechanisms including (1) enhancement of repressive histone mark H3K27me3 through down-regulation of the HDMs JMJD3 and UTX (Figure 1C, 1D and 1E); (2) enhancement of heterochromatin markers SUV39H1 (Figure 2A, 2B and 2C), H3K9me3 (Figure 2A, 2B and 2D) and HP1α (Figure 2A, 2B and 2C). SUV39H1 is involved in gene-specific transcriptional repression through tri-methylation of H3K9 at specific loci (Ait-Si-Ali et al., 2004). Meanwhile H3K9me3 is usually found in silent heterochromatin regions and inactive promoters (Kouzarides, 2007) where it provides a high affinity binding sites for HP1α which subsequently recruits other chromosomal proteins to induce chromatin packaging and gene silencing (Lachner et al., 2001; Melcher et al., 2000). Since formation of heterochromatin involves in oncogene- and drug-induced senescence mechanisms (Narita et al., 2003), we explored senescence mechanism and found that Triptolide induces a senescence-like phenotype in PC-3 cells which was characterized by production of senescence-associated-β-galactosidase (Figure3A), presence of senescence-associated heterochromatic foci in cell nuclei (Figure 3B) , cell cycle arrest in S-phase (Figure 3C) and activation of the pRB1 pathway (Figure 3C and 3D). Therefore, the anti-tumor effect of Triptolide may be explained at least partly by its ability to induce senescence. Our finding is in line with previous reports observing that blockade of apoptosis using potent caspase/ apoptosis inhibitor zVAD-fmk failed to prevent Triptolide-induced cell death in breast cancer cells MCF-7, thus suggesting that other mechanisms must be involved in the anti-tumor effect of Triptolide (Tan et al., 2011). Our findings further suggest that induction of heterochromatin formation contributes to other anti-PCa effects of Triptolide, including suppression of cell viability (Figure 4C); cell cycle arrest (Figure 4D), apoptosis induction (Figure 4E) and suppression of gene expression (Figure 4A and 4B). Our result also support the conclusions from recent studies suggesting that the targeting of XPB protein (Wang et al., 2011) cannot account for all the biological activities of Triptolide (Huang et al., 2012; Smurnyy et al., 2014). In this study we found that Triptolide also suppresses gene expression by promoting heterochromatin formation and deposition on the promoters of E2F1 target genes (Figure 4A and 4B). Whether the presence of E2F1 binding site on the promotor region is a prerequisite for gene targeting by Triptolide remains to be determined and may explain why certain genes escape Triptolide-induced transcription suppression.

In summary, our study showed that Triptolide is a potential epigenetic anti-PCa drug. The anti-PCa activity of Triptolide may be partially attributed to its ability to induce heterochromatin formation. We propose a model (Figure 5) whereby Triptolide enhances the levels of H3K27me3 and H3K9me3 by down-regulating JMJD3/UTX and up-regulating SUV39H1 respectively. Triptolide also upregulates HP1α which then promotes heterochromatin formation, suppression of genomic transcription, and induction of PCa cell senescence.

Funding Statement

This work was supported by the Cameroon-China bilateral cooperation fellowship granted to Ousman Tamgue and the National Natural Science Foundation of China grant (61178084) To Ming Lei. The publication cost for this scientific paper is covered by the South African DST-NRF Innovation postdoctoral fellowship granted to Ousman Tamgue

Statement conflict of interest

The authors disclose no conflict of interest.

Acknowledgments

We are thankful to Kristian Helin for the kind gift of pCMV-HA-JMJD3 plasmid (Addgene plasmid # 24167), the members of the key laboratory of agricultural molecular biology at Northwest A&F University and the laboratory of cytokines and diseases, IDM, division of immunology, University of Cape Town, South Africa for provision of the access to equipment and the critical review of this manuscript.

References

Ait-Si-Ali S, Guasconi V, Fritsch Let al (2004): A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. *EMBO J*, **23**, 605-15.

Bhaumik SR, Smith E, Shilatifard A (2007). Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol*, **14**, 1008-16.

Feinberg AP (2004). The epigenetics of cancer etiology. *Semin Cancer Biol*, **14**, 427-32.

Gao F, Ji GY, Gao ZW, et al (2014): Direct ChIP-bisulfite sequencing reveals a role of H3K27me3 mediating aberrant hypermethylation of promoter CpG islands in cancer cells. *Genomics*, **103**, 204-10.

Huang W, He T, Chai C, et al (2012): Triptolide inhibits the proliferation of prostate cancer cells and down-regulates SUMO-specific protease 1 expression. *PLoS One*, **7**, e37693.

Hubner MR, Spector DL (2010). Role of H3K27 demethylases Jmjd3 and UTX in transcriptional regulation. *Cold Spring Harb Symp Quant Biol*, **75**, 43-9.

Jenuwein T, Allis CD (2001). Translating the histone code. *Science*, **293**, 1074-80.

Kim KH, Roberts CWM (2016). Targeting EZH2 in cancer. *Nat Med*, **22**, 128-34.

Kouzarides T (2007). Chromatin modifications and their function. *Cell*, **128**, 693-705.

Kupchan SM, Court WA, Dailey RG, et al (1972). Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from Tripterygium wilfordii. *J Am Chem Soc*, **94**, 7194-5.

Lachner M, O'Carroll N, Rea S, Mechtler K, Jenuwein T (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, **410**, 116-20.

Lennartsson A, Ekwall K (2009). Histone modification patterns and epigenetic codes. *Biochim Biophys Acta*, **1790**, 863-8.

Manzo SG, Zhou ZL, Wang YQ, et al (2012). Natural product triptolide mediates cancer cell death by triggering CDK7-dependent degradation of RNA polymerase II. *Cancer Res*, **72**, 5363-73.

Melcher M, Schmid M, Aagaard L, et al (2000). Structure-function analysis of SUV39H1 reveals a dominant role in heterochromatin organization, chromosome segregation, and mitotic progression. *Mol Cell Biol*, **20**, 3728-41.

Mellor J (2005). The dynamics of chromatin remodeling at promoters. *Molecular Cell*, **19**, 147-57.

Narita M, Nunez S, Heard E, et al (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell*, **113**, 703-16.

Phillips PA, Dudeja V, McCarroll JA, et al (2007). Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. *Cancer Res*, **67**, 9407-16.

Smurnyy Y, Cai M, Wu H, et al (2014). DNA sequencing and CRISPR-Cas9 gene editing for target validation in mammalian cells. *Nat Chem Biol*, **10**, 623-U152.

Tamgue O, Chai CS, Hao L, et al (2013): Triptolide inhibits Histone Methyltransferase EZH2 and modulates the expression of its target genes in prostate cancer cells. *Asian Pac J Cancer Prev*, **14**, 5663-9.

Tan BJ, Tan BH, Chiu GN (2011). Effect of triptolide on focal adhesion kinase and survival in MCF-7 breast cancer cells. *Oncol Rep*, **26**, 1315-21.

Titov DV, Gilman B, He QL, et al (2011). XPB, a subunit of TFIIH, is a target of the natural product triptolide. *Nat Chem Biol*, **7**, 182-8.

Vispe S, DeVries L, Creancier L, et al (2009): Triptolide is an inhibitor of RNA polymerase I and II-dependent transcription leading predominantly to down-regulation of short-lived mRNA. *Mol Cancer Ther*, **8**, 2780-90.

Waldmann T, Schneider R (2013). Targeting histone modifications - epigenetics in cancer. *Curr Opin Cell Biol*, **25**, 184-9.

Wang Y, Lu J-j, He L, Yu Q (2011) Triptolide (TPL) inhibits global transcription by inducing proteasome-dependent degradation of RNA polymerase II (Pol II). *PLoS One*, **6**, e23993.

Zhao F, Chen Y, Zeng LL, et al (2010). Role of triptolide in cell proliferation, cell cycle arrest, apoptosis and histone methylation in multiple myeloma U266 cells. *Eur J Pharmacol*, **646**, 1-11.

Zhu W, Hu H, Qiu P, Yan G (2009). Triptolide induces apoptosis in human anaplastic thyroid carcinoma cells by a p53-independent but NF-kappaB-related mechanism. *Oncol Rep*, **22**, 1397-401.