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Original Research

β-ionone Inhibits Epithelial-Mesenchymal Transition (EMT) in Prostate Cancer Cells by Negatively Regulating the Wnt/β-Catenin Pathway

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Abstract

Background: β-ionone is a terminal cyclic analog of beta-carotenoids widely found in plants. In recent years, accumulating evidence has shown that β-ionone exerts antitumor effects on various malignant tumors. However, limited studies have revealed the role of β-ionone in regulating the epithelial-mesenchymal transition (EMT) of prostate cancer (PCa) cells. This study aimed to investigate the effect of β-ionone on the EMT process of PCa, focusing on Wnt/β-catenin signaling pathway. **Methods**: After exposure to β-ionone, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the Brdu proliferation assay. The Transwell and wounding healing were used to investigate the migration and invasion abilities of PCa cells. Expression of proteins involved in the EMT process (E-cadherin, N-cadherin, vimentin) and proteins in the Wnt/β-catenin pathway (β-catenin, GSK3-β, and p-GSK3-β) were explored by western blotting. The effects of β-ionone on β-catenin degradation were explored by cycloheximide tracking assay and *in vitro* ubiquitination assay. Nude mouse xenograft model was served as the model system *in vivo*. **Results**: The migration, invasion, and EMT process of PCa Human PC-3 prostate adenocarcinoma cells (PC3) and Human 22RV1 prostate adenocarcinoma cells (22RV1) cells were significantly inhibited after β-ionone treatment. In addition, β-ionone also inhibited the growth and EMT process of subcutaneous xenograft tumors in nude mice. The study also found that β-catenin, which promotes EMT, was downregulated after β-ionone treatment. Further mechanistic studies revealed that β-ionone inhibited the Wnt/β-catenin pathway by accelerating the ubiquitination and degradation of β-catenin in PCa, thus inhibiting the downstream migration, invasion, and EMT processes. **Conclusions**: These findings demonstrate that β-ionone may be a potential natural compound targeting the Wnt/β-catenin pathway for the treatment of PCa.

Keywords: β-ionone; prostate cancer; epithelial-mesenchymal transition; Wnt/β-catenin pathway; ubiquitination

1. Introduction

Prostate cancer (PCa) is the second most common malignancy and the fifth leading cause of death from malignancy in males. There are about 1.27 million new cases worldwide each year [1]. The etiology of PCa is still unclear. Established risk factors include age, family history of cancer, and certain genetic mutations [2]. Although the mortality of PCa is decreasing annually, its treatment remains a challenge due to the heterogeneity and aggressiveness of prostate tumors [3]. Metastasis remains the leading cause of death in most PCa patients after surgery and androgen deprivation therapy (ADT).

Natural components extracted from plants are commonly used in medical research [4]. In recent years, more and more natural anticancer compounds have been discovered. Compared with traditional synthetic drugs, plant-derived drugs usually have the advantages of less toxicity, better tolerance, low price, and easy availability [5]. β-Ionone is a terminal analog of β-carotene and is an important intermediate for many chemicals [6]. β-ionone is a natural flavor mainly found in fruits and grains, and numerous studies have confirmed that it has certain anti-inflammatory, antioxidant and anti-tumor effects [7–9]. It has been confirmed that the growth of tumor cells can be inhibited by β-ionone-mediated cell cycle arrest, anti-oxidation, and promotion of apoptosis in gastric cancer [10], liver cancer [11], breast cancer [12] and PCa [13]. Previous studies in our laboratory also found that β-ionone acted as a ligand for prostate-specific G-protein coupled receptor (PSGR), which could then activate p38 and Jun N-terminal Kinase (JNK), leading to phosphorylation of the Ser650 residue of the androgen receptor (AR), and preventing AR from entering the nucleus, thereby inhibiting the spread and development of PCa cells [14]. However, the exact molecular mechanism by which β-ionone functions in PCa cells remains largely unknown.

The Wnt/β-catenin pathway remains inactivated in normal cells. Under pathological conditions, the increase of Wnt protein can activate the Wnt/β-catenin pathway, and the β-catenin protein will be separated from the axin-Adenomatous Polyposis Coli protein-Casein Kinase 1-Glycogen Synthase Kinase-3β (axin-APC-CK1-GSK-3β) complex and cannot be ubiquitinated and degraded. β-catenin accumulates in the cytoplasm, then enters the nucleus and binds to T-cell factor/lymphoid enhancing factor (TCF/LEF) to activate Cyclin D1, MYC Proto-Oncogene (MYC), matrix metalloproteinase 7 (MMP-7), N-cadherin and other downstream target genes [15,16]. The activated Wnt/β-catenin pathway is involved in the occurrence and development of various malignant tumors [17]. In recent years, many compounds have been found to target this pathway to inhibit tumor proliferation and progression. Shi *et al.* [18] reported that capsaicin could promote the ubiquitination and degradation of β-catenin in melanoma cells, thereby inhibiting the cell migration and invasion. Wu *et al.* [19] found that 2'-hydroxyflavonoids could downregulate the expression levels of p-GSK-3β and β-catenin in PCa, thereby inhibiting cell migration, invasion and epithelial-mesenchymal transition (EMT). However, no studies have shown whether β-ionone is involved in the regulation of this pathway.

In this study, we found that β-ionone inhibited the migration, invasion, and EMT of PCa cells. Mechanistically, β-ionone negatively regulated the Wnt/β-catenin pathway by promoting the ubiquitination and degradation of β-catenin, thereby inhibiting the downstream EMT process and the migration/invasiveness of PCa cells. Our study clarified the regulatory role of β-ionone in the Wnt/β-catenin pathway for the first time and provided a new approach to target this pathway to inhibit PCa progression.

2. Materials and Methods

2.1 Cell Culture and β-ionone Treatment

Human prostate cancer cell Human PC-3 prostate adenocarcinoma cells (PC3), Human 22RV1 prostate adenocarcinoma cells (22RV1), prostatic hyperplasia cell Human Benign Prostatic Hyperplasia Cell Line (BPH-1) and embryonic kidney cell 293T was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin antibiotics, and 0.1 mg/mL streptomycin (Gibco; Thermo Fisher Scientific, Inc.). In addition, all cells were cultured in a humidified 37 °C incubator with 5% CO_2. β-ionone (I12603, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich; Merck KGaA) to make it a storage solution with a final concentration of 200 mM. PC3, 22RV1. BPH-1 cells were treated with the indicated concentrations of β-ionone for different times or the same time at gradient concentrations, and an equivalent volume of DMSO was used as a control.

2.2 MTT Assays

PCa cells were planted into 96-well plates at 8×10^3/well and grown to 60%–80% confluency, then cultured with a gradient concentration of β-ionone for 24 or 48 h. A medium containing 10% MTT (5 mg/mL; Sigma-Aldrich; Merck KGaA) was added to each well and cultured for 4 h. Then, the supernatant was removed and added 250 μL DMSO into each well. The 96-well microplate reader (Bio-Rad, Hercules, USA) was used to detect the absorbance at the wavelength of 490 nm.

2.3 EdU Staining

Cell proliferation was detected using Click-tm EdU Cell Proliferation Kit (NO. C0075S, Beyotime Bio, Shanghai, China). Cells in the logarithmic phase are seeded into 6-well plates and incubated overnight in a 37 °C incubator by 4×10^5/well. The cells were treated with β-ionone (200 mM) or DMSO for another 24 h incubation. Then the plates were added with Edu (10 μM) and returned to the incubator for 2 h, followed by immobilization of cells with 0.4% paraformaldehyde and 0.3% Triton X-100 to increase cell membrane permeability. After PBS washing, each well of the plates was supplemented with the click additive solution for 30 min and stain the nuclei with Hoechst-33,342. Finally, a fluorescence microscope was used to observe and photographed the results.

2.4 Wound-Healing Assays

PCa cells were seeded in 6-well plates and grown in culture dishes to form a 90%–100% confluent monolayer of adherent cells. A 200 μL pipette tip was then used to score a scratch on the monolayer to create a linear wound. After washing the plates twice with pre-chilled PBS, the subsequent culture was performed in a serum-free medium containing 180 μM β-ionone. DMSO was used as the control. The wound closure was observed by an inverted microscope ($\times$ 100) at 0, 12, and 24 hours). The wound healing rate is calculated according to the following formula: wound healing rate = (β-ionone treated group gap closure rate/control group gap closure rate) $\times$ 100.

2.5 Migration and Invasion Transwell Assays

Transwell chambers (12 mm in diameter, 8 μm in pore size, Corning, Beijing, China) were used to detect the migration and invasiveness of cells after β-ionone treatment. Transwell chambers were pre-covered with 30 μL of diluted Matrigel (Sigma-Aldrich; Merck KGaA) for invasion assays. 4×10^5 cells were dispersed in 200 μL of serum-free medium containing β-ionone or DMSO and placed in the upper chamber. The upper chamber was placed into the lower chamber filled with 800 μL of RPMI-1640 containing 10% FBS, after 24 h of incubation, he mini-cells were

washed with PBS and fixed with 4% formalin at room temperature. They were then stained with crystal violet (0.1% in absolute ethanol) for 15 minutes. Five fields of view were randomly selected and photographed by a microscope to calculate the number of cells that had migrated or invaded (magnification, $\times$100).

2.6 Western Blotting

Total protein lysates were collected with lysis buffer containing protease inhibitors and phosphatase inhibitors, then the collected lysates were centrifuged at 15,000 rpm for 15 min at 4 °C. A 30 μg sample of total protein lysates were electrophoresed on an 8% or 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel and transferred to PVDF membranes. Polyvinylidene fluoride (PVDF) membranes were blocked with 5% Bovine serum albumin (BSA) for 1 h at room temperature, followed by incubation with the following specific primary antibodies diluted in 5% BSA (dilution 1:2000) at 4 °C overnight: Rabbit vimentin (cat. no. 5471), total β-catenin (cat. no. 8480), phosphorylated glycogen synthase kinase (GSK)3β (Ser9; cat. no. 5558), total GSK3β (cat. no. 12456) and Vinculin (cat. no. 4970); which purchased from Cell Signaling Technology, Inc (Boston, MA, USA). Antibodies against epithelial (E)-cadherin (cat. no. ab15148), neural (N)-cadherin (cat. no. ab76057) and Histone H3 (cat. no. ab176842) were purchased from Abcam (San Diego, CA, USA). PVDF membranes were incubated with enzyme-conjugated secondary antibody for 1 hour at room temperature and images were obtained using the ECL system (Thermo Fisher Scientific, Rochester, NY, USA). Vinculin protein level was used as the endogenous control, and the level of the target protein was compared with vinculin in the same group, and then the expression levels of the target protein was analyzed in each group.

2.7 Plasmid Transfections

To overexpress β-catenin in PCa, the β-catenin cDNA was cloned into the pcDNA3.1 vector. For transfection, plasmids and Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) were separately added to serum-free medium and mixed 1:1, using empty vector as a control. Twenty-four hours after transfection, the transfection efficiency was detected by qRT-PCR and western blotting, and an appropriate concentration of β-ionone was added for subsequent operations.

2.8 Immunofluorescence

Cells were seeded in 6-well plates with coverslips and allowed to grow attached to the slides. After grown to a suitable density on the coverslip, the cells were treated with β-ionone for 24 h and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were washed with PBS and treated with 0.5% Triton™ X-100 solution for 15 min to increase cell permeability. The slides were washed with PBS and incubated overnight at 4 °C with an anti-β-catenin primary antibody (cat. no. 8480; dilution, 1:200; Cell Signaling Technology, Inc). The next day, the slides were washed with PBS, mixed with goat anti-rabbit IgG H&L fluorescein isothiocyanate (FITC) (cat. no. ab6717; cat. no. 1:200; Abcam), and incubated for 1 h at room temperature. The cells were stained with DAPI (1 μg/mL) for 5min again, sealed with anti-quenching resin, and the expression and distribution of β-catenin were detected by a confocal laser microscope.

2.9 RNA Extraction and qRT-PCR

Total cellular RNA was extracted with TRIzol reagent, and 1 μg of RNA was reverse transcribed with the Superscript III transcriptase (Invitrogen, Grand Island, NY) to obtain cDNA. qRT-PCR was performed to determine the mRNA expression level of β-catenin using the Bio-Rad CFX96 system. The PCR primer sequence of β-catenin was 5'-AAAGCGGCTGTTAGTCACTGG-3' (forward) and 5'-CGAGTCATTGCATACTGTCCAT-3' (reverse) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse). Using GAPDH as an internal reference, relative changes in gene expression were normalized against GAPDH.

2.10 Turnover Assays and Protein Ubiquitination

PC3 cells pretreated with β-ionone and DMSO were incubated with 50 μg/mL cycloheximide (CHX, Sigma-Aldrich) for a specified time in turnover assays. β-catenin protein level was detected by western blotting. The degradation pathway of β-catenin was further confirmed by treating PC3 cells with MG132 (proteasome inhibitor, Sigma-Aldrich) and β-ionone. 293T cells were transfected with His-Ub and Flag-β-catenin plasmids to detect the ubiquitination of β-catenin *in vitro*, After transfection for 24 h, 293T cells were cultured with newly replaced RPMI-1640 medium containing 180 μM β-ionone or an equal volume of DMSO for 16 h, and then 20 μg/mL of proteasome inhibitor MG132 was added for another 8 h. Then 293T cells were collected and washed with pre-cooled PBS to obtain 1 ml of cell suspension, of which 100 μL was used as the input group for routine protein extraction. The remaining 900 μL cell suspension was crushed with an ultrasonic pulverizer and then added with Ni-NTA agarose beads, and then incubated at room temperature for 3 h. The Ni-NTA agarose beads were collected by centrifugation, and the supernatant was discarded. After washed several times, the beads were added with appropriate Immunoprecipitation Buffers (IP buffer) with protein sample loading buffer, and then denatured at 95 °C for 10 minutes. The ubiquitination level of and the target protein was detected by western blotting.

2.11 Xenograft Animal Model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and their care was in accordance with institution guidelines. Ten 4-week-old male nude mice (weight 15–20 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University, raised in a pathogen-free environment, and given a normal diet. PC3 cells (4×10^6 cells mixed with same volume of matrigel) were injected subcutaneously into the left hind flanks of 10 mice. After the subcutaneous transplanted tumor was successfully established, the mice were randomly divided into two groups: the treatment group was treated with β-ionone (75 mg/kg) diluted with corn oil, and the control group was treated with an equal volume of corn oil, Each mouse was intraperitoneally injected once every 3 days and the tumor size were measured. The tumor volume was calculated as following formula: Tumor volume (mm^3) = (length) $\times$ $(width)^2 \times \pi/6$. Nude mice were sacrificed after 3 weeks of treatment, and tumors were excised, weighed, and measured in volume. A small amount of tumor tissue was taken for western blotting and immunohistochemical staining to detect the expression of the target protein in the tissue. All animal experiments were conducted under the guidance of the Committee for Animal Protection and Utilization of Xi'an Jiaotong University, and executed according to standard ethical guidelines (2020-G-208).

2.12 Target Prediction for β-ionone

The potential targets of β-ionone were explored and assessed utilizing public databases such as Swiss target prediction (https://www.swisstargetprediction.ch/), Similarity ensemble approach (https://sea.bkslab.org/) and SuperPred (https://prediction.charite.de/subpages/target_prediction.php). In all these databases species of target origin was limited to Homo Sapiens once the target has been predicted. The top 10 targets of the prediction results of each of the three websites were captured and intersected. Further prediction was conducted in PLIP (https://plip-tool.biotec.tu-dresden.de/) to binding residues of β-ionone with these targets. Finally the software AutoDock Vena and Pymol were applied for visualization the molecular docking of these targets and β-ionone.

2.13 Statistical Analysis

All experiments were performed in three independent replicates. All statistical analyses were performed using GraphPad Prism 8.2 software (GraphPad Software Inc., San Diego, CA, USA). Student's *t*-test was used for comparisons between two groups, and one-way ANOVA was used to assess mean differences between three or more groups, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

3. Results

3.1 β-ionone Inhibits PCa Cells Migration and Invasion

To assess the effect of β-ionone on PCa and non-tumor cell viabilities, PC3, 22RV1 and BPH-1 cells were treated with gradient concentrations of β-ionone solution for 24 h or 48 h. The results showed that when the concentration of β-ionone was $\leq$200 μM, there was no significant effect on the viabilities of PC3, 22RV1 and BPH-1 cells. The concentration of β-ionone $\geq$200 μM was found to affect the viabilities of PC3 and 22RV1. And β-ionone inhibited the growth of BPH-1 cells only when the concentration of β-ionone reached 400 μM (Fig. 1A). The results of EdU staining after treating cells with β-ionone (200 μM) similarly confirmed the results of MTT (Fig. 1B). Therefore, the concentration of 60/120/180 μM β-ionone was selected to treat PC3, 22RV1 and BPH-1 cells for subsequent experiments. In view of the highly invasive characteristics of prostate cancer, we investigated whether β-ionone affects cell migration and invasion of PCa. Wound healing experiments showed that β-ionone significantly delayed the rate of wound closure. However, the same concentration of β-ionone did not affect the migration of BPH-1 (Fig. 1C). The results of the transwell assays further showed that β-ionone inhibited the migration and invasion abilities of PC3 and 22RV1 cells (Fig. 1D,E).

3.2 β-ionone Suppresses EMT in PCa Cells

EMT plays a crucial role in tumor metastasis and progression. To investigate whether the inhibition of migration and invasion of PCa cells by β-ionone is related to EMT, we detected the expression of EMT markers using western blotting. The results showed that the β-ionone treatment increased the expression level of E-cadherin while downregulating the expression of N-cadherin and Vimentin in a concentration and time-dependent manner (Fig. 2A,B). To further demonstrate the effect of β-ionone on EMT, we treated cells with EMT-induced TGF-β1. The results indicated that β-ionone could reverse TGF-β1-induced EMT in PCa cells (Fig. 2C). In addition, the results of migration and invasion assays demonstrated that cell migration and invasion enhanced by TGF-β1 could be inhibited by β-ionone (Fig. 2D,E). These results confirmed that β-ionone could inhibit PCa cell migration and invasion by suppressing EMT.

3.3 β-ionone Suppresses Wnt/β-Catenin Pathway

The Wnt/β-catenin signaling pathway has been found to act as an upstream pathway of EMT and promote PCa progression. Therefore, we further determined whether β-ionone inhibition of EMT and migration was related to the Wnt/β-catenin pathway. The results demonstrated the expression levels of β-catenin and p-GSK-3β were downregulated in PCa cells treated with β-ionone (Fig. 3A). After overexpression of β-catenin in PC3, β-ionone still downregulated the expression level of β-catenin protein

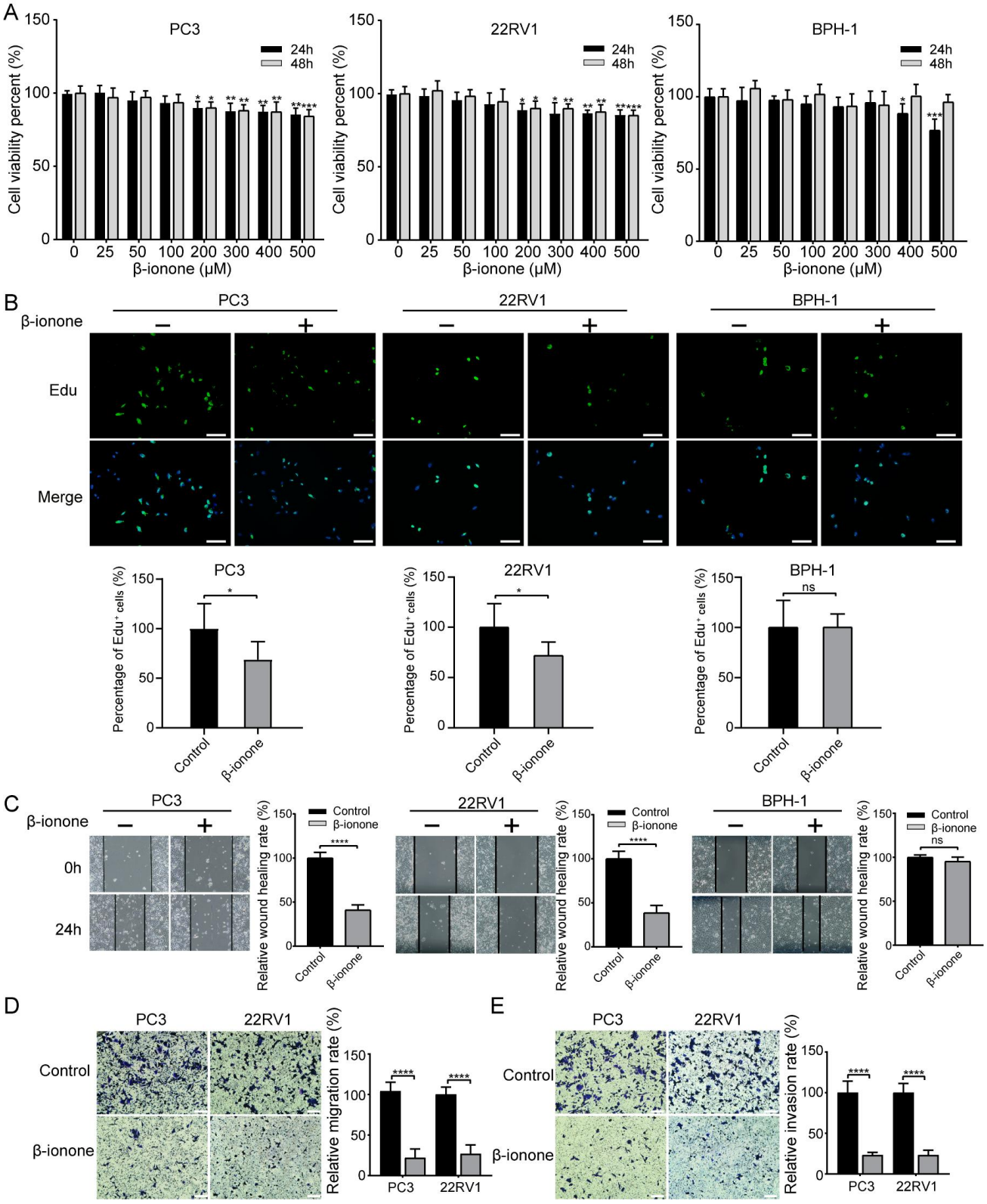

Fig. 1. β-Ionone inhibits migration and invasion of prostate cancer cells. (A) MTT assays were performed to determine the cell viabilities of PC3, 22RV1 and BPH-1 treated with different concentrations of β-ionone for 24 and 48 h. (B) EdU staining explore the Edu-positive rate of cells after 24 h of β-ionone treatment. (C) Wound healing assays were performed on PC3, 22RV1 and BPH-1 cells treated with DMSO or 180 μM of β-ionone. (D) Transwell migration and (E) invasion assays were used to investigate the migration and invasion abilities of PC3 and 22RV1 cells after treatment with DMSO or 180 μM β-ionone. Magnification, $\times$100. Scale bar, 20 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

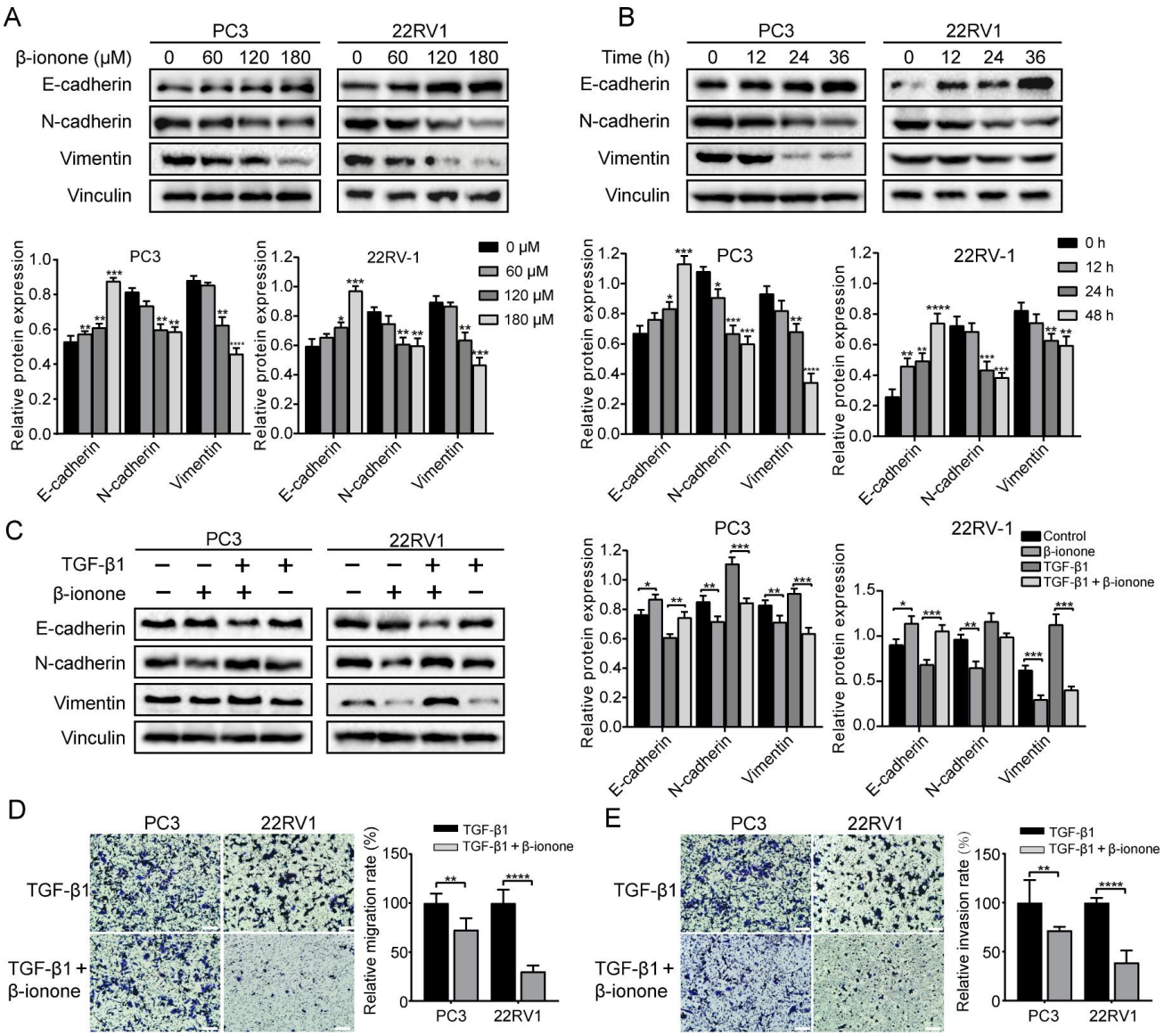

Fig. 2. β-ionone inhibits EMT in prostate cancer cells. The expressions of E-cadherin, N-cadherin and vimentin were detected by Western blotting in prostate cancer cells PC3 and 22RV1 were treated with gradient concentrations of β-ionone for 24 h (A) or 180 μM of β-ionone for 0, 12, 24, and 36 h (B) by Western blotting. (C) After treating PC3 and 22RV1 with 180 μM β-ionone or (and) 5 ng/mL TGF-β1 for 24 h, the variation trend in EMT markers were detected by western blotting. (D) Transwell migration and (E) invasion assays were used to determine the migration and invasion abilities of PC3 and 22RV1 cells after treatment with 180 μM β-ionone or (and) 5 ng/mL TGF-β1. Magnification, $\times$100. Scale bar, 20 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

(Fig. 3B). At the same time, β-ionone also decreased the expression of β-catenin in the cytoplasm and nucleus (Fig. 3C). Immunofluorescence analysis further confirmed the decreased expression of intracellular β-catenin (Fig. 3D), and a negative control group was used to exclude nonspecific binding (**Supplementary Fig. 1**). Hence, β-ionone can inhibit EMT by negatively regulating the Wnt/β-catenin signaling pathway in prostate cancer cells.

3.4 β-ionone Inhibits EMT of PCa by Regulating Ubiquitination and Degradation of β-catenin

Previous studies have demonstrated that ubiquitin-proteasomes maintain an inactive state of the Wnt/β-catenin pathway by degrading β-catenin. The mRNA level of β-catenin did not change significantly after the β-ionone treatment of cells (Fig. 4A), so we sought to consider its ubiquitination and degradation.

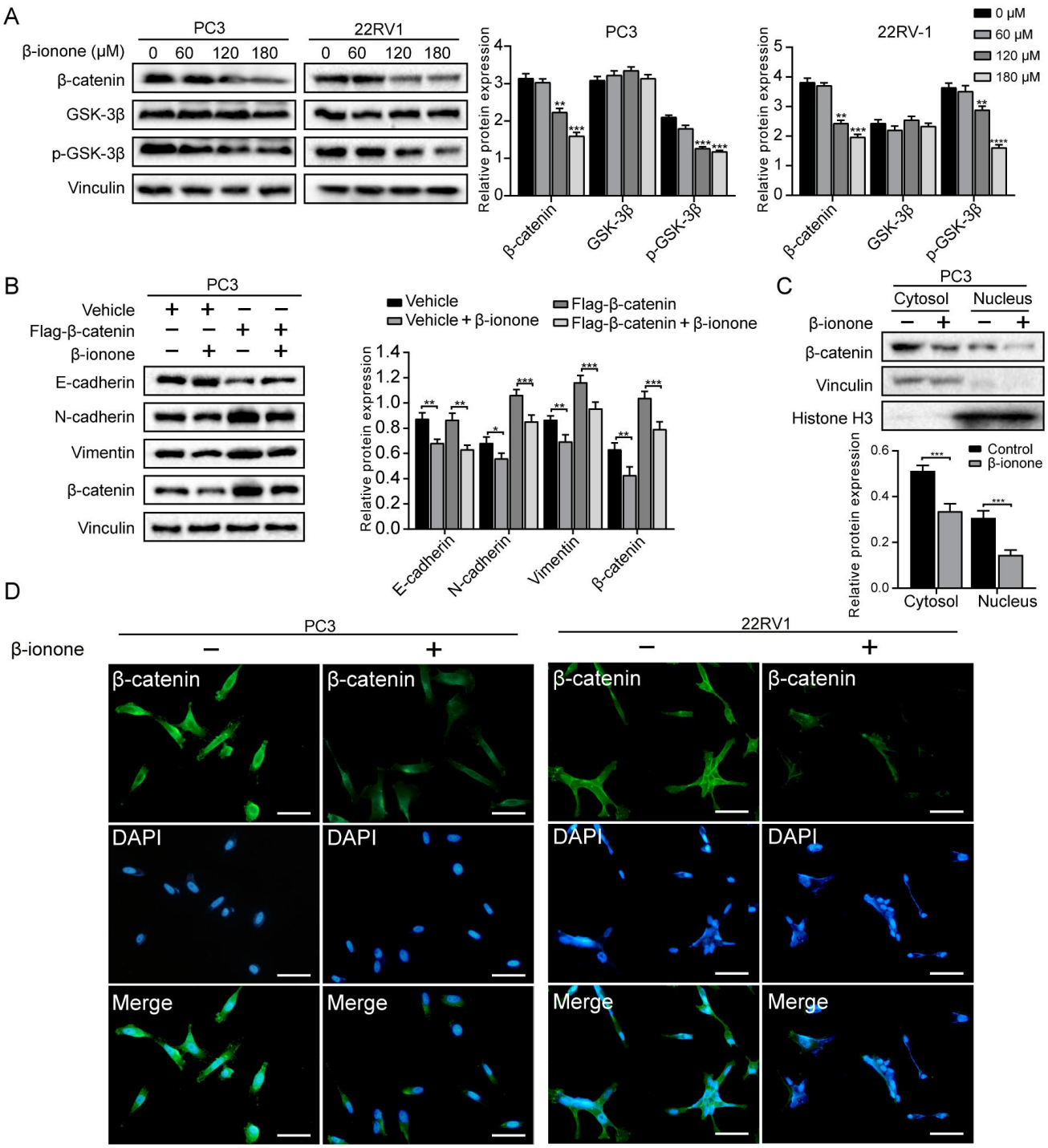

Fig. 3. β-ionone inhibits the Wnt/β-catenin pathway in prostate cancer cells. (A) The expression of β-catenin, GSK-3β and p-GSK-3β were detected in prostate cancer cells PC3 and 22RV1 treated with gradient concentrations of β-ionone for 24 h. (B) Intracellular β-catenin was overexpressed and cells were treated with 180 μM β-ionone or DMSO, and relevant protein expression levels of the EMT were assayed by Western blot. (C) Nucleoplasmic protein separation and (D) Cell immunofluorescence detection of the effect of β-ionone treatment on the expression levels of β-catenin in the cytoplasm and nucleus. Magnification, $\times$100. Scale bar, 20 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

As expected, β-ionone accelerated the degradation of the β-catenin protein (Fig. 4B,C), which was slowed by proteasome inhibition with MG132 (Fig. 4D). The ubiquitination assays further confirmed that β-ionone significantly increased the ubiquitination level of β-catenin (Fig. 4E). In summary, β-ionone promotes the ubiquitination and degradation of β-catenin, thereby inhibiting the Wnt/β-catenin pathway.

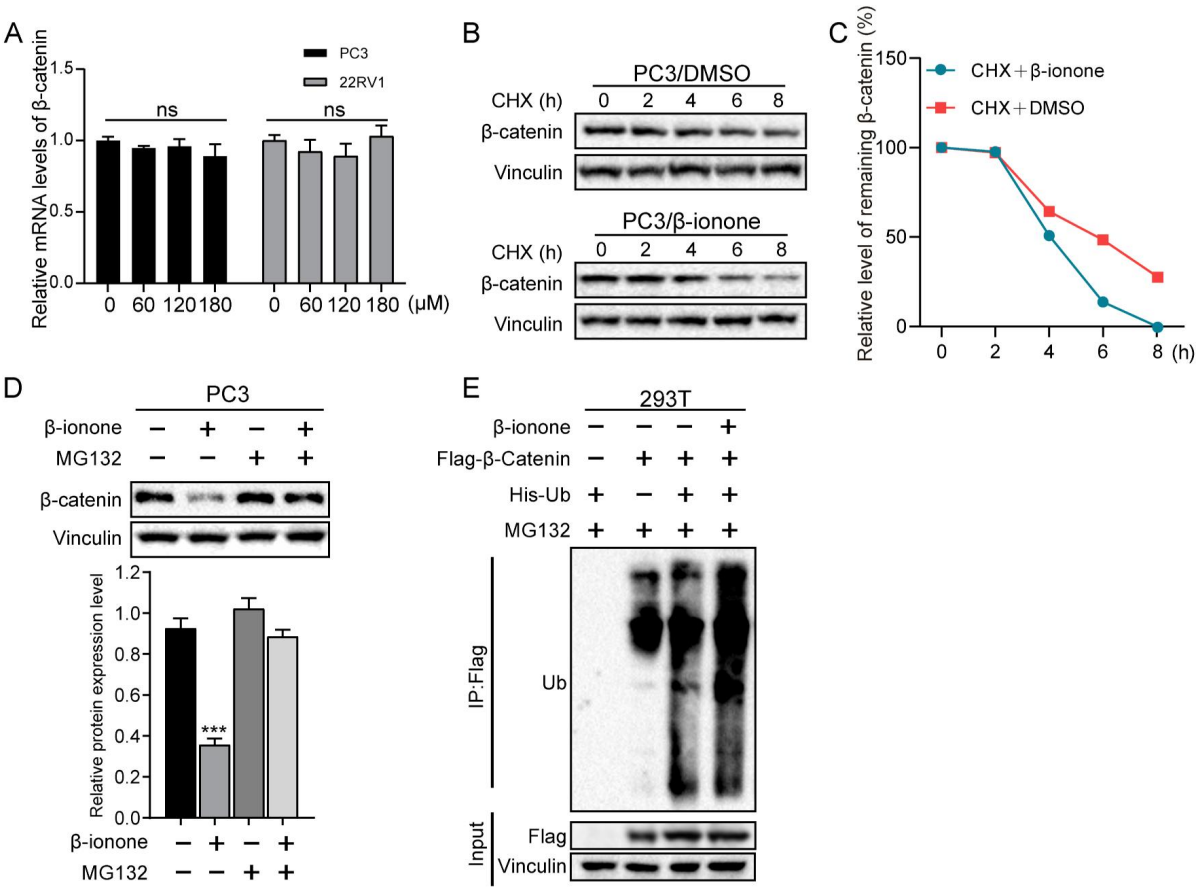

Fig. 4. β-ionone inhibitis the Wnt/β-catenin signaling pathway by regulating its ubiquitination and degradation. (A) Prostate cancer cells PC3 and 22RV1 were treated with gradient concentrations of β-ionone for 24 h (A) to detect the mRNA levels of β-catenin. ns $p > 0.05$. (B,C) Protein synthesis was inhibited using CHX and the effect of 180 μM β-ionone on the rate of β-catenin degradation in cells was determined. (D) Effects of β-ionone or DMSO on β-catenin protein levels after treatment with MG132. (E) It was investigated the effect of 180 μM β-ionone treatment on the ubiquitination level of β-catenin in 293T cells by Immunoprecipitation. ***$p < 0.001$.

3.5 β-ionone Inhibits PCa EMT and Tumor Growth in Vivo

To verify the results *in vitro* , we used human prostate cancer PC3 cells to construct subcutaneous xenograft tumors in nude mice as an *in vivo* model. According to the previous studies, the dose of β-ionone was set at 75 mg/kg, and there were no abnormal changes in diet and body weight of the two groups of mice during the treatment. In the subcutaneous xenografts of nude mice, β-ionone showed an inhibitory effect on the proliferation of xenografts (Fig. 5A–C). We speculate that it may be related to its anti-inflammatory effect *in vivo*, which needs to be further explored. Western blot showed that the expression levels of β-catenin and Vimentin were down-regulated in tumor tissues in the β-ionone treatment group, while promoting the expression of E-cadherin (Fig. 5D). Immunohistochemical results showed that protein expression the positive rate of β-catenin, Vimentin and Ki67 protein in the treatment group was lower than in the control group, indicating that β-ionone may inhibit tumor proliferation *in vivo*. The related proteins of Wnt/β-catenin and EMT are consistent with the western blot analysis (Fig. 5E). In conclusion, these results are consistent with the results *in vitro*, indicating that β-ionone inhibits PCa EMT *in vivo* by down-regulating the Wnt/β-catenin pathway.

3.5 Molecular Docking

To explore the possible targets of β-ionone, we used the website Swiss target prediction, Similarity ensemble approach and SuperPred for predictions. The intersection of the top ten prediction results of each website was obtained, and finally the proteins encoded by the three genes of RXRA, RXRB, and RXRG were most likely to be used as targets for β-ionone. Further analysis using PLIP revealed that β-ionone could bind to 243A, 244A, 249A, and 316A of RXRA-encoded proteins, 504A, 505A, and 508A of RXRB-encoded proteins, and 274B, 275B, and 278B residues of RXRG-encoded proteins through hydrophobic interaction (**Supplementary Fig. 2**).

4. Discussion

PCa has become the most common urological malignancy in males. In recent years, due to the increased use of

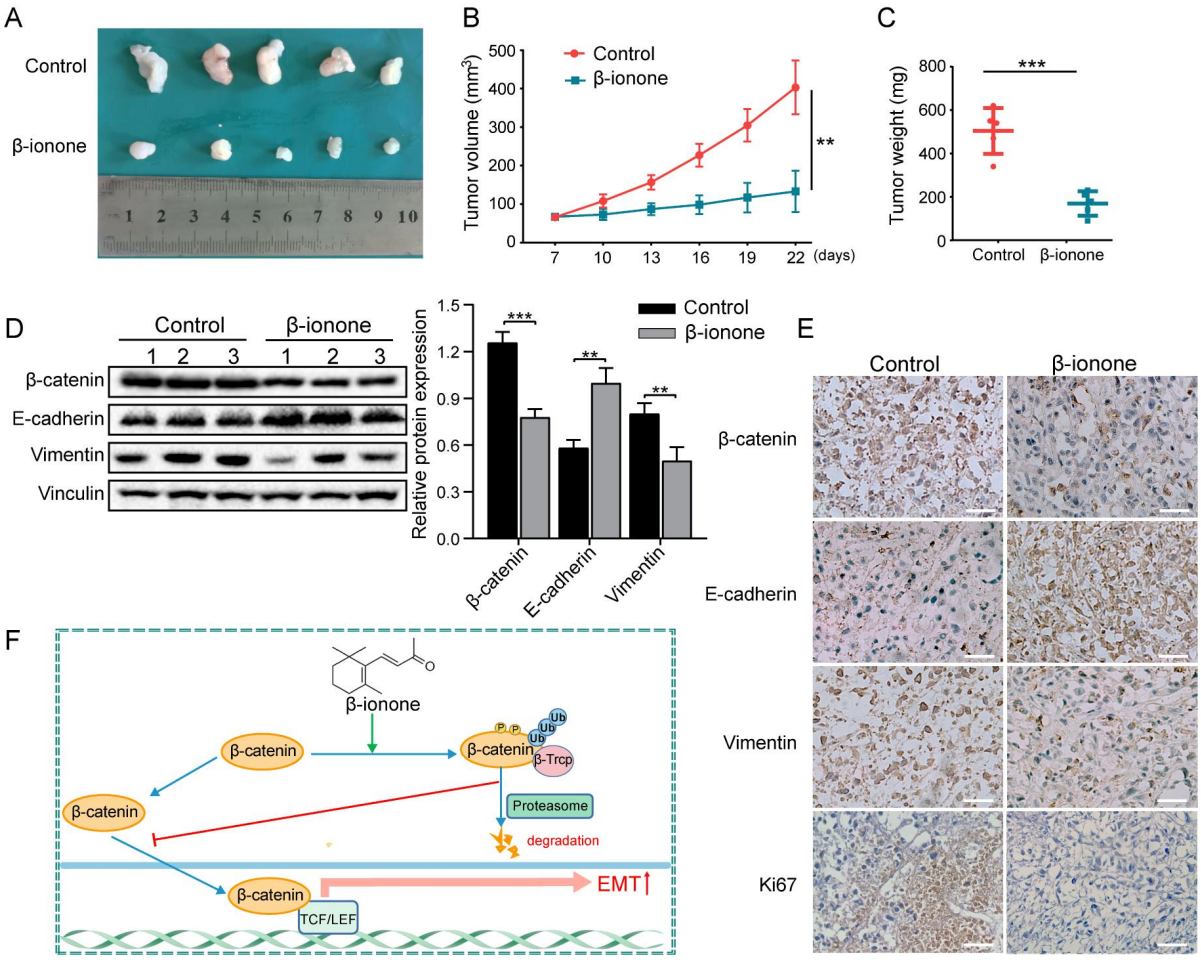

Fig. 5. ***β*-ionone inhibits the Wnt/*β*-catenin pathway and EMT *in vivo*.** (A) PC3 cells were injected subcutaneously into male nude mice, and tumors were dissected after treatment with *β*-ionone or corn oil for 22 days. (B) Tumor volume was measured every three days in corn oil and *β*-ionone treated mice. (C) Mice were sacrificed in 22 days and tumor weights were weighed. (D) The expression levels of *β*-catenin, E-cadhrin, and Vimentin in subcutaneous xenografts of mice were detected by Western blot and Immunohistochemical analysis (E). Immunohistochemical analysis of the expression levels of *β*-catenin, E-cadhrin, Vimentin and Ki67 in subcutaneous xenografts. (F) Pattern diagram was drawn to depict *β*-ionone inhibiting EMT process in prostate cancer cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

neoadjuvant endocrine therapy, radical surgery, radiotherapy, and adjuvant endocrine therapy, the prognosis of patients with PCa has improved significantly [20]. However, tumor metastasis is still the main cause of death in patients with advanced PCa. It is thus urgently needed to explore novel and effective anti-metastatic agents against advanced PCa for a better prognosis.

β-ionone has been foung to have strong anti-inflammation, anti-bacterial, and anti-tumor activity [21]. Jones *et al.* [22] found that *β*-ionone treatment significantly inhibited the viability of PCa LNCaP, PC3 and DU145 cells. In DU145 and PC3 cells, *β*-ionone down-regulated the expression of cyclin-dependent kinase (CDK4) and cyclin D1, and induced cell cycle arrest in the G1 phase. In terms of cell proliferation, Xie *et al.* [14] also reported that *β*-ionone could act as a PSGR ligand to activate PSGR, which then activated p38 and JNK. Activation of p38 and JNK blocks AR entry into the nucleus, thereby inhibiting the proliferation of PCa cells [23]. In addition, the inhibitory effect on AR and PSGR receptor-positive LNCaP and C4-2 cell proliferation was more pronounced.

Currently, there are few studies on the effects of *β*-ionone on migration, invasion and EMT in PCa cells. Sanz *et al.* [24] found that *β*-ionone, a PSGR agonist, can promote the metastasis and spread of LNCaP cells in subcutaneous xenograft mice model. Our study indicated that *β*-ionone could significantly inhibit cell migration, invasion ability and EMT in PCa PC3 and 22RV1 cells. The reason for the different results may be possibly attributed to the higher expression level of PSGR in LNCaP cells [14]. Activation of PSGR can regulate Mitogen-Activated Protein Kinase (MAPK) and NF-*κ*B pathways to participate in tumor metastasis by targeting Intercellular Adhesion Molecule 1 (ICAM1), RELB Proto-Oncogene (RELB) and Interleukin

1 Beta (IL1B) [25]. Therefore, in PSGR-positive PCa cells, β-ionone can suppress the proliferation of PCa cells by promoting cell apoptosis and activating PSGR to inhibit AR translocation, and on the other hand, activation of PSGR by β-ionone can also promote cell migration and invasion. In our study, PCa PC3 and 22RV1 cell lines serving as the model system *in vitro* and *in vivo* hardly express PSGR [14], so PSGR/MAPK/ NF-κB-mediated cell migration and invasion might be limited in those cell lines. After exposure to β-ionone in PC3 and 22RV1 cells, the migration and invasion aiblitiy of PCa cells were inhibited, and the western blotting also showed that E-cadherin was up-regulated, while the epithelial phenotypic markers N-cadherin and Vimentin were down-regulated. These results suggest that β-ionone may act on PCa through other PSGR-independent signaling pathways, which warranted further in-depth studies.

These results suggest that β-ionone may act on PCa through other pathways. It is well known that GSK-3β in the classical Wnt/β-catenin pathway can promote phosphorylation and degradation of β-catenin. The activity of GSK-3β is regulated by phosphorylation of its Ser-9 and Tyr-216, and phosphorylation of Ser-9 will reduce the activity of GSK-3β [26]. The p-GSK-3β phosphorylation site detected in our study is Ser-9 and our results showed that β-ionone reduced Ser-9 phosphorylation on GSK-3β and enhanced degradation of β-catenin, which is consistent with previous studies. In addition, it has been previously reported that β-ionone can inhibit the PI3K/Akt pathway [27]. Akt can promote the phosphorylation of Ser-9 of GSK-3β [28], so this may also be one of the reasons for the decrease in GSK-3β phosphorylation level and enhanced degradation of β-catenin in this study. To further explore the possible targets of β-ionone, several online prediction tools including Swiss target prediction, SEA, and SuperPred were applied. We found that both Retinoid X Receptor Alpha (RXRA), Retinoid X Receptor Beta (RXRB), and Retinoid X Receptor Gamma (RXRG) may be possible receptors for β-ionone (**Supplementary Fig. 2**). The retinoic acid X receptors (RXRs) family are nuclear receptors that mediate the biological effects of retinoic acid by participating in retinoic acid-mediated gene activation. Although there is currently a lack of evidence linking β-ionone to the RXR family, previous studies have shown that activation of RXRA (RXRα) is associated with the degradation of β-catenin. In 2008, it was reported that retinol could induce RXRα to bind to β-catenin and promoted its degradation [29]. Similarly, Liang *et al*. [30] found that RXRα can bind to β-catenin, and that RXRα overexpression can promote the ubiquitination of β-catenin in colorectal cancer. Combining this evidence with our predictions, we suspect that β-ionone may promote the degradation of β-catenin by activating the expression of RXRA. These conjecture also need to be confirmed by more further experiments.

Although the PC3 and 22RV1 cells used in our study have mesenchymal changes as tumor cells themselves, they still express epithelial markers (E-cadherin), and the EMT process also can be induced and enhanced by TGF-β. The above facts indicate that there is still some EMT process in these two tumor cells, so it is feasible for us to study the inhibitory effect of β-ionone on EMT. The Wnt/β-catenin pathway is a vital pathway regulating the occurrence of EMT in tumors, and the expression level of its critical molecule β-catenin is correlated with tumor progression and prognosis [31]. Yu *et al*. [32] found that activation of the Wnt/β-catenin pathway can up-regulate the expressions of Nkx3.1 and Probasin in epithelial cells to promote prostate hyperplasia and induce the development of low-level PIN to high-level PIN in studies involving prostatic hyperplasia and intraepithelial prostate neoplasia. Some researchers have also found that some drugs, such as Wogonoside [33] and Oldhamianoside II [34] could inhibit the activation of the Wnt/β-catenin pathway and thus inhibit EMT in PCa cells. There is also crosstalk between the AR signal and the Wnt/β-catenin signal. For androgen-dependent PCa cells, AR inhibits Wnt signaling by inhibiting WNT5A and LEF1 expression. During androgen deprivation therapy, AR's inhibition of Wnt/β-catenin signaling is weakened, and activated Wnt signaling, which in turn activates AR, causing androgen-independent growth of PCa [35]. Similarly, the AR and Wnt/β-catenin signaling pathways in CRPC stimulate each other to activate specific target genes and promote androgen-dependent growth and progression in PCa cells [36]. Due to this crosstalk mechanism, inhibiting the Wnt/β-catenin pathway in Castration-Resistant Prostate Cancer (CRPC) was reported to overcome its resistance to Enzalutamide and to reduce the incidence of tumor metastasis [37].

In summary, current research has confirmed that the Wnt/β-catenin pathway plays an important role in prostate cancer cell invasion and EMT. This study investigated whether β-ionone affects EMT through the Wnt/β-catenin pathway. The results showed that the expressed levels of p-GSK-3β and β-catenin were significantly decreased in PC3 and 22RV1 cells treated with β-ionone, and the content of β-catenin in the nucleus was significantly decreased. Subsequent turnover and protein ubiquitination showed that β-ionone could promote the ubiquitination and degradation of β-catenin in PCa PC3 and 22RV1 cells. Targeting Wnt/β-catenin pathway by β-ionone thus provided a novel approach for PCa treatment.

5. Conclusions

In conclusion, this study is the first to discover the potential role of β-ionone on the Wnt/β-catenin signaling pathway: β-ionone can promote the ubiquitination and degradation of β-catenin in PCa cells, and negatively regulate the Wnt/β-catenin pathway, thus suppressing cell migration, invasion, and EMT. Therefore, β-ionone may serve as a potential drug for the treatment of PCa.

Abbreviations

EMT, epithelial-mesenchymal transition; PCa, prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; PSGR, prostate-specific G-protein coupled receptor; TCF/LEF, T-cell factor/lymphoid enhancer factor.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

TH, and JZ designed the research study. QF, TQ and BL performed the research. WD, YW and YF provided help and advice on laboratory techniques. QF, TQ and BR analyzed the data and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal experiments were conducted under the guidance of the Committee for Animal Protection and Utilization of Xi'an Jiaotong University, and executed according to standard ethical guidelines (2020-G-208).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2712335.

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: a Cancer Journal for Clinicians. 2020; 70: 7–30.

[2] Rebello RJ, Christoph O, Knudsen KE, Loeb S, Johnson DC, Reiter R E, *et al*. Prostate cancer (Primer). Nature Reviews: Disease Primers. 2021; 7: 9.

[3] Gundem G, Van Loo P, Kremeyer B, Alexandrov LB, Tubio JMC, Papaemmanuil E, *et al*. The evolutionary history of lethal metastatic prostate cancer. Nature. 2015; 520: 353–357.

[4] Buyel JF. Plants as sources of natural and recombinant anticancer agents. Biotechnology Advances. 2018; 36: 506–520.

[5] Kalhori MR, Khodayari H, Khodayari S, Vesovic M, Jackson G, Farzaei MH, *et al*. Regulation of Long Non-Coding RNAs by Plant Secondary Metabolites: A Novel Anticancer Therapeutic Approach. Cancers. 2021; 13: 1274.

[6] Lu Y, Yang Q, Lin Z, Yang X. A modular pathway engineering strategy for the high-level production of β-ionone in Yarrowia lipolytica. Microbial Cell Factories. 2020; 19: 49.

[7] Yang J, Mu W, Cao Y, Liu G. Synthesis and biological evaluation of β-ionone oriented proapoptosis agents by enhancing the ROS generation. Bioorganic Chemistry. 2020; 104: 104273.

[8] Ansari M, Emami S. B-Ionone and its analogs as promising anticancer agents. European Journal of Medicinal Chemistry. 2016; 123: 141–154.

[9] Kang C, Jayasooriya RGPT, Choi YH, Moon S, Kim W, Kim G. B-Ionone attenuates LPS-induced pro-inflammatory mediators such as no, PGE2 and TNF-α in BV2 microglial cells via suppression of the NF-κB and MAPK pathway. Toxicology *in Vitro*. 2013; 27: 782–787.

[10] Dong H, Zhang S, Sun W, Liu Q, Ibla JC, Soriano SG, *et al*. B-Ionone arrests cell cycle of gastric carcinoma cancer cells by a MAPK pathway. Archives of Toxicology. 2013; 87: 1797–1808.

[11] Abd-Elbaset M, Mansour AM, Ahmed OM, Abo-Youssef AM. The potential chemotherapeutic effect of β-ionone and/or sorafenib against hepatocellular carcinoma via its antioxidant effect, PPAR-γ, FOXO-1, Ki-67, Bax, and Bcl-2 signaling pathways. Naunyn-Schmiedeberg's Archives of Pharmacology. 2020; 393: 1611–1624.

[12] Dong H, Wang K, Chang X, Jin F, Wang Q, Jiang X, *et al*. Beta-ionone-inhibited proliferation of breast cancer cells by inhibited COX-2 activity. Archives of Toxicology. 2019; 93: 2993–3003.

[13] Kypta RM, Waxman J. Wnt/β-catenin signalling in prostate cancer. Nature Reviews Urology. 2012; 9: 418–428.

[14] Xie H, Liu T, Chen J, Yang Z, Xu S, Fan Y, *et al*. Activation of PSGR with β-ionone suppresses prostate cancer progression by blocking androgen receptor nuclear translocation. Cancer Letters. 2019; 453: 193–205.

[15] Hiremath IS, Goel A, Warrier S, Kumar AP, Sethi G, Garg M. The multidimensional role of the Wnt/β-catenin signaling pathway in human malignancies. Journal of Cellular Physiology. 2022; 237: 199–238.

[16] Clevers H, Nusse R. Wnt/β-Catenin Signaling and Disease. Cell. 2012; 149: 1192–1205.

[17] Koni M, Pinnarò V, Brizzi MF. The Wnt Signalling Pathway: A Tailored Target in Cancer. International Journal of Molecular Sciences. 2020; 21: 7697.

[18] Shi S, Li C, Zhang Y, Deng C, Liu W, Du J, *et al*. Dihydrocapsaicin inhibits cell proliferation and metastasis in melanoma via down-regulating β-catenin pathway. Frontiers in Oncology. 2021; 11: 648052.

[19] Wu S, Huang J, Hui K, Yue Y, Gu Y, Ning Z, *et al*. 2'-Hydroxyflavanone inhibits epithelial-mesenchymal transition, and cell migration and invasion via suppression of the Wnt/β-catenin signaling pathway in prostate cancer. Oncology Reports. 2018; 40: 2836–2843.

[20] Litwin MS, Tan H. The Diagnosis and Treatment of Prostate Cancer: A Review. Journal of the American Medical Association. 2017; 317: 2532.

[21] Paparella A, Shaltiel-Harpaza L, Ibdah M. β-Ionone: Its Occurrence and Biological Function and Metabolic Engineering. Plants. 2021; 10: 754.

[22] Jones S, Fernandes NV, Yeganehjoo H, Katuru R, Qu H, Yu Z, *et al*. B-Ionone Induces Cell Cycle Arrest and Apoptosis in Human Prostate Tumor Cells. Nutrition and Cancer. 2013; 65: 600–610.

[23] Gioeli D, Black BE, Gordon V, Spencer A, Kesler CT, Eblen ST, *et al*. Stress Kinase Signaling Regulates Androgen Receptor Phosphorylation, Transcription, and Localization. Molecular Endocrinology. 2006; 20: 503–515.

[24] Sanz G, Leray I, Grébert D, Antoine S, Acquistapace A, Muscat A, *et al*. Structurally related odorant ligands of the olfactory re-

ceptor or51E2 differentially promote metastasis emergence and tumor growth. Oncotarget. 2017; 8: 4330–4341.
[25] Li Y, Li Q, Li D, Gu J, Qian D, Qin X, *et al*. Exosome carrying PSGR promotes stemness and epithelial-mesenchymal transition of low aggressive prostate cancer cells. Life Sciences. 2021; 264: 118638.
[26] Wu D, Pan W. GSK3: a multifaceted kinase in Wnt signaling. Trends in Biochemical Sciences. 2010; 35: 161–168.
[27] Liu Q, Dong H, Sun W, Liu M, Ibla JC, Liu L, *et al*. Apoptosis initiation of β-ionone in SGC-7901 gastric carcinoma cancer cells via a PI3K-AKT pathway. Archives of Toxicology. 2013; 87: 481–490.
[28] Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A. Glycogen synthase kinase 3: more than a namesake. British Journal of Pharmacology. 2009; 156: 885–898.
[29] Dillard AC, Lane MA. Retinol Increases beta-catenin-RXRalpha binding leading to the increased proteasomal degradation of beta-catenin and RXRalpha. Nutrition and Cancer. 2008; 60: 97–108.
[30] Liang J, Tang J, Shi H, Li H, Zhen T, Duan J, *et al*. MiR-27a-3p targeting RXRα promotes colorectal cancer progression by activating Wnt/β-catenin pathway. Oncotarget. 2017; 8: 82991–83008.
[31] Zhang Y, Wang X. Targeting the Wnt/β-catenin signaling pathway in cancer. Journal of Hematology & Oncology. 2020; 13: 165.
[32] Yu X, Wang Y, Jiang M, Bierie B, Roy-Burman P, Shen MM, *et al*. Activation of beta-Catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. Prostate. 2009; 69: 249–262.
[33] Wei C, Jing J, Zhang Y, Fang L. Wogonoside Inhibits Prostate Cancer Cell Growth and Metastasis via Regulating Wnt/β-Catenin Pathway and Epithelial-Mesenchymal Transition. Pharmacology. 2019; 104: 312–319.
[34] Li K, Zhan X, Sun J, Wang T, Dong H, Jing F, *et al*. Oldhamianoside II inhibits prostate cancer progression via regulation of EMT and the Wnt/β-catenin signaling pathway. Oncology Letters. 2018; 15: 9457–9463.
[35] Luo J, Wang D, Wan X, Xu Y, Lu Y, Kong Z, *et al*. Crosstalk Between AR and Wnt Signaling Promotes Castration-Resistant Prostate Cancer Growth. OncoTargets and Therapy. 2020; 13: 9257–9267.
[36] Pakula H, Xiang D, Li Z. A Tale of Two Signals: AR and WNT in Development and Tumorigenesis of Prostate and Mammary Gland. Cancers. 2017; 9: 14.
[37] Zhang Z, Cheng L, Li J, Farah E, Atallah NM, Pascuzzi PE, *et al*. Inhibition of the Wnt/β-Catenin Pathway Overcomes Resistance to Enzalutamide in Castration-Resistant Prostate Cancer. Cancer Research. 2018; 78: 3147–3162.