1α,25-Dihydroxyvitamin D3 restrains stem cell-like properties of ovarian cancer cells by enhancing vitamin D receptor and suppressing CD44

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Abstract. Scientific evidence linking vitamin D with various cancer types is growing, but the effects of vitamin D on ovarian cancer stem cell-like cells (CSCs) are largely unknown. The present study aimed to examine whether vitamin D was able to restrain the stemness of ovarian cancer. A side population (SP) from malignant ovarian surface epithelial cells was identified as CSCs, in vitro and in vivo. Furthermore, 1α ,25-dihydroxyvitamin D₃ [1α ,25(OH)₂D₃] treatment inhibited the self-renewal capacity of SP cells by decreasing the sphere formation rate and by suppressing the mRNA expression levels of cluster of differentiation CD44, NANOG, OCT4, SOX2, Krüppel-like factor 4 and adenosine triphosphate binding cassette subfamily G member 2. Additionally, 1α ,25(OH)₂D₃ treatment decreased the expression of Cyclin D1, whereas it increased the expression of β -catenin and vitamin D receptor (VDR). Notably, immunofluorescence staining verified that 1a,25(OH)₂D₃ promoted the expression of β -catenin in the cytoplasm. Furthermore, vitamin D₃ delayed the onset of tumor formation derived from injection of ovarian CSCs to nude mice, by reducing CD44 and enhancing β -catenin expressions *in vivo*. In conclusion, 1 α ,25(OH)₂D₃ restrains the stem cell-like properties of ovarian cancer cells by enhancing the expression of VDR, by promoting the expression of β -catenin in the cytoplasm, and by suppressing

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the expression of CD44. These findings provide a novel insight into the functions of vitamin D in diminishing the stemness of cancer CSCs.

Introduction

Ovarian cancer is the fourth most frequent gynecologic malignancy and the leading cause of tumor-associated mortality in the USA (1). Epithelial ovarian cancer, which accounts for ~90% of ovarian cancers, is generally diagnosed at an advanced stage (2). Furthermore, the prognosis and five-year survival have not improved significantly owing to the high recurrence rate (3). Therefore, it is urgent to identify the underlying mechanisms and to develop alternative therapeutic strategies for this type of cancer.

The ovarian surface epithelium (OSE) is the recognized source of epithelial ovarian cancer (2,4). Several characteristics of epithelial ovarian carcinomas imply it is a cancer stem cell-driven disease. First, the stem properties of OSE have been recognized previously (5), and the cancer-prone stem cell niche was also identified at the helium area of ovary (6). Second, epithelial ovarian cancer can differentiate into several subtypes that recapitulate the histology of other normal gynecologic tissues (2). Third, the high recurrence rate following the initial successful treatment may derive from a small number of cells within the cancer population, which are: i) Capable of repopulating the entire tumor and ii) exhibit cytoprotective mechanisms on somatic stem cells (6-10). Furthermore, the presence of cancer stem cell-like cells (CSCs) in patients is reported to be associated with poor survival and chemoresistance (11,12). Therefore, targeting CSCs may be a potential therapeutic approach to epithelial ovarian cancer.

Emerging evidence suggests that vitamin D deficiency is strongly associated with the risk of various human cancers, including colorectal, breast, prostate and ovarian cancer (13). 1α ,25-dihydroxyvitamin D₃ [1α ,25(OH)₂D₃], the active metabolite of vitamin D₃, functions by binding to vitamin D receptor (VDR). 1α ,25(OH)₂D₃, or analogues of 1α ,25(OH)₂D₃, may serve as anticancer agents owing to their ability to suppress proliferation, invasion, metastasis and angiogenesis, and to induce apoptosis (14-17). Furthermore, 1α , $25(OH)_2D_3$ is reported to inhibit the proliferation of prostate cancer stem cells by inducing cell cycle arrest and senescence (18). Furthermore, results from in vitro and xenograft studies indicated that the vitamin D analogue (BXL0124) may decrease mammosphere growth by reducing cluster of differentiation CD44 expression levels (14,19,20). There is preliminary evidence that 1α ,25(OH)₂D₃ could be used to cure and inhibit prostate and breast CSCs (21). Our previous study demonstrated that 1α ,25(OH)₂D₃ inhibited the migration of human ovarian cancer cells by increasing the expression of VDR and suppressing epithelial-mesenchymal transition (17). However, the effects of vitamin D₃ on ovarian CSCs remain largely unknown. Side population (SP) cells have been presented as functional markers of CSCs (22-26). The present study investigated whether 1α , 25(OH)₂D₃ was able to inhibit the stem cell-like phenotype of SP cells isolated from mouse OSE (MOSE) cells, and determined its possible underlying mechanisms. The present findings indicated that $1\alpha_2(OH)_2D_3$ suppressed the properties of ovarian CSCs by enhancing VDR expression and reducing CD44 level, which may provide the novel strategy for treating epithelial ovarian cancer.

Materials and methods

Cell culture and reagents. MOSE cells were isolated as described by Roby et al (27). Briefly, the 4 mice are 6-8 week old and ~20 g. All mice were housed with full of food and water under controlled conditions of temperature at 21±2°C, relative humidity at 55±5% and a 12:12 h light-dark cycle. Ovaries from female breeder mice (BALB/c) were resected and, following removal of the residual remnants of the oviducts, were incubated in 1.5 ml tubes for 30 min with trypsin in 5% CO₂ at 37°C. Following incubation, tubes were removed from the incubator and inverted 10 times. The ovaries were transferred to a clean 1.5 ml tube for the second digestion with trypsin, as above. Cells were collected, pelleted by 1,000 x g/min for 5 min at 4°C, resuspended in DMEM/F12 medium containing 20 ng/ml mEGF, 20 ng/ml mouse basic fibroblast growth factor, $2 \mu g/ml$ insulin and $4 \mu g/ml$ heparin sodium and seeded onto 35 mm dishes. On the 2nd day, the culture dishes were not removed, because moving them is conducive to cell growth and therefore the stability of the microenvironment around the cells can be maintained. On the 3rd day, growth medium was changed according to the cell growth. MOSE cells were continuously passaged in vitro. MOSE cells maintained proliferation and underwent spontaneous neoplastic transformation when subcultured for >80 passages in vitro (see Supplementary data). The spontaneous malignant transformation of MOSE cells in late stage (M-L cells) were maintained in Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F12) containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, 10 ng/ml mouse epithelial growth factor (mEGF) and 1% insulin-transferrin-selenium in an atmosphere of 95% humidity and 5% CO₂ at 37°C. The MOSE cells have been tested for mycoplasma and human cell lines contamination by STR profiling and were demonstrated to be clean (see Figs. S1 and S2; Table SI). The reagents for cell culture were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Vitamin D₃ (40,000 IU/ml) and 1α ,25(OH)₂D₃ were purchased from Shanghai General Pharmaceutical Company, Ltd. (www.cpshgp.com/english/; Shanghai, China) and Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), respectively. In the present study, cells were treated by 10 nM 1α ,25(OH)₂D₃ *in vitro*, and mice were was administrated by vitamin D₃. *In vivo*, vitamin D₃ is successively hydroxylated by the 25-hydroxylase and 1α -hydroxylase, and forms 1α ,25(OH)₂D₃, the active form of vitamin D₃.

Isolation of SP cells. M-L cells (1x10⁶ cells/ml) were re-suspended in DMEM/F12 medium containing 2% FBS and 4% penicillin-streptomycin, and stained with 10 µg/ml Hoechst 33342 (Sigma-Aldrich; Merck KGaA) in the presence or absence of 50 µg/ml verapamil (Sigma-Aldrich; Merck KGaA) at 37°C for 90 min with intermittent shaking every 10 min. Following incubation, the cells were centrifuged by 1,000 x g/min for 5 min at 4°C and washed with cold phosphate-buffered saline (PBS). The SP and non-side population (NSP) cells were isolated and collected by MoFloAstrios EQ fluorescence activated cell sorting (Summit 6.2; FACS, www.beckmancoulter.cn/ls-discovery/flow/researchflow/moflo-astrios.html; Beckman Coulter, Inc., Brea, CA, USA). The Hoechst dye was excited with a UV laser at 346 nm and its fluorescence emissions were measured with 630/22 (Hoechst 33342 Red) and 424/44 filters (Hoechst Blue). For purification, isolated SP cells were cultured in serum-free DMEM/F12 medium containing 20 ng/ml mEGF, 20 ng/ml mouse basic fibroblast growth factor, 1:50 B27, $2 \mu g/ml$ insulin, 4 μ g/ml heparin sodium and 6 mg/ml glucose for 10 days and were subsequently re-suspended in StemPro Accutase Cell Dissociation Reagent at 37°C for 10 min. The NSP cells were cultured in DMEM/F12 medium containing 20 ng/ml mEGF, 20 ng/ml mouse basic fibroblast growth factor, $2 \mu g/ml$ insulin and 4 μ g/ml heparin sodium. The reagents in serum-free medium were purchased from Thermo Fisher Scientific, Inc. Subsequently, the cells were centrifuged by 1,000 x g/min for 5 min at 4°C and maintained in the fresh serum-free medium until further use.

Detection of CD44 and CD117. The SP and NSP cells were washed, re-suspended and separately incubated with fluorescence-conjugated antibodies against CD44 (1:1,000; cat. no. 555478), CD117 (1:1,000; cat. no. 555714) and IgG (1:1,000; cat.nos.555749 and 555742; BD Biosciences, San Jose, CA, USA) in 4°C for 10 min. The appropriate concentrations of each antibody were recommended by the manufacturer. The cells were washed and analyzed by CytomicsTM FC 500 from Beckman Coulter, Inc. In brief, unstained cells, CD44⁺, CD117⁺ and double-stained control cells were used to mark the four quadrants in a dot-plot for unstained, CD44⁺, CD117⁺ and double-positive populations. The software used for the analysis is CXP Analysis, which is the part of FC500 flow cytometer (Beckman Coulter, Inc.).

Sphere-formation assay. The SP cells were re-suspended in StemPro Accutase Cell Dissociation Reagent at 37°C for 10 min, terminated by the serum-free DMEM/F12 medium. Subsequently, 1,000 cells/well were plated in the 96-well plates and cultured in serum-free medium for 10 days. The spheres with a minimum size of 50 μ m were counted under a brightfield microscope (CKX41F; Olympus Corporation, Tokyo, Japan) equipped with a digital camera. The sphere-formation rate was expressed as the following formula: (Number of spheres formed/number of plated cells) x 100.

Reverse transcription-quantitative polymerase chain reaction (*RT-qPCR*). Total RNA extractions from 1x10⁶ cells SP or NSP cells were performed using TRIzol[®] reagent (Life Technologies; Thermo Fisher Scientific, Inc.). For the synthesis of first-strand cDNA, total RNA was reverse-transcribed by the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). Primer sequences are available in Table I. qPCR reactions were conducted with the SYBR-Green I Nucleic AcideGel stain (Roche Diagnostics) according to manufacturer's protocol. Thermocycling parameters were: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. Relative expression levels were calculated using the $2^{-\Delta \Delta Cq}$ method by Livak and Scmittgen (28). GAPDH was used as normalization control.

Western blotting. Approximately 10⁶ cells were scraped off with radioimmunoprecipitation buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China). Subsequently, the lysates were centrifuged at 7,500 x g for 30 min at 4°C and the supernatants were collected. Protein concentrations were quantified by the Bicinchoninic Acid assay (Beyotime Institute of Biotechnology). Proteins (30 μ g/ sample) were separated by 10% SDS-PAGE and were transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk in PBS + 1% Tween-20 and incubated with primary antibodies against VDR (1:500; cat. no. 12550), Cyclin D1 (1:1,000; cat. no. 2978), β-catenin (1:1,000; cat. no. 8480), Sex-determining region Y (SOX2; 1:1,000; cat. no. 23064), NANOG (1:1,000; cat. no. 8822), octamer-binding protein (OCT4; 1:1,000; cat. no. 2840) and GAPDH (1:1,000; cat. no. 5174) at 4°C overnight. Subsequently, the membranes were incubated for 1 h at room temperature with 1:3,000 anti-mouse IgG, HRP-linked antibody (cat. no. 7076) and anti-rabbit IgG, HRP-linked antibody (cat. no. 7074). All antibodies were purchased from Cell Signaling Technology, Inc. (CST; Danvers, MA, USA). Protein bands were visualized using Enhanced Chemiluminescence Detection (Merck KGaA); to quantify the level of protein expression, densitometric analysis was performed according to the manufacture's protocol (Gbox Chemi-XR 5; Syngene, Frederick, MD, USA). The specific/individual protein expressions (GeneSnap image acquisition software, GeneTools image analysis software; Syngene) should be normalized with their respective GAPDH loading control first. Subsequently, when drafting the histograms, the NSP or control groups should be set to '1' and all other expression compared with that.

Immunofluorescence staining. Approximately 1x10⁵ cells were plated onto coverslips until they reached an optimal density of 70-80%. The cells were fixed with 4% paraformaldehyde at 4°C for 20 min, permeabilized with 0.1% Triton X-100 for 15 min at 4°C and blocked with 1% FBS for 1 h at room temperature. Subsequently, the cells were

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence $(5' \rightarrow 3')$
GAPDH	F: TTGATGGCAACAATCTCCAC
	R: CGTCCCGTAGACAAATGGT
CD44	F: AGCGGCAGGTTACATTCAAA
	R: CAAGTTTTGGTGGCACACAG
NANOG	F: AGGGTCTGCTACTGAGATGCTCTG
	R: CAACCACTGGTTTTTCTGCCACCG
OCT4	F: AGAAGGAGCTAGAACAGTTTGC
	R: CGGTTACAGAACCATACTCG
CD133	F: TAGAGGGAAGTCATTCGGCT
	R: CCCAAGATACCTTCAATGCTG
SOX2	F: AAAGCGTTAATTTGGATGGG
	R: ACAAGAGAATTGGGAGGGGT
KLF4	F: CAGTGGTAAGGTTTCTCGCC
	R: GCCACCCACACTTGTGACTA
NOTCH1	F: CTGAGGCAAGGATTGGAGTC
	R: GAATGGAGGTAGGTGCGAAG
NOTCH2	F: TGTGCCGTTGTGGTAGGTAA
	R: TGCTGTGGCTCTGGCTGT
ABCG2	F: TCGCAGAAGGAGATGTGTTGAG
	R: CCAGAATAGCATTAAGGCCAGG
CD117	F: CGGTCGACTCCAAGTTCTACAAG
	R: GTTGCAGTTTGCCAAGTTGGAGT
β-catenin	F: ATGGCTTGGAATGAGACTGC
	R: CTCCATCATAGGGTCCATCC
c-Myc	F: CAACGTCTTGGAACGTCAGA
	R: TCGTCTGCTTGAATGGACAG
Cyclin D1	F: TGTTCGTGGCCTCTAAGATG
	R: ACTCCAGAAGGGCTTCAATC
VDR	F: TGACCCCACCTACGCTGACT
	R: CCTTGGAGAATAGCTCCCTGTACT

ABCG2, ATP binding cassette subfamily G member; CD, cluster of differentiation; F, forward; KLF, Krüppel-like factor; OCT, octamer-binding protein; R, reverse; SOX, Sex-determining region Y; VDR, vitamin D receptor.

incubated with primary antibody of β -catenin (CST; 1:100; cat. no. 8480) overnight at 4°C. Following this, the cells were labeled with anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor[®] 488-conjugated; CST; 1:1,000, cat. no. 4412) in the dark room for 1.5 h at room temperature and washed with PBS three times. Nuclei were stained with DAPI for 20 min at room temperature. The signals were detected using a TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems GmbH, Wetzlar, Germany).

X-ray and heavy particle resistance of SP cells. Approximately 1x10⁵ cells were irradiated with 2, 4, 6, 8 or 10 Gy of X-rays using a Pantac HF-320S X-ray generator (Shimadzu Co., Kyoto, Japan) or carbon-ion beams accelerated by the Heavy

ion medical accelerator in Chiba in the National Institute of Radiological Sciences (Chiba, Japan). Following irradiation, NSP cells were plated in triplicate in 60-mm dishes for assays of clonogenicity. After culturing for 14 days at 37°C, the colonies were fixed with 75% alcohol and stained with 0.3% methyl violet for 20 min at room temperature. Colonies containing >50 cells were counted as the survivors. Additionally, SP cells in serum-free medium were plated (1,000 cells/well) in 96-well plates for sphere-formation assay. The spheroid formation rates were calculated as the percentage of the spheres $>50 \,\mu\text{m}$. At least three parallel samples were scored in six replicates conducted for each aforementioned irradiation condition. Cell survival fractions were obtained from fitting the surviving fraction to a linear-quadratic model expressed by the following formula: SF = exp - $(\alpha D + \beta D^2)$, where SF is the survival fraction, exp means 'exp-function', α and β are the constant of the fitted curve by the results, and D is the irradiation dose.

Tumorigenesis. BALB/c nude mice (age, 4-6 weeks, 18-20 g) were purchased from Soochow University Laboratory Animal Center (Suzhou, China). Mice were provided with water and food ad libitum and were housed at five animals per cage. All mice were housed under controlled conditions of temperature at 21±2°C, relative humidity at 55±5% and a 12:12 h light-dark cycle. All surgical procedures and care administered to the animals were approved by the Institutional Animal Care and Use Committee (approval number is ECSU-201800049). In the orthotopic model, 10 μ l cell suspension (1x10⁴ NSP or SP cells) mixed with Matrigel (1:1; BD Biosciences; cat. no. 356234) were injected orthotopically into the one ovary (n=2 mice/group). In the subcutaneous model, it has been reported that SP cells has stronger tumorigenicity than NSP cells (22,24). Therefore, different number of SP and NSP cells were injected into mice. One hundred μ l cell suspension containing 1x10⁴ SP cells or 1x10⁶ NSP cells mixed with Matrigel were injected subcutaneously into flanks of the mice (n=4 mice/group), respectively. The subcutaneous model was used to evaluate the effect of vitamin D₃ on tumorgenicity of SP cells. The mice in the vitamin D₃-treatment group were injected with a single dose of vitamin D₃ (1,000 IU/week) intramuscularly. The animals were sacrificed at the indicated time intervals (4-12 weeks) when tumor nodules were identified on their body surfaces. The anesthesia of the mice used was 1% pentobarbital sodium (50 mg/kg), prior to the injection of cells to their ovary. The euthanasia/sacrifice of the mice was performed by 3% pentobarbital sodium (150 mg/kg), followed by cervical dislocation to ensure the death of mice. Tumor growth was monitored by measuring 2 perpendicular diameters. Tumor volumes were calculated according to the formula: $0.5 \times a \times b^2$, where a and b are the largest and smallest diameter, respectively.

Statistical analysis. Statistical analysis was performed using the paired Student's t-test for the difference between two groups. One-way analysis of variance was used where multiple comparisons were made, followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Categorizing MOSE cells. The capacity of anchorage-independent growth was determined, which in an in vitro hallmark of neoplastic transformation of cells (29). Early (<20 passages) and intermediate (21-80 passages) MOSE cells were unable to form colonies in soft agar. Notably, late-passage (>81) MOSE cells were capable of forming >30 mm colonies. Compared with MOSE cells at earlier stages, late-passage MOSE cells exhibited an increased plating efficiency and growth rate, another proliferative parameter that is often associated with neoplastic change. Furthermore, the tumor formation rates were significantly increased in vivo (27,30). M-L cells formed tumors up to 100%, whereas M-E and M-I cells did not form tumors. Therefore, three sequential stages of transformed MOSE cells were defined as M-E (≤ 20 passages; early), M-I (21-80 passages; intermediate) and M-L (≥81 passages; late) cells, respectively.

Identification of ovarian cancer stem cells-like cells. Our previous study demonstrated that the acquisition of stemness was closely associated with malignant transformation of MOSE cells (unpublished data). SP cells are an important hallmark for the definition of the stem cell-like characteristics (22-26); therefore, whether SP cells may be used to enrich ovarian CSCs was investigated. Malignant MOSE (M-L) cells were separated by FACS into two populations. SP cells actively pump the Hoechst 33342 dye out of the cells, while NSP cannot and therefore this is a way to isolate these two cell populations (22-26). The percentage of SP cells in M-L cells reached up to 24.4% (Fig. 1A). After culturing for 3-4 days in serum-free culture medium, NSP cells gradually grew by adherence, and acquired epithelial morphology; however, SP cells formed larger spheres (Fig. 1B). Results from the sphere formation assay revealed that the sphere-forming rates of SP cells were significantly higher compared with those of the NSP cells (Fig. 1C).

To further verify the stem cell-like properties of SP cells, the expressions of CD44+ and CD117+, two well-known ovarian CSCs markers, were assessed by flow cytometry. In SP cells, the number of CD44⁺ (99.8%) and CD117⁺ (21.2%) cells were significantly higher compared with NSP cells (Fig. 1D and E). RT-qPCR results demonstrated that the mRNA levels of CD44 and CD133 in SP cells were increased, compared with the NSP cells (Fig. 1F). Notably, the mRNA expression levels of multipotent genes, such as SOX2, Krüppel-like factor (KLF)4, NOTCH1 and NOTCH2 were increased in SP cells, whereas NANOG expression was decreased, compared with NSP cells (Fig. 1G). Similarly, the protein expression levels of SOX2 and OCT4 were also significantly increased in SP compared with NSP, and the protein expression of NANOG was decreased. (Fig. 1H). In addition, the mRNA expression levels of ATP binding cassette subfamily G member (ABCG)2 and c-Myc were also significantly elevated in SP cells (Fig. 1G). Notably, the protein levels of VDR, β-catenin and Cyclin D1 in SP cells were lower compared with those in NSP cells (Fig. 1I).

Radioresistance is also a remarkable phenotype of CSCs (31,32). Thus, the survival fraction of cells irradiated by X-rays and carbon ions was determined using clonogenic and



Figure 1. Identification of SP cells with ovarian cancer stem cell-like properties *in vitro* and *in vivo*. (A) Results of FACS indicated that the SP cells accounted for 24.40% of the M-L cells. (B) Morphology of SP and NSP cells cultured in serum-free medium. (C) Sphere-forming rates of SP and NSP cells in different passages. (D, E) Results of flow cytometry assay indicated that CD44 and CD117 accounted for 99.8 and 21.2% of the SP cells, respectively, which was significantly higher compared with NSP cells. (F and G) Relative mRNA expression levels of (F) CD44, CD117 and CD133, and (G) OCT4, NANOG, SOX2, KLF4, NOTCH1, NOTCH2, c-Myc and ABCG2 in NSP and SP cells were examined by reverse transcription-quantitative polymerase chain reaction. (H and I) Relative protein expression levels of (H) SOX2, NANOG and OCT4, and (I) β -catenin, VDR and Cyclin D1 in SP and NSP cells were examined by western blotting. (J) Cell survival fractions with radiation dose of X-rays or carbon ions as the abscissa and the survival rate as the ordinate. (K) Representative images showing the tumorigenicity of NSP and SP cells *in vivo*. Data are presented as the mean ± standard deviation; n=3 in (A-J) and n=4 in (K); *P<0.05 and **P<0.01 vs. NSP. ABCG2, ATP binding cassette subfamily G member 2; CD, cluster of differentiation; KLF, Krüppel-like factor; NSP, non-side population; OCT, octamer-binding protein; SOX, Sex-determining region Y; SP, side population; VDR, Vitamin D receptor.

sphere-forming assay. The dose-response curves demonstrated that the survival fraction of SP cells was higher compared with

the NSP cells, which indicated that SP cells exhibited resistance to both X-rays and carbon ions (Fig. 1J).



Figure 2. 1α ,25(OH)₂D₃ inhibits cancer stem cell-like phenotype. (A) Effects of VD on the sphere formation of SP and NSP cells cultured in suspension medium. (B) Effects of VD on the number and diameter of spheres in SP cells. (C and D) Examination of changes in the mRNA expression levels of (C) multipotent genes and (D) ABCG2 in SP cells treated with VD were determined by reverse transcription-quantitative polymerase chain reaction. (E) Examination of changes in the protein expression levels of SOX2, OCT4 and NANOG in SP cells treated with VD were determined by western blot. Data are presented as the mean ± standard deviation; n=3; *P<0.05 and **P<0.01 vs. CTR. ABCG2, ATP binding cassette subfamily G member; CTR, Control; NSP, non-side population; OCT, octamer-binding protein; SOX, Sex-determining region Y; SP, side population; VD, 1α ,25(OH)₂D₃.

To further determine the tumorigenesis of SP and NSP cells *in vivo*, subcutaneous (n=4 mice/group) and orthotopic (n=2 mice/group) models of ovarian cancer were established in nude mice injected with SP or NSP. Neither of the two mice in the orthotopic NSP groups formed tumors; of the two mice that were orthotopically implanted with SP cells, one died at post-implantation, the other one grew a tumor with volume of 49.95 mm³ by 36 days post-implantation (data not shown). In the subcutaneous model, different numbers of NSP and SP cells were injected. None of the four mice injected with 1x10⁶NSP cells formed any tumor by 12 weeks post-injection; however, two of the four mice injected with 1x10⁴ SP cells formed tumors within 30 days post-injection, the volumes of which was 0.018 and 48 mm³ (Fig. 1K). Taken together, these *in vitro* and *in vivo* results demonstrated that the SP cells isolated from oncogenic transformation-MOSE cells exhibited properties of CSCs with self-renewal capability, multipotency and radioresistance.

 1α ,25(OH)₂D₃ inhibits stem cell-like phenotype of SP cells. To verify whether active vitamin D₃ is able to inhibit the stemness of CSCs, SP cells were treated with 10 nM 1 α ,25(OH)₂D₃.



Figure 3. $1\alpha,25(OH)_2D_3$ regulates the VDR/ β -catenin signaling pathway to inhibit the stemness of SP cells. (A-C) Relative changes in the mRNA levels of VDR, β -catenin and Cyclin D1 were examined in SP cells treated with 10 nM $1\alpha,25(OH)_2D_3$. (D-G) Relative changes in the protein levels of VDR, β -catenin and Cyclin D1 were examined in SP cells treated with various concentration of $1\alpha,25(OH)_2D_3$. (H) Representative images of SP cells untreated and treated with $1\alpha,25(OH)_2D_3$, (H) Representative images of SP cells untreated and treated with $1\alpha,25(OH)_2D_3$ showing β -catenin expression by confocal laser scanner microscope; nuclei were visualized by DAPI staining. Arrow pointed to the β -catenin in cytoplasm. Data are represented as the mean \pm standard deviation; n=3; *P<0.05 vs. CTR. CTR, control; VD, $1\alpha,25(OH)_2D_3$ NSP, non-side population; SOX, Sex-determining region Y; SP, side population; VDR, vitamin D receptor.

Notably, a few 1α ,25(OH)₂D₃-treated SP cells gradually grew by adherence following the treatment for three days, compared with untreated SP cells. Furthermore, the number and diameter of spheres formed were reduced in 1α ,25(OH)₂D₃-treated SP cells (Fig. 2A and B). Consistent with this phenotype, the mRNA expression levels of the multipotent genes NANOG, OCT4, SOX2, KLF4 and ABCG2 were downregulated in 1α ,25(OH)₂D₃-treated SP cells, compared with the untreated SP cells (Fig. 2C and D). However, the protein levels of NANOG, OCT4 and SOX2 were not significantly reduced in SP cells treated with 1α ,25(OH)₂D₃ (Fig. 2E). These results indicated that 1α ,25(OH)₂D₃ may be able to suppress the self-renewal capability and expression of multipotent gene expressions including NANOG, OCT4, SOX2, KLF4 in ovarian CSCs, although this needs to be verified.

 1α ,25(OH)₂D₃ increases β -catenin expression but decreases Cyclin D1 expression in SP cells. We know that 1α ,25(OH)₂D₃ exhibits its biological effects mainly through binding to VDR (13). It was determined that the protein and mRNA expression levels of VDR were increased in SP cells treated by 1α ,25(OH)₂D₃ (Fig. 3A, D and E). Subsequently, whether



Figure 4. Effects of vitamin D_3 on tumorigenesis of SP cells *in vivo*. (A) Tumorigenicity of SP cells treated with vitamin D_3 *in vivo*. (B) Western blot analysis of VDR, Cyclin D1, CD44 and β -catenin and (C-F) quantification of their protein expression levels. Data are presented as the mean \pm standard deviation; n=4; *P<0.05 and ***P<0.001 vs. CTR. CD, cluster of differentiation; CTR, control; NSP, non-side population; SP, side population; VD, Vitamin D; VDR, vitamin D receptor.

 1α ,25(OH)₂D₃ engaged in modulating VDR-mediated genes of ovarian CSCs was determined. The results of RT-qPCR demonstrated that 1α , 25(OH)₂D₃ had the tendency to increase β-catenin but significantly decreased Cyclin D1 mRNA expression levels in SP cells compared with Control cells (Fig. 3B and C). Western blot results demonstrated that 1α ,25(OH)₂D₃ treatment enhanced β -catenin and reduced Cyclin D1 protein expression levels in a dose-dependent manner (Fig. 3F and G). However, immunofluorescence staining indicated that the expression of β -catenin was mainly located in the nuclei of vehicle-treated SP cells, and β-catenin was blocked in cytoplasm following treatment with 1α ,25(OH)₂D₃ (Fig. 3H) Combined with the suppression of stem cell-like properties demonstrated in Fig. 2, these results implicated that 1α ,25(OH)₂D₃ may inhibit the stemness of SP cells through increasing VDR and cytoplasmic β -catenin and decreasing Cyclin D1 expression levels.

Vitamin D_3 inhibits the tumorigenic phenotype of SP cells in vivo. To further determine whether vitamin D₃ had suppressive effects on tumorigenesis of SP cells, ~1x10⁴ SP cells were subcutaneously inoculated into nude mice. Tumor formation was monitored by palpation and direct body dissection. In two of the four vehicle-treated Control mice tumor nodules were palpable as early as 30 days post-inoculation. By contrast, two of the four mice treated with vitamin D₃ exhibited signs of tumor growth at 45 days post-inoculation (Fig. 4A). To determine whether vitamin D_3 also modulates the expression of stemness-associated proteins in vivo, total protein was extracted from tumor tissues and analyzed by western blotting (Fig. 4B). The results indicated that vitamin D₃ treatment led to increased VDR and decreased Cyclin D1 protein expression levels, but the changes were not significant (Fig. 4C and D). Furthermore, vitamin D₃ decreased the expression levels of CD44, whereas it increased the expression levels of β -catenin in vivo compared

with Control mice (Fig. 4E and F). These results demonstrated that vitamin D_3 may have delayed the onset of tumor formation derived from injection of ovarian CSCs by reducing CD44 and increasing β -catenin expression, but this needs to be validated further.

Discussion

Accumulating evidence demonstrates that CSCs serve crucial roles in the development of chemoresistance, tumor relapse and metastasis in patients with ovarian cancer (33,34). The present study reports that vitamin D_3 not only inhibits self-renewal capability and multipotent gene expressions of ovarian CSCs *in vitro*, but it may also delay the onset of tumor formation. Furthermore, 1α ,25(OH)₂ D_3 treatment increased both the mRNA and protein expression levels of VDR and β -catenin, whereas it decreased the expression of Cyclin D1 and certain stemness-associated genes, including CD44, NANOG, OCT4, SOX2, KLF4 and ABCG2 in ovarian CSCs *in vitro*. Notably, the reduced expression of CD44 was demonstrated *in vitro* and *in vivo*.

Owing to repeating disruption and repairing with ovulation-associated remodeling, OSE with stem-like properties have been identified (5), and the junction area of hilum and oviduct contains cancer-prone stem cell niche (6). Ovarian CSCs with multipotent and self-renewal capability are usually purified using the surface markers CD44, CD117 and CD133, or functional marker such as isolation of SP and ALDH⁺ cells (22,35,36). It is reported that SP cells isolated from SKOV-3 cells displayed stem-like phenotype (25,37,38). In the present study, SP cells were isolated from malignant transformation MOSE cells, and demonstrated that both mRNA and protein expression levels of SOX2 and Oct4 were increased in SP cells. Additionally, they exhibited self-renewal capability and radioresistance in vitro, and an increased tumor growth in vivo. Notably, both mRNA and protein expression levels of VDR were reduced in SP cells. In general, high VDR expression is associated with reduced mortality and improved prognosis in breast and prostate tumors (39,40). 1α ,25(OH)₂D₃ activates and represses its target genes, such as RXRA, SMAD3, CCND3, by combining with VDR. Previous studies showed that 1α , 25(OH)₂D₃ inhibit proliferation and angiogenesis, and induce apoptosis of cancer cells in human cancers including ovarian cancer (41-43). However, it is not clear whether 1α ,25(OH)₂D₃ could reduce stemness of CSCs or promote differentiation through binding to VDR.CD44, a marker for stem cells of several cancers, serves an important role in ovarian CSCs (44,45). Additionally, a number of studies have reported that 1α , 25(OH)₂D₃ has potent effects on prostate and breast cancer stem cells, and demonstrated that 1α ,25(OH)₂D₃ and its analogue inhibited the proliferation of prostate and breast stem cells by inducing senescence and by decreasing CD44 expression levels (14,18-20). The present study results also indicated that 1α ,25(OH)₂D₃ suppressed the self-renewal capacity of ovarian CSCs by reducing CD44 expression levels, as well as the expressions of multipotent genes, such as Oct4, SOX2, NANOG, KLF4 and ABCG2 in vitro. Notably, 1α , $25(OH)_2D_3$ treatment decreased the mRNA expression levels of Oct4, SOX2 and NANOG, but not their protein levels. Previous studies have indicated that Oct4, SOX2 and NANOG often function in combination with each other to be involved in self-renewal and proliferation (46,47). CD44v3, a CD44 variant isoform, was reported to interact with Oct4-SOX2-NANOG (48). At the transcriptional level, Oct4, SOX2 and NANOG form a positive autoregulatory loop which is important for the maintenance of the undifferentiated state. At the post-translational level, non-coding RNAs are emerging as a key player in the control of cell proliferation and cell fate determination during differentiation (48). For example, microRNA-302 is controlled by a promoter containing Oct4-SOX2-NANOG-binding sites in human head and neck squamous cell carcinoma (48). It is worth exploring whether 1α ,25(OH)₂D₃-decreased CD44 may weaken the interaction with Oct4-SOX2-NANOG, which may result in no changes at the protein level, even though the mRNA expression levels of Oct4, SOX2 and NANOG were significantly decreased. In addition, 1α , $25(OH)_2D_3$ increased the β -catenin expression levels and decreased Cyclin D1 expression.

β-Catenin is a dual function protein, involved in both stemness and contribution to metastasis. On the one hand, β -catenin, as an important molecule of the Wnt signaling pathway, sustains stem cell renewal ability by maintaining multipotency in certain cell types (49). On the other hand, β-catenin, as a proto-oncogene, drives metastasis formation by translocation to the nucleus. Alterations in the localization and expression levels of β-catenin are associated with many cancers, including hepatocellular, colorectal, lung, breast, ovarian and endometrial cancer (50-55). Furthermore, the level of β -catenin can be modulated by VDR in colon cancer cells (56). In addition, 1α , $25(OH)_2D_3$ suppresses the expression of Cyclin D1 in epidermal carcinoma (57). These studies indicated that β -catenin and Cyclin D1 are both downstream target genes of 1α , 25(OH)₂D₃. In the present study, 1α , $25(OH)_2D_3$ increased the expression of VDR and decreased the expression of Cyclin D1 at mRNA and protein levels. Notably, 1α , 25(OH)₂D₃ significantly increased the expression of β -catenin, which was inconsistent with previous studies. However, immunofluorescence staining verified that 1α ,25(OH)₂D₃ only increased the expression of β -catenin in cytoplasm, consistent with Pálmer's study (58), which may result in the decrease of Cyclin D1. These results demonstrated that 1α ,25(OH)₂D₃ inhibited the stemness of CSCs through blocking the localization of β -catenin in cytoplasm.

However, there are several limitations in the present study. The number of mice used in the present study was not sufficient, which may have reduced the statistical power. Since the previous studies showed that SP cells exhibited more tumorigenicity than NSP cells in subcutaneous model in vivo (22,24), in the present study, 1x106 NSP cells were subcutaneously injected into mice. Two of the four mice injected with 1×10^4 SP cells formed tumors within 30 days post-injection, but none of the four mice injected with 1x106 NSP cells formed any tumor by 12 weeks. Additionally, whether SP cells would display more tumorgenicity than NSP cells in the orthotopic model need to be investigated. Unfortunately, the successful rate of the orthotopic operation was low and could barely get enough orthotopic mice for further study. In addition, the direct or indirect interaction between VDR and β -catenin, which is also very interesting and may be a novel underlying mechanism, was not investigated.

In conclusion, the present study demonstrated that 1α ,25(OH)₂D₃ restrained stem cell-like properties of ovarian cancer cells by enhancing VDR and had the tendency to promote cytoplasmic β -catenin, and reducing CD44 expression levels. These results may provide a novel strategy for vitamin D₃ in diminishing the stemness of CSCs. Future studies utilizing human ovarian cancer tissues to examine the role of vitamin D₃ in stem cell-like cell self-renewal may extend our understanding of ovarian cancer biology, which may lead to chemotherapeutic agents that may suppress stemness and improve the clinical outcome for patients with epithelial ovarian cancer.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BL and LL conceived and designed the experiments. LL and MJ performed the experiments. LL, MJ and YH analyzed the data. YH contributed to the material and analysis tools. MJ and BL drafted and revised the manuscript. All authors have read and approved the final manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All surgical procedures and care administered to the animals were approved by the Institutional Animal Care and Use Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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