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Original Paper

Pristimerin Inhibits Prostate Cancer Bone Metastasis by Targeting PC-3 Stem Cell Characteristics and VEGF-Induced Vasculogenesis of BM-EPCs

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Key Words
Pristimerin • Bone metastasis • Prostate cancer cells • Stemness • Endothelial progenitor cells • Vasculogenesis

Abstract
Background/Aims: Prostate cancer (PCa) is one of the most common malignant cancers and a major leading cause of cancer deaths in men. Cancer stem-like cells are shown to be highly tumorigenic, pro-angiogenic and can significantly contribute to tumor new vessel formation and bone marrow derived-EPCs (BM-EPCs) are shown to recruit to the angiogenic switch in tumor growth and metastatic progression, suggesting the importance of targeting cancer stem cells (CSCs) and EPCs for novel tumor therapies. Pristimerin, an active component isolated from Celastraceae and Hippocrateaceae, has shown anti-tumor effects in some cell lines in previous studies. However, the effect and mechanism of Pristimerin on CSCs and EPCs in PCa bone metastasis are not well studied. ***Methods:*** The effect of Pristimerin on PC-3 stem cell characteristics and metastasis were detected by spheroid formation, CD133 and CD44 protein expression, matrix-gel invasive assay and colony-formation assay in vitro, VEGF and pro-inflammatory cytokines expression by ELISA assay, and tumor tumorigenicity by X-ray and MR in NOD-SCID mice model in vivo. In addition, we also detected the effect of Pristimerin on VEGF-induced vasculogenesis and protein expression of BM-EPCs. ***Results:*** Pristimerin could significantly inhibit spheroid formation and protein expression of CD133 and CD44, reduce VEGF and pro-inflammation cytokines expression of PC-3 cell, and prevent the xenografted PC-3 tumor growth in the bone of nude mice. The present data also showed that Pristimerin significantly inhibited VEGF-induced vasculogenesis of BM-EPCs by suppressing the EPCs functions including proliferation, adhesion, migration, tube formation and inactivation the

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phosphorylation of VEGFR-2, Akt and eNOS. ***Conclusion:*** These data provide evidence that Pristimerin has strong potential for development as a novel agent against prostate bone metastasis by suppressing PC-3 stem cell characteristics and VEGF-induced vasculogenesis of BM-EPCs.

Introduction

Prostate Cancer (PCa) is the most common male malignancy in western countries and represents a major disease burden in the world. The principal problem arising from PCa is its propensity to metastasize to bone. Once tumors metastasize to bone, they are virtually incurable and result in significant disease morbidity prior to a patient's death [1-4]. Despite the high incidence and serious consequences of skeletal metastasis of prostate cancer, the mechanism underlying this osteotropism is unclear.

Recent identification of prostate cancer stem cells (CSCs) has provided a new insight into prostate carcinogenesis [5-8]. CSCs display unlimited proliferation potential, ability to self-renew, and capacity to generate a progeny of differentiated cells that constitute the major tumor population [9-11]. CSCs are the critical drivers of tumor progression and metastasis [12, 13]. Moreover, the highly resistant nature of CSCs to different chemotherapies suggested that these cells may also contribute to treatment failure and disease relapse [14]. Most CSCs have been identified by cell-surface markers for the corresponding normal tissue stem/progenitor cells. Prostate CSCs were first identified in prostate tumors using $CD44^+/\alpha_2ß_1^{hi}/CD133^+$ as the cell surface markers [15]. Using similar approaches, CSCs have also been identified in prostate cancer cell lines PC-3 and LNCaP [16, 17].

Metastasis and neovascularization can be affected by many factors. Pro-inflammatory cytokines, including interleukin-1ß (IL-1ß,), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-α) could be prometastatic or proangiogenic and their deregulated expression directly correlate with the metastatic potential of several human carcinomas [18]. Therefore, anti-inflammatory drug may be an ideal strategy to inhibit PCa bone metastasis.

It may seem counterintuitive to expect that CSC, which by definition represent a very small minority of cells in a tumor, could make a significant contribution to tumor vasculogenesis. Indeed, transient bursts of angiogenic activity can be sufficient to initiate sustained tumor new vessel formation [19]. Therefore, it is possible that CSC provide the signals necessary to trip the "angiogenic switch" early during the growth of primary and/or metastatic tumors. An emerging area of research suggests that CSCs may also support tumor vasculogenesis that are partly dependent on increased expression of VEGF [20].

In cancer patients with metastasis, neovascularization-mediated progression of micrometastasis to lethal macrometastasis is the major cause of mortality [1]. Many studies have suggested that endothelial progenitor cells (EPCs) residing in the bone marrow can be recruited to tumor in response to tumor-derived cytokines, where they contribute to vascular development by incorporating into the walls of nascent capillaries [21]. CSCs have been shown to be highly pro-angiogenic compared with the majority of tumor cells [7]. VEGF is expressed in PCa at a high level [22-24], and its expression correlates with increasing grade, vascularity, and tumorigenicity [24, 25]. Meanwhile, VEGF can promote vasculogenesis of EPC at the site of tumor neovascularization [26], in which EPCs differentiate into mature endothelial cells, and incorporate into growing tumor vessels [27, 28]. Notably, recent studies have shown that bone marrow derived-EPCs (BM-EPCs) are recruited to the angiogenic switch in tumor growth and metastatic progression [29-31].

Recent studies have shown that a number of botanical compounds have anti-CSC effect. Pristimerin, a naturally occurring quinonemethide triterpenoid compound isolated from Celastraceae and Hippocrateaceae, attracts considerable interest due to its potential chemopreventive and chemotherapeutic properties [32-34]. Pristimerin has anti-inflammatory, antioxidant, antimalarial, and insecticidal activities and possess growth

inhibitory effect on a series of human cancer cell lines such as breast cancer, cervical cancer, multiple myeloma tumors, and prostate cancer [35-38]. However, the potential efficacy of Pristimerin against PCa bone metastasis remains unknown.

The aim of this study was to investigate whether Pristimerin, can inhibit the activies of human bone metastatic prostate cancer PC-3 cells and supress bone tumorigenicity of PC-3 cells *in vivo*, and, if so, through what mechanisms.

Materials and Methods

Reagents

The purity of Pristimerin (purity e98%, Enzo Life Sciences, Lausen, Switzerland) used for experiments. Pristimerin was dissolved in dimethyl sulfoxide (DMSO, sigma, Saint Louis, USA) to a stock concentration of 50 mM and then aliquoted and stored at -20°C.

Culture conditions of PC-3 cell line

Human prostatic adenocarcinoma PC-3 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in F-12 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), in a 37°C, 5% CO_2 environment at constant humidity.

Isolation and cultivation of EPCs

Bone marrow was collected from the drill holes of the pedicle during internal spine fixation of patients with lumbar degenerative diseases (15 patients, age range 59–72 years, mean age 63.28 years). Informed consent for bone marrow collection was obtained from the patients, and all procedures were performed in accordance with the guidance and approval of a research ethics committee in the First Affiliated Hospital of Sun Yat-sen University (NO.2008-55). The procedures for isolation, cultivation and identification of human EPC cultures followed previously published methods [39].

Spheroid formation assay

The spheroid formation assay was modified from a previously reported protocol [40]. Briefly, PC-3 cells were pre-treated with Pristimerin (0, 0.4, 0.8, 1.6 μM) for 24h and then plated at the density of 400 cells/well on 6-well poly-HEMA (Sigma Aldrich, St. Louis, USA) -coated plates maintained in F-12 medium supplemented with B27 (1:50, Invitrogen, Carlsbad, CA, USA), 20 ng/mL EGF (BD Biosciences, San Jose, CA, USA), and 20 ng/mL bFGF (Invitrogen, Carlsbad, CA, USA) for 14 d. After 14 d, the number of prostaspheres (tight, spherical, nonadherent masses >100 μm in diameter) were counted, and images of prostaspheres were captured under the light microscope. For serial passage of primary spheres, the primary spheres were treated with Pristimerin for the above doses for 24 h and subsequently collected, dissociated with trypsin, and resuspended in F12 medium. After 14 d, the number of prostaspheres (tight, spherical, nonadherent masses >100 μm in diameter) were counted, and images of prostaspheres were captured under the light microscope. Sphere formation efficiency = colonies/input cells×100%.

In vitro invasion assay

The invasion assay was done by using Transwell chamber consisting of 8 mm membrane filter inserts (Corning, NY, USA) coated with Matrigel (ECMatrix™, BD Biosciences, Heidelberg, Germany) as previously described [40]. Briefly, PC-3 cells were cultivated and treated with indicated concentrations of Pristimerin for 24 h and cells were trypsinized and suspended in serum-free medium. Then 1.5×10^5 cells were added to the upper chamber, whereas lower chamber was filled with medium with 10% FBS. After incubated for 48 h, cells were invaded through the coated membrane to the lower surface, in which cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min at room temperature. The cell count was done under the microscope (100×).

Colony formation assay

Colony formation assay was performed as previously described [40, 41]. Brifely, PC-3 cells were cultivated and treated with indicated concentrations of Pristimerin for 24h before they were plated onto

a 65-mm Petri dish at 300 cells as single cells for 12 days, and colonies were stained with crystal violet. Plating efficiency = number of colonies (≥50 cells per colony) per input cells x 100%.

Enzyme-linked immunosorbent assay (ELISA)

Briefly, PC-3 cells grown in 24-well plates were treated with (0, 0.2, 0.4, 0.8 μM) of Pristimerin for 48 h. Cell supernatant was collected and centrifuged at 2,000 x g at 4°C for 10 min to remove cell debris, and then used for ELISA assay of VEGF (Excell, Shanghai, China), IL-1ß (R&D Systems, Minneapolis, MN, USA), IL-6 (R&D Systems, Minneapolis, MN, USA), IL-8 (R&D Systems, Minneapolis, MN, USA), and TNF-α (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Cell viability assay

Cell viability was determined by 2-(2-methoxy-4-nitrophenyl)-3-(4- nitrophenyl)-5-(2,4 -disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay kit (CCK-8, Dojindo, Japan). BM-EPCs (5×10^3 cells/well) were treated with Pristimerin (0, 0.2, 0.4, 0.8 μM) and/or without VEGF for 36 h. CCK-8 was used according to the manufacturer's instructions. WST-8 was added into each well and incubated for 4 h. The absorbance at 450 nm was measured using a microplate reader.

Cell adhesion assay

Cell-matrix adhesion assay. The cell-matrix adhesion assay was performed as previously described [42]. Briefly, human BM-EPCs were cultured for 24 h at 90% confluency and treated with Pristimerin (0, 0.2, 0.4, 0.8 μM) and/or without VEGF for 24 h as above. Then, BM-EPCs at 1×104 cells/well were plated onto fibronectin-coated 96-well culture plates and incubated for 30 min at 37°C. Thereafter, non-adherent cells were removed by washing three times with PBS. The adherent cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and stained with 0.1% crystal violet for 30 min at room temperature. Adherent cells were counted using a phase contrast microscope by independent, blinded investigators.

Cell-cell adhesion assay. For cell-cell adhesion assay, BM-EPCs were seeded at a density of 1×104 cells/ well in 96-well plate until confluency. Meanwhile, another group of BM-EPCs were cultured for 24 h at 90% confluency and treated with Pristimerin (0, 0.2, 0.4, 0.8 μM) and/or without VEGF, for 24 h as above. Then, the EPCs were labeled with 5-Chloromethylfluorescein Diacetate (CellTracker™ Green CMFDA, Invitrogen) and seeded at a density of 1×104 cells/well to the plate which containing 100% confluent EPC monolayer. The plate was incubated at 37°C for 30 min. Thereafter, non-adherent cells were removed by washing three times with PBS. Adherent cells were counted under the fluorescence microscope (OLYPUMS IX81, JAPAN) by independent blinded investigators.

EPCs migration assay

A transwell migration assay was performed as described previously [43]. Briefly, BM-EPCs (8×10^4 cell/well) along with the Pristimerin (0, 0.2, 0.4, 0.8 μM) were seeded into the upper chambers. The bottom chambers were filled with 600 μL EBM-2 supplemented with 10 ng/mL VEGF. The transwell system was incubated for 6-8 h in 5% CO_2 at 37°C. Thereafter, non-migrating cells on the upper surface of the filter were removed using a cotton-tipped swab, and cells on the lower surface were stained with 1% crystal violet. Images were taken using an inverted microscope (Magnification, ×100, OLYPUMS IX81, JAPAN).

Capillary-like tube formation assay

Tube formation was assessed as described previously [39]. Briefly, EPCs were pretreated with Pristimerin (0, 0.2, 0.4, 0.8 μM) for 30 min, and then seeded onto the Matrigel layer in 96–well plates at a density of 1×10^4 cells with or without 10 ng/mL VEGF. Capillary-like tubes were imaged after 6-12 h incubation using an inverted microscope (Olympus; magnification, ×100). All side branches were counted by three independent investigators in a blinded manner.

Western blot

To determine the effects of Pristimerin on the VEGFR2–dependent signaling pathway, BM-EPCs were first starved in serum-free EBM-2 for 6 h, and then pretreated with or without Pristimerin for 30 min, followed by the stimulation with 50 ng/mL of VEGF for 10 min (for VEGFR2 activation). Total protein was

extracted from the cultured cells using radioimmunoprecipitation lysis buffer with protease inhibitors, and the protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Proteins were resolved on sodium dodecyl sulfate (SDS) polyacrylamide gel by electrophoresis and then transferred onto Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ). Then the membranes were blocked by 5% non-fat dry milk and incubated with primary antibodies against CD133 (Miltenyi Biotech, Auburn, CA), CD44 (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-VEGFR2, phospho-eNOS, phospho-Akt, VEGFR2, eNOS, Akt and α-tubulin (Cell Signaling Technology) for 2 h at room temperature. After washing with TBST, the membrane was incubated with anti-rabbit IgG secondary antibodies, and the signals were visualized by chemiluminescence (ECL kit, Amersham, Piscataway, NJ). All immunoblots showed are representative of at least three independent experiments.

In vivo tumorigenicity assay

Intra-tibial injection model was used to determine whether Pristimerin can inhibit bone metastasis tumorigenicity. Six male severe combined immunodeficient (SCID) mice of 3-4 weeks old were purchased from HFK Bio-Technology.CO. LTD (Beijing, China). Before inoculation, PC-3 cells were treated with 1.6 μM Pristimerin for 24 h and resuspended at the density of 3×10^5 cells per 40 μL, and injected into the tibia using a drilling motion. On the 5th week, hind limbs were radiographed using a Faxitron X-ray machine (Faxitron X-ray Corp, USA) to detect the bone lesions. Bone lesions were evaluated and calculated where 0 grade for no lesion, 1 for minor lesions, 2 for small lesions, 3 for significant lesions with minor break of margins, and 4 for significant lesions with major break in peripheral lesions. Meanwhile, MRI (3.0 T MRI, GE Signa EXCITE HD) was used to detect the tumorigenicity. Subsequently, mice were sacrificed, and tibias were collected, decalcified and fixed in formalin for further histologic analysis.

Statistical analysis

All data are expressed as the mean± standard deviation from at least three individual experiments. Statistical comparisons between groups were performed by one-way ANOVA followed by Student's t-test using SPSS 16.0 software package. P values less than 0.05 were considered statistically significant.

Results

Pristimerin suppresses stem cell characteristics of PC-3 in vitro

The ability to form prostaspheres in non-adherent culture is one of the characteristics of prostate CSCs [44-46]. To test if Pristimerin treatment can inhibit prostate CSC properties, prostasphere formation of PC-3 was studied in the presence or absence of Pristimerin. As shown in Fig. 1A and 1C, PC-3 cells cultured for 14 d under non-adherent conditions resulted in formation of prostaspheres, but the addition of Pristimerin drastically suppressed prostasphere formation. To further prove that Pristimerin is effective in inhibiting prostasphere formation, primary prostaspheres with enriched CSC population were dissociated and re-seeded into non-adherent culture plate to allow for the formation of secondary prostaspheres. Consistent with the result from the primary spheroid formation assay, Pristimerin treatment significantly suppressed the number of prostaspheres found in PC-3 cells, and the higher dosage of Pristimerin (1.6 μM) completely eliminated all the secondary prostaspheres. To test if the anti-cancer effect of Pristimerin is through targeting of CSC properties, we then investigated whether Pristimerin treatment affects the expression of prostate CSC markers in PC-3 cell line, which has been reported to contain CSCs [47]. PC-3 cells were treated with 0.4, 0.8 and 1.6 μM Pristimerin for 24 h, the protein expression of CD133 and CD44 was examined by Western blot. As shown in Fig. 1E, expression of CD44 was significantly down-regulated after Pristimerin treatment in a dose-dependent manner. Downregulation of CD133 was also observed after Pristimerin treatment, although the effect was less obvious. All in all, the above results suggest that Pristimerin is able to significantly suppress the CSC properties of PC-3 cells.

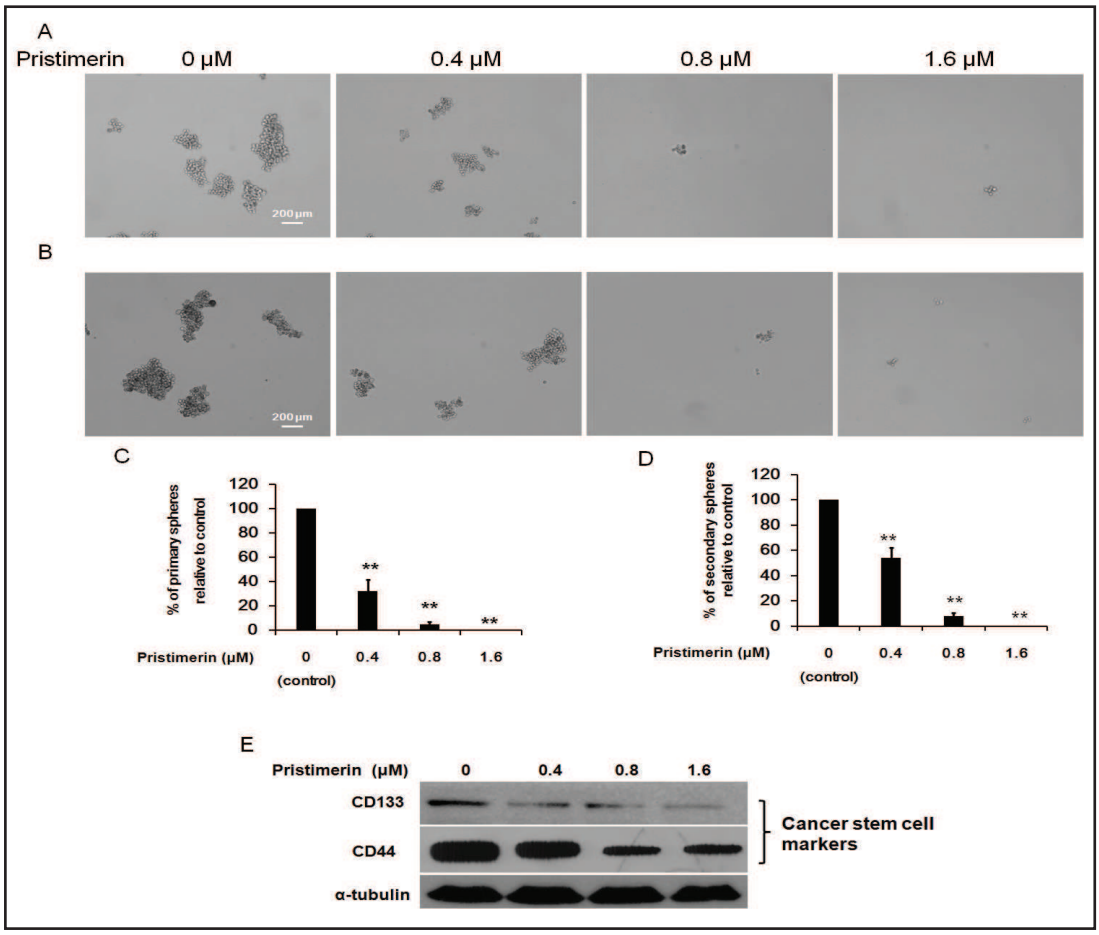

Fig. 1. Effects of Pristimerin on PC-3 CSC properties *in vitro*. (A) and (C) Spheroid formation assay was performed with PC-3 cells. Two hundred of cells were seeded onto polyHEMA pre-coated plates and treated with Pristimerin at different concentrations (0, 0.4, 0.8 and 1.6 μM) for 14 d, and the number of prostaspheres formed was counted. Note that Pristimerin treatment efficiently suppresses the spheroid formation ability of PC-3 cells. Image of the prostaspheres was captured under microscope. Note that no prostaspheres can be found in cells treated with 1.6 μM of Pristimerin. (B) and (D) Pristimerin inhibited the formation of secondary prostaspheres. Primary prostaspheres were dissociated and re-seeded into polyHEMA pre-coated plate. Pristimerin was added 24 h after the plating. Note that prostasphere formation was inhibited in a dose-dependent manner of Pristimerin. (E) Pristimerin down-regulates prostate CSC markers in PC-3 cells. Western blotting of prostate CSC markers CD44 and CD133 in PC-3 cells after Pristimerin treatment. Note that Pristimerin significantly down-regulates both stem cell markers in a dose-dependent manner. Data are presented as mean ± SD, $^{**}P<0.01$.

Pristimerin reduces prostate cancer bone metastasis in vitro

To investigate the role of Pristimerin in the development and progression of PCa bone metastasis, matrix-gel invasion assay and colony formation assays were performed *in vitro*. The invasive property of PC-3 cells was examined by Transwell-Matrigel penetration assay, which depicted significantly fewer cells were penetrated through the gel-membrane when PC-3 cells treated by Pristimerin in a dose-dependent manner (Fig. 2 A and C). To determine efficiency of Pristimerin in inhibiting colony formation of PC-3 *in vitro*, colony-forming assay was performed. As shown in Fig. 2B and D, the number of colonies (% plating efficiency) were reduced in PC-3 cells pre-treated by Pristimerin in a dose-dependent manner. All in all, the above results suggest that Pristimerin is able to significantly suppress the bone metastasis of PC-3 *in vitro*.

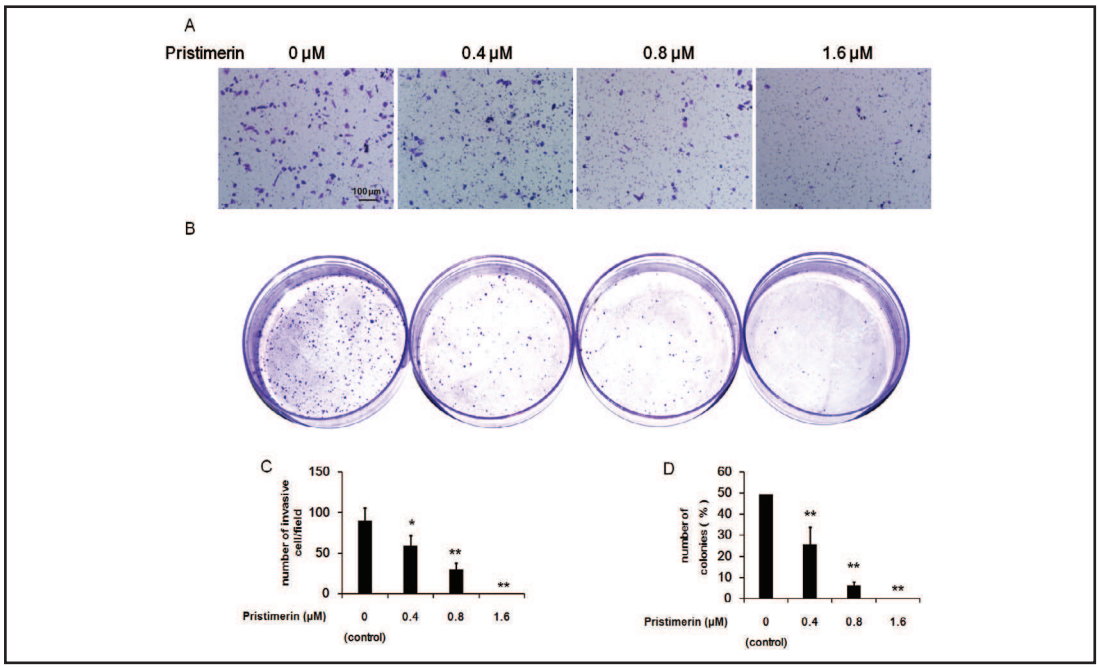

Fig. 2. Pristimerin repressed the bone metastasis of PC-3 cells *in vitro*. (A) and (C) The invasive properties of indicated cells were tested in invasion assay in a Transwell insert coated with Matrigel. Penetrated cells were counted and analyzed in histogram. Compared with the control group, Pristimerin significantly repressed the invasive ability of PC-3 cell. (B) and (D) Colony-formation exists in PC-3 cells. Compared with the control group, Pristimerin significantly repressed the number of colonies. Data are expressed as mean ± SD, **P*<0.05, ***P*<0.01.

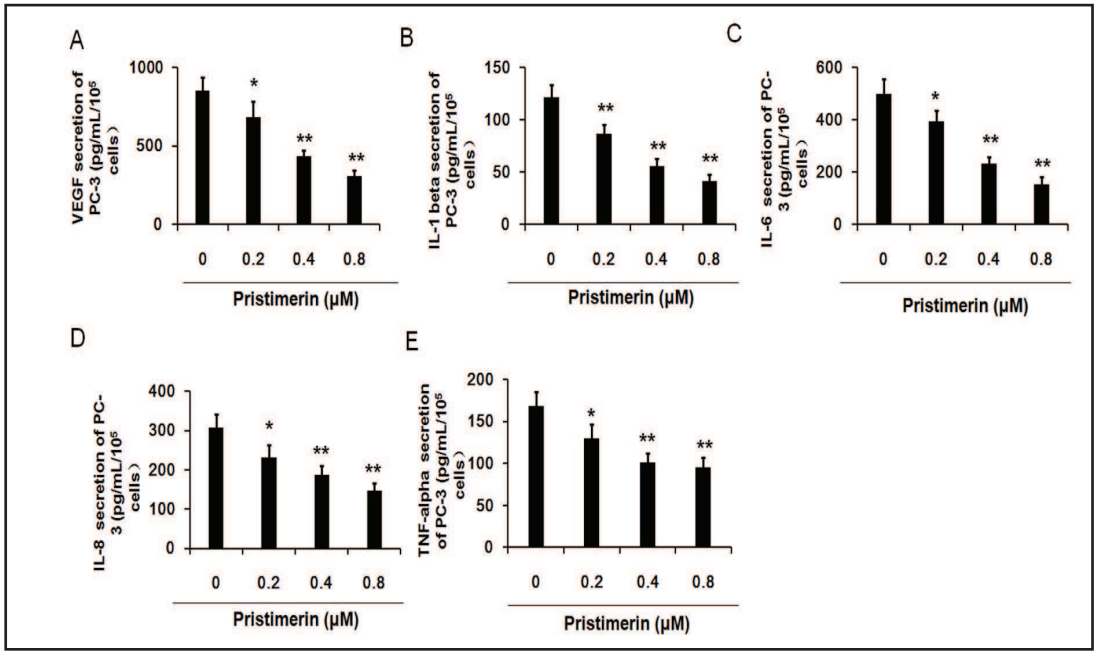

Fig. 3. Pristimerin inhibited VEGF and pro-inflammatory cytokine production in PC-3 cells. (A) Compared with the control group, VEGF secretion in PC-3 CM was inhibited by the pre-treated Pristimerin in a dose-depended manner (* P<0.05, ** P<0.01). (B-E) Pro-inflammatory cytokines such as IL-1ß, IL-6, IL-8, and TNF-α level were estimated by ELISA method according to the manufacturers' recommendations. (B) IL-1ß, (C) IL-6, (D) IL-8, and (E) TNF- α. Data are expressed as mean ± SD, * p<0.05, ***P*<0.01.

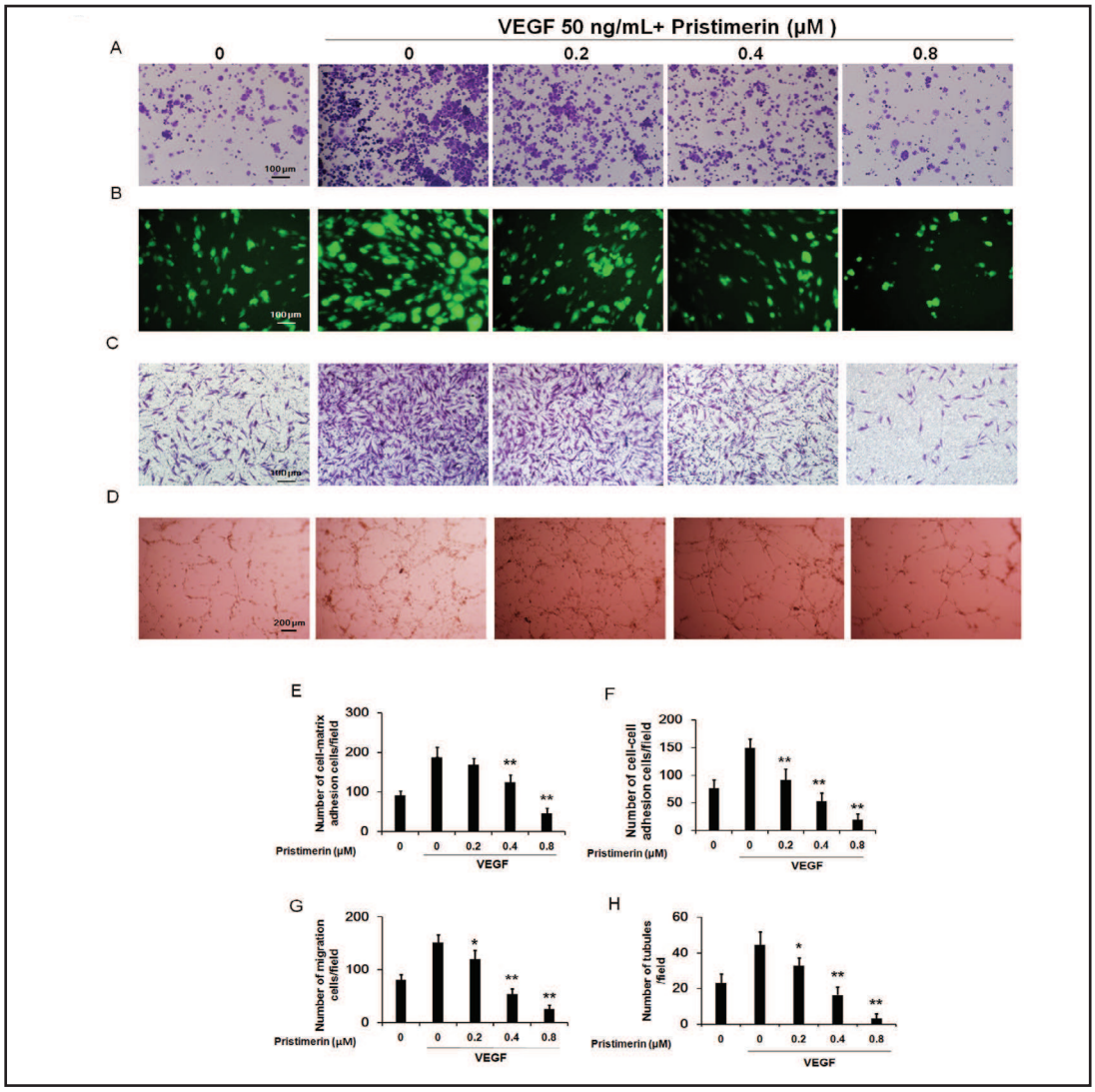

Fig. 4. Pristimerin suppressed the VEGF-induced functional activity of BM-EPCs. (A, E) Pristimerin inhibited VEGF-induced cell-matrix adhesion significantly at a low dose of 0.2 μM. Adherent cells were photographed (magnification, ×100) and quantified by manual counting. (B, F) Pristimerin inhibited VEGF-induced cell-cell adhesion significantly at a low dose of 0.2 μM. (C, G) Pristimerin inhibited VEGF-induced chemotactic motility of BM-EPCs significantly at 0.4 and 0.8 μM. Migrated cells were photographed (magnification, ×100) and quantified by manual individual cell counting. (D, H) Pristimerin inhibited VEGF-induced capillary structure formation significantly at a low dose of 0.2 μM. After incubation, BM-EPCs were fixed, and tubular structures were photographed (magnification, ×100) quantified by manual counting. The results are representative of at least three individual experiments. (I) Cell viability was inhibited significantly by Pristimerin at a low dose of 0.2 μM for 24 h. The results are representative of at least three independent experiments. Data are expressed as mean ± SD, * p<0.05, ***P*<0.01.

Pristimerin inhibits production of VEGF and pro-inflammatory cytokines in PC-3 cells

To further investigate the role of Pristimerin in VEGF and pro-inflammation cytokines expression in supernatant, ELISA assay was used. VEGF plays an important role in vasculogenesis by promoting BM-EPC proliferation, migration, and differentiation. As shown in Fig. 3A, Pristimerin treatment caused a dose-dependent and remarkable decrease in VEGF secretion. Furthermore, the pro-inflammatory cytokines production of IL-1ß (Fig. 3B), IL-6 (Fig. 3C), IL-8 (Fig. 3D), and TNF-α (Fig. 3E) were also significantly inhibited by Pristimerin in PC-3 cells in a dose-dependent manner. Taken together, our results suggested that

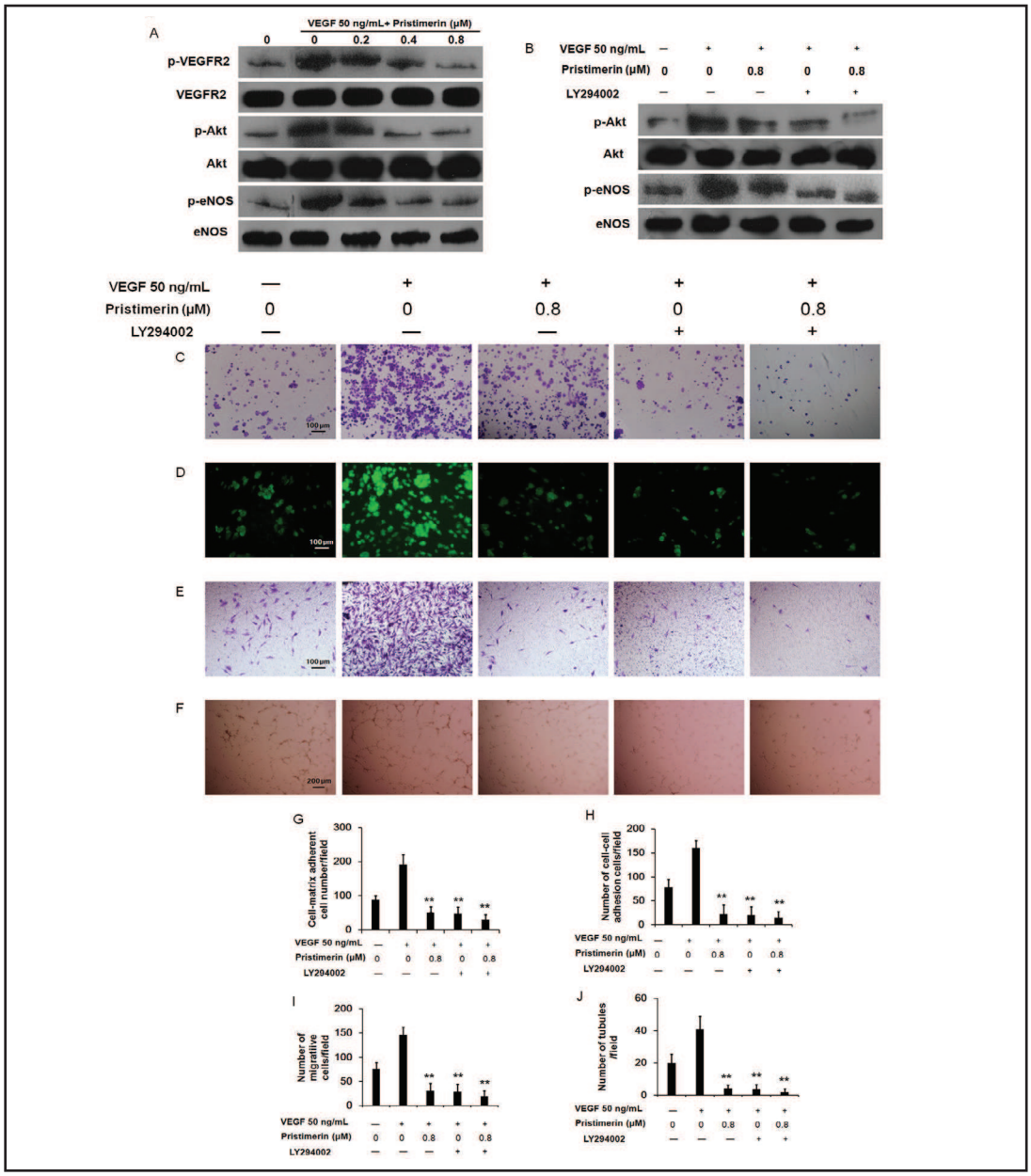

Fig. 5. (A) Pristimerin inhibited the VEGF-triggered activation of VEGFR-2 and Akt pathways in BM-EPCs. Cells were pretreated with Pristimerin for 30 min and incubated with VEGF for 10 min. VEGFR-2, Akt and eNOS were detected with phospho-specific antibodies by Western blot. Pristimerin suppressed the phosphorylation of p-VEGFR-2, p-Akt and p-eNOS triggered by VEGF in BM–EPCs. (B-J) BM-EPCs were pretreated with either LY294002 (20 μM) for 60 min, then cultured with VEGF or 0.8 μM Pristimerin, and protein expression was determined by Western blot. Cell-matrix adhesion, cell-cell adhesion, cell migration and capillary structure formation were determined by the methods described above. Data are expressed as mean ± SD, $^{**}P<0.01$.

Pristimerin was effective in reducing the VEGF and pro-inflammatory cytokines production in the supernatant of PC-3 cell.

Pristimerin inhibits VEGF-induced vasculogenesis of BM-EPCs

To investigate the role of Pristimerin in VEGF-induced vasculogenesis of BM-EPCs, cell viability, cell migration, cell adhesion and capillary structure formation assays were

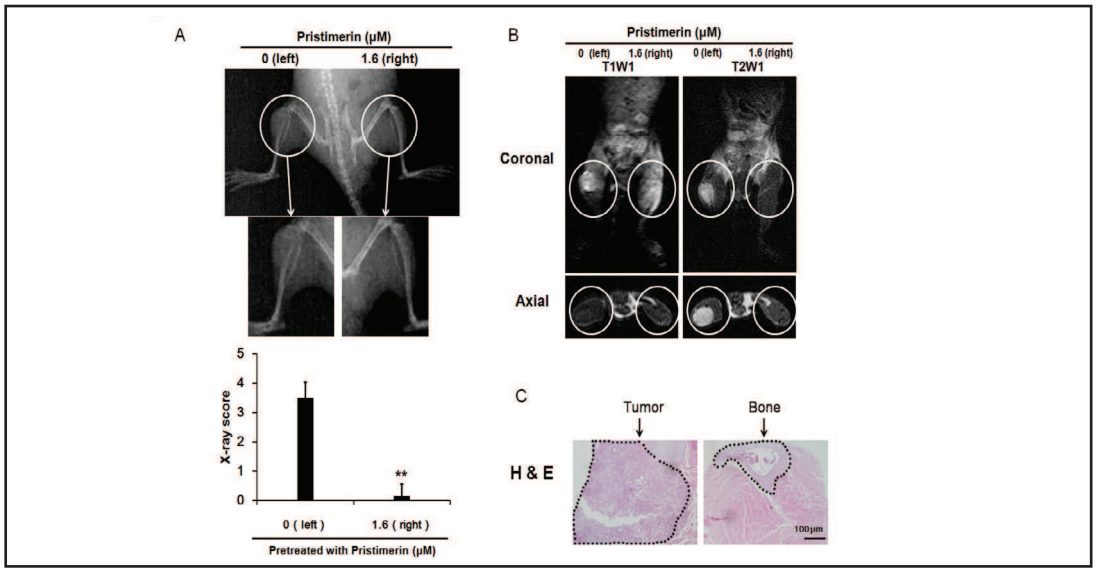

Fig. 6. Pristimerin repressed the tumorigenicity of PC-3 cells in bone. (A)Male SCID mice were inoculated with Pristimerin pre-treated PC-3 cells (left 0 μM and right 1.6 μM) through the intra-tibial route. Skeletal lesions in radiographs are demonstrated. (B) MRI show pre-treated with Pristimerin (1.6 μM) inhibited tumorigenicity of PC-3 cells *in vivo*. (C) Histologic analysis was carried out by H&E-staining. Data are expressed as mean ± SD, $^{**}P<0.01$.

performed *in vitro*. To observe the possible cytotoxicity of Pristimerin on the cells, we examined whether Pristimerin modulates VEGF-induced cell viability of BM-EPCs by CCK-8 assay. As shown in Fig. 4I, incubation of BM-EPCs with Pristimerin for 24 h exhibited a dose-dependent reduction in the number of BM-EPCs, with significant inhibition first occurring at a low dose of 0.2 μM. To investigate the possibility that Pristimerin alters the adhesiveness of EPCs, cells were incubated with Pristimerin for 24 h. After replating on fibronectin-coated dishes for 30 min, EPCs pre-exposed to Pristimerin exhibited a significant decrease in the number of adhesive cells in a dose-dependent manner, with significant inhibition first occurring at 0.4 μM and greater inhibition at 0.8 μM (Fig. 4A and E). Additionally, Pristimerin could markedly inhibit cell-cell adhesion with a much lower concentrations of 0.2 μM (Fig. 4B and F). It is known that cell mobility and maintenance of cell survival signaling are essential for capillary tube formation. To assess the anti-angiogenic properties of Pristimerin *in vitro*, we examined the cellular response of Pristimerin-treated BM-EPCs to VEGF-induced migration using a transwell assay. Pristimerin reduced the migrative ability of BM-EPCs in a dose-dependent manner, with significant inhibition first occurring at 0.2 μM and greater inhibition at 0.4 and 0.8 μM (Fig. 4C and G). To further determine the effect of Pristimerin on vasculogenesis, we examined how Pristimerin regulates capillary tubule formation of BM-EPCs. When EPCs were seeded on Matrigel, robust tube-like structures were formed in the presence of VEGF. However, treatment with 0.2, 0.4 or 0.8 μM of Pristimerin abolished the VEGF-induced tubule formation of BM-EPCs (Fig. 4D and H). These data suggest that Pristimerin has strong ability to inhibit VEGF-induced vasculogenesis of BM-EPCs *in vitro*.

Pristimerin inhibits activation of VEGFR2 and Akt/eNOS induced by VEGF in BM-EPCs

Interaction of VEGFR-2 with VEGF leads to the activation of various downstream signaling molecules responsible for EPC survival. To further delineate the mechanisms that contribute to the vasculogenesis inhibition effect of Pristimerin, we examined the signaling molecules involved in the VEGF pathway using Western blot analysis. As shown in Fig. 5A, phosphorylation of VEGFR-2 induced by VEGF was suppressed by Pristimerin in a dose-dependent manner. Thus, the anti-angiogenic property of Pristimerin may be at least partially due to VEGFR-2 inhibition. Upon examination of the key pathway components that regulated

the endothelial cell function in angiogenesis, we found that Pristimerin considerably suppressed the VEGF-induced phosphorylation of Akt and eNOS in BM-EPCs, while the total protein levels remained constant (Fig. 5A). These results suggest that Pristimerin may inhibit VEGF-induced vasculogenesis of BM-EPCs through blocking of these signaling pathways.

Furthermore, we used the highly specific PI3K inhibitor LY294002 to assess the role of the PI3K/Akt pathway in angiogenesis. Unexpectedly, LY294002 inhibited not only Akt, but also notably phosphorylated eNOS (Fig. 5B). The present data also showed that the suppression of PI3K abolished the attenuated effect by Pristimerin on Akt/eNOS expression. Cell-matrix adhesion, cell-cell adhesion, cell migration and tube formation ability were significantly decreased when VEGF was co-administered with LY294002 (Fig. 5C-J), and inhibition of PI3K activity by LY294002 abolished the inhibitory effect on the cell functional activities imposed by Pristimerin. Taken together, these observations demonstrate that Pristimerin relies upon PI3K/Akt/eNOS to offer anti-angiogenic effect in BM-EPCs.

Pristimerin inhibits tumorigenicity in vivo

To further investigate the role of Pristimerin in tumorigenicity of PCa bone metastasis *in vivo*, an intra-tibial injection mouse model was used. The tumorigenicity were assessed by MRI, and the extents and areas of skeletal lesions were assessed by X-ray scores. Four weeks after intra-tibial inoculation, skeletal lesions of all animals in the left tibias were remarkably larger than those in the right tibias (Fig. 6A), which demonstrated PC-3 cells pre-treated by Pristimerin had less skeletal invasive ability compared with control. Histological examinations using H&E-stainning of the tissues showed that, when the Pristimerin-treated groups still retained normal tubular structures (Fig. 6B). MRI analysis displayed that PC-3 cells pretreated by Pristimerin revealed no tumor development (cannot be detected by MRI) and almost no bone invasion compared with control group (Fig. 6B). Histologic analysis was carried out by H&E-staining in which tumors were lined out by dashed line as shown in the left panel and the normal bone tissue was lined out by dashed line in the right panel (microscope 40×, Fig. 6C). We could find out that bone was destroyed by the invasion of the tumor, but pretreatment by Pristimerin inhibited the bone destruction remarkably. Taken together, our results suggested that Pristimerin was effective in reducing the tumorigenic potential of bone metastasis in prostate cancer bone metastasis PC-3 cells.

Discussion

The evaluation of naturally occurring dietary compounds may indicate novel approaches for the treatment of prostate cancer, which remains one of the most lethal cancers despite tremendous scientific efforts. In our present work, for the first time, we systematically evaluated the chemopreventive effect of Pristimerin to bone metastasis and VEGF-induced vasculogenesis of BM-EPCs.

First, we found that pristimerin exerted significant inhibitory effects on stem-like properties of PC-3 cells. CSCs may be the critical drivers of tumor progression and metastasis [12, 13]. Previously, many studies have used CD133 and CD44 as CSC characteristics. CD44+ PCa cells were shown to have the stem-like properties of increased tumorigenic, clonogenic, and metastatic potential [17]. Although CD44 does not seem to belong to the stemness genes, such as OCT4 and KLF4, that are central for maintaining stem cell characteristics, CD44 can contribute to the activation of stem cell regulatory genes and can be a target of these genes [48]. More importantly, a recent study has demonstrated that the transcriptional reprogramming led by nuclear CD44 has an active role in transforming cancer cells to a CSC-like phenotypem [49]. Our results suggest that Pristimerin might play a significant role in the bone metastasis progression of PCa by regulating CSC characteristics. These prostate CSCs not only express high level of CD133 and CD44, but also are highly tumorigenic when compared to the non-CSC population. In the present study, we found that Pristimerin inhibited tumor sphere formation of PC-3 cells and repressed expression of CSC markers including

CD133 and CD44 in PC-3 cells. Meanwhile, Pristimerin significantly inhibit bone metastasis *in vitro* and tumorigenicity *in vivo*. These findings demonstrate that Pristimerin negatively regulate the CSCs properties of PC-3 cells from PCa bone metastasis. And once stem cell characteristics were inhibited by Pristimerin, bone metastasis of prostate cancer PC-3 cells *in vitro* and tumorigenic *in vivo* were substantially suppressed. Importantly, this is the first report to demonstrate the effect of Pristimerin on suppression of stem cell characteristics leading to chemopreventive effect to bone metastasis of prostate cancer.

Several inflammatory cytokines have been linked with tumorigenesis, which suggests that inflammation is associated with cancer development [50]. TNF-α is a potent pleiotropic proinflammatory cytokine produced by macrophages, neutrophils, fibroblasts, keratinocytes, NK cells, and tumor cells [51]. The role of TNF-α has been linked to all steps of tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis and B cells, and tumor cells [52, 53]. IL-1β promotes the production of angiogenic proteins from host stromal or infiltrating cells in tumor microenvironment enhancing tumor growth and metastasis [54, 55]. IL-6 and IL-8 are multifunctional cytokines, which showed profound angiogenic potential both ex vivo and *in vivo*, and are able to promote tumor growth and metastasis notably. Our results showed that Pristimerin treatment significantly decreased the elevated levels of VEGF and proinflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α in PC-3 cells, which suggested that the anti-tumor effect of Pristimerin is associated with anti-inflammation in cancer.

Over the past 30 years, inhibition of new blood vessel formation has been accepted as an effective strategy to treat human cancer. BM-derived cells contribute to tumor neovasculature and, when modified to express a vessel formation inhibitor, can restrict tumor neovascularization. Extensive research has led to the identification and isolation of regulators of vasculogenesis, some of which represent therapeutic targets, such as VEGF. VEGF is expressed by prostate cancer at a high level, and its expression correlates with increasing grade, vascularity, and tumorigenicity [24, 25]. Because VEGF can alter the marrow microenvironment from a quiescent state to a highly pro-angiogenic and pro-tumorigenic environment, it is important for the regulation of angiogenesis as well as cell function. Many studies have suggested that EPCs residing in the bone marrow can be recruited to the tumor in response to tumor derived cytokines, where they contribute to vascular development by incorporating into the walls of nascent capillaries [20, 21]. EPCs provide both instructive (release of pro-angiogenic cytokines, such as VEGF) and structural (vessel incorporation and stabilization) functions that contributing to the initiation of tumor neovasculogenesis. EPC recruitment may play a fraction. VEGF exerts its biological effects by binding to its receptor tyrosine kinases, expressed on BM-EPCs. VEGFR2 is the primary receptor mediating the angiogenic activity of VEGF through distinct signal transduction pathways that regulate BM-EPCs proliferation, migration, differentiation and tube formation. Interruption of VEGFR2 signaling is thought to be necessary for tumor angiogenesis and macroscopic solid tumor growth [56]. Activation of VEGFR2 leads to the activation of various downstream signal transduction proteins, including extracellular signal-related kinase Akt and Akt-dependent eNOS phosphorylation [57, 58], which promotes the growth, migration, differentiation and cell viability of BM-EPCs in neovasculogenesis. Induction of apoptosis by Pristimerin involved activation of caspases, mitochondrial dysfunction, inhibition of anti-apoptotic nuclear factor-κB (NF-κB) and Akt in ovarian carcinoma cells and pancreatic cancer cells [59, 60]. Pristimerin also induces apoptosis in prostate cancer cells predominately through the mitochondrial apoptotic pathway by inhibiting antiapoptic Bcl-2 through a reactive oxygen species-dependent ubiquitin-proteasomal degradation pathway [61]. Recent evidence shows that Pristimerin promotes degradation and inhibits phosphorylation of protein kinases in the Raf/MEK/ERK and PI3K/AKT/mTOR signaling pathways [62]. In the present study, we found that Pristimerin inhibited the elevated level of VEGF in PC-3 cells and could inhibit VEGF-induced vasculogenesis of BM-EPCs. And VEGF-induced phosphorylation of VEGFR-2, Akt and eNOS were also dramatically suppressed by Pristimerin. This finding indicates that Pristimerin inhibited the VEGF secretion of PC-3 and BM-EPCs, as a result,

reduced the instructive functions of BM-EPCs, suggesting that Pristimerin targeting VEGF/VEGFR-2 signaling is powerful and potential agent in cancer therapy.

Taken together with previous studies, Pristimerin has been identified as a candidate therapeutic agent to prevent bone metastasis. Specifically, Pristimerin may have therapeutic value in cancers where PC-3 stem cell characteristics and VEGF-induced vasculogenesis of BM-EPCs are stimulated. Furthermore, Pristimerin might be used for therapeutics in combination with anti-cancer agents and health foods to prevent and block progression of tumor development.

Conclusions

In conclusion, the present study highlights the ability of stemness inhibition on PC-3 cells and anti-angiogenic differentiation on BM-EPCs imposed by Pristimerin. Furthermore, Pristimerin also prevent the xenografted PC-3 tumor growth *in vivo*. In characterizing the mechanism of action, we discovered that inactivation of the PI3K/Akt/eNOS pathways is required for Pristimerin-mediated anti-angiogenic effect in BM-EPCs. Results of the present study suggest a better understanding of the mechanism of the anti-stemness and anti-angiogenesis signaling cascade engaged by Pristimerin, that can identify potential targets for intervention to prevent bone metastasis of prostate cancer.

Abbreviations

PCa (prostate cancer); BM-EPC (bone marrow-derived endothelial progenitor cell); CSCs (cancer stem cells); eNOS (endothelial nitric oxide synthase); VEGF (vascular endothelial growth factor); VEGFR-2 (vascular endothelial growth factor receptor 2); TNF-α (tumor necrosis factor-alpha); IL-1ß (interleukin-1ß); IL-6 (interleukin-6); IL-8 (interleukin-8).

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Disclosure Statement

No potential conflicts of interest were disclosed.

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