0026-895X/11/7903-360–367$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 79:360–367, 2011

Vol. 79, No. 3
68403/3665617
Printed in U.S.A.

A Novel Gemini Vitamin D Analog Represses the Expression of a Stem Cell Marker CD44 in Breast Cancer

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Received August 25, 2010; accepted November 16, 2010

ABSTRACT

CD44 is a multifunctional transmembrane protein involved in cell proliferation, angiogenesis, invasion, and metastasis. CD44 is identified as a cancer stem cell marker, and the CD44-positive breast cancer cells are enriched in residual breast cancer cell populations after conventional therapies, suggesting that CD44 may be an important target for cancer prevention and therapy. Therefore, we investigated for the inhibitory effect of a novel Gemini vitamin D analog, 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol (BXL0124), on mammary tumor growth and CD44 expression in MCF10DCIS.com human breast cancer in vitro and in vivo. MCF10DCIS.com cells were injected into mammary fat pads in immunodeficient mice, and BXL0124 was then administered intraperitoneally (0.1 μg/kg body weight) or orally (0.03 or 0.1 μg/kg body weight) 6 days a week for 5 weeks. At necropsy, mammary tumors and blood were collected for evaluating tumor growth, CD44 expression, and serum calcium level. BXL0124 suppressed mammary tumor growth and markedly decreased the expression of CD44 protein in MCF10DCIS xenograft tumors without causing hypercalcemic toxicity. BXL0124 also inhibited the expression of CD44 protein and mRNA as well as the transcriptional activity of the CD44 promoter in cultured MCF10DCIS.com cells. The repression of CD44 expression induced by BXL0124 was blocked by siRNA vitamin D receptor (VDR), indicating that the regulation of CD44 expression by BXL0124 is a VDR-dependent event. The novel Gemini vitamin D analog, BXL0124, represses CD44 expression in MCF10DCIS.com cells in vitro and in xenograft tumors, suggesting an inhibitory role of a Gemini vitamin D derivative on breast cancer stem cells.

Introduction

Breast cancer is the second leading cause of cancer-related deaths among women, with as many as 40% relapsing with metastatic disease after therapy, and conventional therapies have been able to effectively shrink solid tumors but have failed to produce long-term clinical remissions without recurrence and metastasis (Morrison et al., 2008). It has been suggested that cancer stem cells, which represent a subset of tumor cells, are responsible for the origin and maintenance of tumors. Moreover, cancer stem cells are believed to be the main cause of metastasis and recurrences of cancer because of their strong tumor-initiating abilities and resistance to conventional therapies (Al-Hajj et al., 2004; Sheridan et al., 2006; Morrison et al., 2008).

A transmembrane glycoprotein, CD44, first known to be involved in cell-cell interaction and cell adhesion, has been identified as a key cell-surface marker for cancer stem cells in pancreas cancer, prostate cancer, head and neck squamous

This work was supported in part by the National Institutes of Health National Cancer Institute [R01-CA127645]; the National Institutes of Health National Institute of Environmental Health Sciences [Grant ES005022]; and the Trustees Research Fellowship Program at Rutgers, the State University of New Jersey.

J.Y.S. and H.J.L. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.110.068403.

ABBREVIATIONS: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; BXL0124, 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideutero-butyl)-23-yne-26,27-hexafluoro-cholecalciferol; CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; CD44v3, CD44 containing variant domain 3; CD44v6, CD44 containing variant domain 6; DAPI, 4,6-diamidino-2-phenylindole; DCIS, ductal carcinoma in situ; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; qPCR, quantitative polymerase chain reaction; SCID, severe combined immunodeficiency; siRNA, small interfering RNA; VDR, vitamin D receptor.

cell carcinoma, and breast cancer (Al-Hajj et al., 2003; Collins et al., 2005; Li et al., 2007; Prince et al., 2007). Induction of CD44 expression in human breast cancer cell lines has been shown to enhance self-renewal, mammosphere growth, and drug resistance, demonstrating functional roles of CD44 in breast cancer stem cells (To et al., 2010). CD44 is also known as an important mediator for the response of cells to their cellular microenvironment (Ponta et al., 2003). Al-Hajj et al. (2003) first identified breast cancer stem cells from human breast cancer specimens, which are rich in $CD44^+/CD24^{-/low}$ cells, and showed that this distinct population of cells had the exclusive ability to form tumors in mice. Later, Fillmore and Kuperwasser (2008) demonstrated that human breast cancer cells contain a defined subpopulation of $CD44^+/CD24^{-/low}$ cells, which can drive tumorigenesis and regenerate tumor cell heterogeneity. A clinical study also indicated that $CD44^+/CD24^{-/low}$ cells were enriched in residual breast cancers after conventional therapies (Creighton et al., 2009).

The MCF10DCIS.com cell line is one of the derivatives of the MCF10A series, which is a unique human model of breast tumor progression reflecting basal-like breast cancer (Miller et al., 2000; Worsham et al., 2006). It is noteworthy that studies have shown the strong association between basal-like phenotype of breast cancer cells and presence of the $CD44^+/CD24^{-/low}$ cells (Fillmore and Kuperwasser, 2007). Behbod et al. (2009) showed that MCF10DCIS.com cells also contained $CD44^+/CD24^{-/low}$ subpopulations that formed a large number of DCIS-like lesions in xenografted mammary tumors and even suggested that the majority of MCF10DCIS.com cells may possess tumor-initiating properties. Moreover, the bipotential progenitor properties of MCF10DCIS.com cells, which give rise to not only epithelial cells but also myoepithelial cells in mouse xenografts, demonstrate the ability of generating heterogeneous cell populations (Hu et al., 2008), suggesting that the MCF10DCIS.com cell line might be a useful model for studying the efficacy of preventive and therapeutic agents for inhibiting breast cancer stem cells.

Our recent report of in vivo and in vitro inhibitory activities of novel Gemini vitamin D analogs on MCF10DCIS.com cells (Lee et al., 2008) led us to study whether $1\alpha,25(OH)_2D_3$ or Gemini vitamin D analogs regulate cancer stem cell markers such as CD44, which eventually results in inhibition of breast tumorigenesis. $1\alpha,25(OH)_2D_3$ is the key hormone in calcium/phosphate homeostasis and bone mineralization, and an epidemiological study indicates an association between low serum vitamin D levels and increased risk for breast cancer (Deeb et al., 2007). However, the clinical application of $1\alpha,25(OH)_2D_3$ for inhibition of breast cancer was limited by hypercalcemic toxicity, which was detected in 20 to 30% of patients in clinical trials (Hellström et al., 1990; Deeb et al., 2007). Therefore, numerous synthetic vitamin D_3 analogs have been developed for better anticancer efficacy with lower toxicity (Hines et al., 2010). Our studies demonstrated that Gemini vitamin D analogs inhibited the formation and growth of estrogen receptor-positive mammary tumors in vivo without hypercalcemic toxicity (Lee et al., 2008) and suppressed mammary tumor growth in the ErbB2-overexpressing transgenic mice (Lee et al., 2010). In the present study, because of the importance of CD44 as a cancer stem cell marker and its suggested functional roles in breast cancer, we examined the effects of Gemini BXL0124 on regulation of CD44 in cultured MCF10DCIS.com cells in vitro and in MCF10DCIS.com xenograft tumors in vivo.

Materials and Methods

Reagents and Cell Culture. $1\alpha,25(OH)_2D_3$ and Gemini vitamin D analog 1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol (BXL0124; Maehr et al., 2009; >95% purity) (Fig. 1A) were provided by BioXell, Inc. (Nutley, NJ) and dissolved in dimethyl sulfoxide (DMSO). For in vivo animal experiments, BXL0124 was diluted in Cremophor/phosphate-buffered saline [1:8 (v/v)] or in sesame oil (Sigma-Aldrich, St. Louis, MO) for intraperitoneal injection or oral administration, respectively. The human MCF10DCIS.com breast cancer cell line was provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) (Miller et al., 2000). MCF10DCIS.com and MCF10CA1a cells were maintained in DMEM/F-12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37°C and 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction. These procedures have been reported previously (Lee et al., 2006). Labeled primers, including glyceraldehyde-3-phosphate dehydrogenase, osteopontin, and CD44, were obtained from Applied Biosystems (Foster City, CA).

Transient Transfection of CD44 and Promoter Assay. The detailed procedures have been described previously (Lee et al., 2009). pCMV-β-galactosidase vector was provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). CD44 promoter- and p53 binding site-mutated CD44 promoter-luciferase reporter vectors were provided by Dr. Robert A. Weinberg (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA). For the transient transfection, vectors were mixed with FuGene6 Transfection Reagent (Invitrogen, Carlsbad, CA), and MCF10DCIS.com cells were incubated with the mixtures for 6 h in serum-free DMEM/F-12 medium. Then, cells were treated with compounds for 24 h in 0.1% bovine serum albumin containing DMEM/F-12 medium. Luciferase activity was measured with a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) and normalized for β-galactosidase activity.

Western Blot Analysis. The procedure was described previously (Lee et al., 2009), and the primary antibody, CD44, which recognizes all CD44 splicing variants, was from Santa Cruz Biotechnology (Santa Cruz, CA); CD44 containing variant domain 3 (CD44v3) and CD44 containing variant domain 6 (CD44v6) was from R&D Systems (Minneapolis, MN); vitamin D receptor (VDR) was from Thermo Fisher Scientific (Waltham, MA); β-actin was from Sigma-Aldrich; and secondary antibodies were from Santa Cruz Biotechnology.

Fluorescence Microscopy. MCF10DCIS.com cells were incubated in glass-bottomed dishes (MatTek, Ashland, MA) with or without BXL0124 (10 nM). Cells were fixed with 4% paraformaldehyde, blocked with 10% bovine serum albumin, and then incubated sequentially with CD44 primary antibody (1:100; Santa Cruz Biotechnology), fluorophore-conjugated secondary antibody (Alexa Fluor 488; Invitrogen), and 4,6-diamidino-2-phenylindole (DAPI). The cells were irradiated with green laser at 488 nm for detection of CD44 and with UV light at 364 nm for nuclear staining by DAPI.

Animal Experiments in the Xenograft Model. The detailed procedure for the injection of MCF10DCIS.com cells was described previously (Lee et al., 2008). To determine mammary tumor growth, MCF10DCIS.com cells were injected into the mammary fat pad area in severe combined immunodeficiency (SCID) mice (four per group), and mice were sacrificed at 1, 2, 3, 4, and 5 weeks after injection. Tumors were collected for further analysis. To test the anticancer activity of Gemini vitamin D analog BXL0124, MCF10DCIS.com cells were xenografted into the mammary fat pad area in nu/nu or SCID mice. Then, vehicle control (0.1 ml) or Gemini vitamin D analog BXL0124 (0.03 or 0.1 μg/kg body weight in 0.1 ml of vehicle) was administered either intraperitoneally or orally 6 days a week

from day 4 until the end of the experiment. Body weight and tumor size were measured twice a week. Five weeks after the injection of MCF10DCIS.com cells, mammary tumors were weighed and collected for further analysis. All animal studies were done in accordance with an institutionally approved protocol.

Determination of Serum Calcium Level. The procedure for the determination of calcium concentration in serum samples (POINTE Scientific, Inc., Canton, MI) was reported previously (Lee et al., 2008).

Immunohistochemistry and Quantification. The procedure for immunohistochemistry was described previously (Lee et al., 2008). The slides were incubated overnight at −4°C with CD44 primary antibody (1:50; Santa Cruz Biotechnology) or proliferating cell nuclear antigen (PCNA) primary antibody (1:1000; BD Pharmingen, San Diego, CA). The numbers of cells according to CD44 membrane staining intensity or PCNA nuclear staining intensity were quantified by using a Scan Scope (Aperio, Vista, CA).

Flow Cytometry. MCF10DCIS.com cells were incubated with DMSO, $1\alpha,25(OH)_2D_3$ or BXL0124 (10 nM) for 24 h. Cells were trypsinized into single cell suspension, counted, washed with phosphate-buffered saline, and stained with antibodies against CD44-allophycocyanin and CD24-PE (BD Pharmingen). The cells (5×10^5 cells/well) were incubated with antibodies for 20 min on ice. Unbound antibodies were washed off, and cells were analyzed on a BD FACSArray Bioanalyzer (BD Biosciences, San Jose, CA).

Knockdown of VDR by siRNA. The detailed procedure was described previously (Paul et al., 2009). MCF10DCIS.com cells were incubated without siRNA or with 1 μM negative control siRNA or each of two VDR siRNAs targeting different sequences in the VDR gene (Thermo Fisher Scientific) for 72 h in Accell siRNA delivery medium (Thermo Fisher Scientific). The cells were followed by incubation with DMSO or BXL0124 (10 nM) for 24 h in cell culture medium.

Statistical Analysis. Statistical significance was evaluated using the Student's t test.

Results

The Novel Gemini Vitamin D Analog, BXL0124, Inhibits MCF10DCIS.com Xenograft Mammary Tumor Growth in SCID and nu/nu Mice without Hypercalcemic Toxicity. We demonstrated previously that novel Gemini vitamin D analogs had better efficacy for growth inhibition of MCF10DCIS.com cells than $1\alpha,25(OH)_2D_3$ in vitro (Lee et al., 2008). In our present study, we investigated the inhibitory effect of a potent Gemini vitamin D BXL0124 (Fig. 1A) on the growth of MCF10DCIS.com xenograft mammary tumors in SCID mice and in nu/nu mice. In nu/nu mice, oral administration of BXL0124 at the dose of 0.1 μg/kg body weight suppressed tumor size and tumor weight by 47 ($p < 0.05$) and 52% ($p < 0.05$), respectively (Fig. 1B). In SCID mice, when BXL0124 was given intraperitoneally at the dose of 0.1 μg/kg body weight, average tumor volume and weight were significantly reduced by 75 ($p < 0.01$) and 66% ($p < 0.01$), respectively (Fig. 1C). When BXL0124 was administered orally at the dose of 0.03 and 0.1 μg/kg body weight, tumor volume and weight were significantly reduced in MCF10DCIS.com xenografted SCID mice (Fig. 1D). Tumor

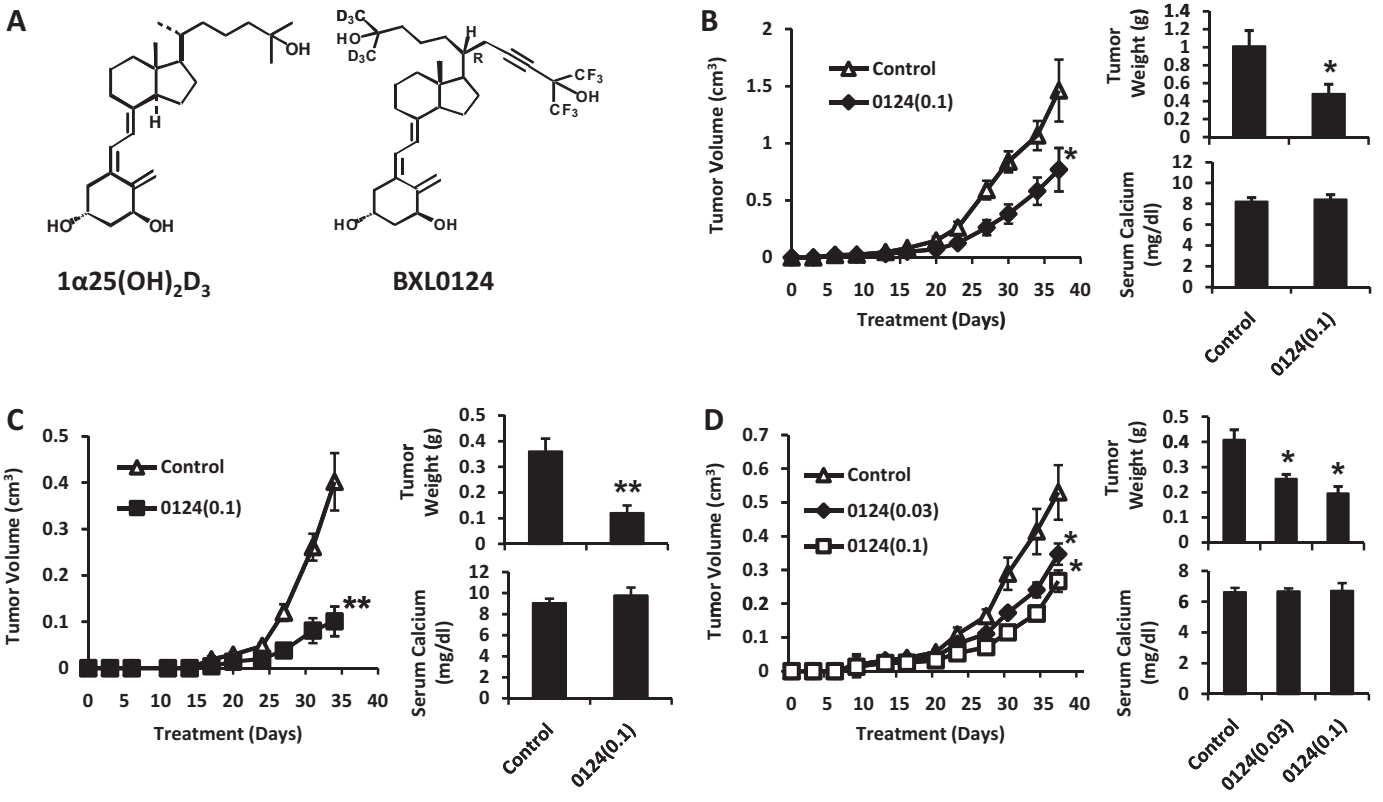


Fig. 1. BXL0124 inhibits MCF10DCIS.com xenograft mammary tumor growth in immunodeficient mice without hypercalcemic toxicity. A, the structures of $1\alpha,25(OH)_2D_3$ and the Gemini vitamin D analog BXL0124. B, BXL0124 (0.1 μg/kg body weight) was administered orally in MCF10DCIS.com xenografted nu/nu mice ($n = 5$) once a day, 6 times per week. Tumor volume was measured twice per week. Tumor weight and serum calcium level were measured at necropsy. C, BXL0124 (0.1 μg/kg body weight) was given intraperitoneally in MCF10DCIS.com xenografted SCID mice ($n = 5$) 6 times a week. Tumor volume was measured twice per week. Tumor weight and serum calcium level were measured at necropsy. D, BXL0124 (0.03 or 0.1 μg/kg body weight) was administered orally in MCF10DCIS.com xenografted SCID mice ($n = 7$) 6 times per week. Tumor volume was measured twice per week. Tumor weight and serum calcium level were measured at necropsy. The data are presented as the mean ± S.E. (statistical analysis: *, $p < 0.05$; **, $p < 0.01$).

volume was reduced by BXL0124 treatment at the dose of 0.03 and 0.1 μg/kg body weight by 36 ($p < 0.05$) and 49% ($p < 0.05$), respectively. Tumor weight was decreased by 37 ($p < 0.05$) and 52% ($p < 0.05$) with 0.03 and 0.1 μg/kg body weight of BXL0124 treatment, respectively (Fig. 1D). In all animal studies, both intraperitoneal injection and oral administration of BXL0124 at the doses tested did not cause any significant changes in body weights and serum calcium levels, indicating no hypercalcemic toxicity at the given doses (Fig. 1, B–D).

CD44 Is Expressed in MCF10DCIS.com Xenograft Mammary Tumors during Tumor Growth. MCF10DCIS.com cells form DCIS-like lesions that spontaneously progress to invasive tumors when they are xenografted into immunodeficiency mice (Miller et al., 2000). H&E staining showed that MCF10DCIS.com cells xenografted into the mammary fat pad of SCID mice produced lesions that are histologically similar to DCIS of human breast cancers by week 2. By week 3, comedo DCIS-like lesions with apoptotic centers were formed, and invasive tumor lesions were also detected. A majority of tumors became invasive by week 4 (Fig. 2A). Throughout the growth of tumors and transition from the DCIS-like lesions to invasive tumors, CD44 proteins were dominantly expressed in the membrane of epithelial cells in MCF10DCIS.com xenograft tumors (Fig. 2A). Tumor growth from weeks 1 to 5 was measured, and the average tumor volumes for weeks 1, 2, 3, 4, and 5 were 0.02, 0.03, 0.05, 0.14, and 0.41 cm^3, respectively (Fig. 2B).

BXL0124 Represses CD44 Expression Level in MCF10DCIS.com Xenograft Tumors In Vivo. BXL0124 was given orally at the dose of 0.1 μg/kg body weight in nu/nu mice (Fig. 1B). All tumors (one tumor for each mouse) were pooled for each group ($n = 5$) and analyzed for CD44 protein expression level by Western blot analysis. The protein expression levels for both the standard form of CD44 (CD44s, the most widely expressed standard isoform of CD44, 85 kDa) and variant forms of CD44 (CD44v, 100~250 kDa) were markedly down-regulated by BXL0124. The repression of CD44v3 and CD44v6 variants expression by BXL0124 treatment was also shown by using antibodies that specifically recognized individual variant forms (Fig. 3A). The expression of PCNA, a marker for cell proliferation, was also significantly repressed by BXL0124 treatment (Fig. 3A). In the histological evaluation using H&E staining of mammary tumors, we confirmed that all mammary tumors from the control and BXL0124 treatment groups were determined to be adenocarcinomas (Fig. 3B). However, both CD44 and PCNA expression levels of MCF10DCIS.com xenograft tumors in nu/nu mice were significantly decreased in BXL0124-treated group compared with the control (Fig. 3C). Three mammary tumors from each group were selected, and three representative areas from each tumor were analyzed to quantify the staining intensity by using Scan Scope. The staining intensities of CD44 and PCNA were scored from 3+ (the strongest staining) to 0+ (negative staining) in each individual epithelial cell automatically. In both the control and BXL0124-treated groups, CD44 protein was localized exclusively on the plasma membrane of epithelial cells in mammary tumors. The distribution of cell fraction by CD44 staining intensity in the control group was 48 (3+), 32 (2+), and 20% (1+), whereas BXL0124-treated group showed 15 (3+), 62 (2+), and 23% (1+) of CD44 staining intensity (Fig. 3C). For PCNA staining, 82% of cells were PCNA-positive in control group,

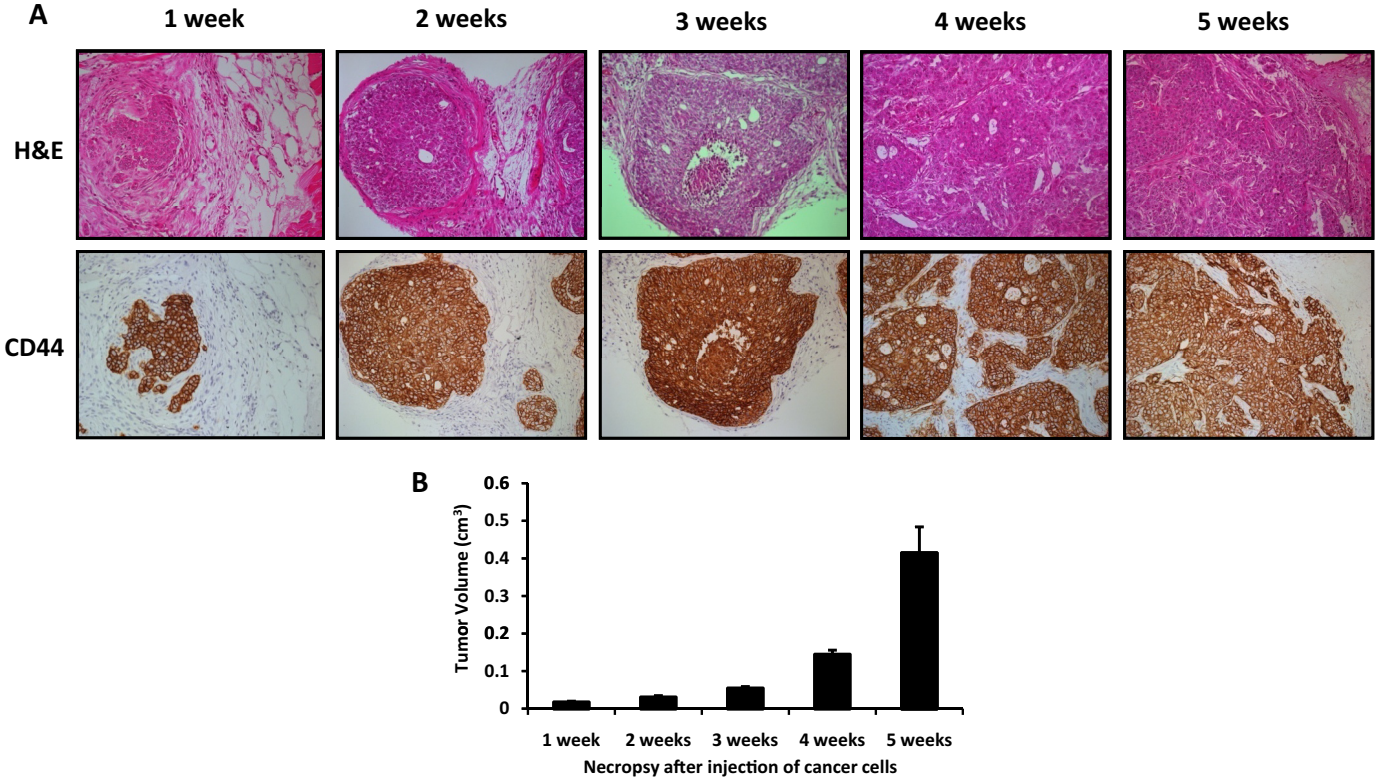


Fig. 2. MCF10DCIS.com xenograft mammary tumors express CD44 during the growth of tumors. A, at day 0, MCF10DCIS.com cells (10^6 cells) were injected into the mammary fat pad area of SCID mice and divided into five groups. Mice ($n = 4$) were sacrificed at 1, 2, 3, 4, and 5 weeks after injection. All tumors were collected and analyzed for H&E and immunohistochemical analysis, and a representative tumor staining for H&E and CD44 is shown for each week (original magnification, 200×). B, tumor volume was measured at necropsy every week. The data are presented as the mean ± S.D. ($n = 4$).

whereas 62% of cells were PCNA-positive in BXL0124-treated group (Fig. 3C). Cells (18%) were PCNA-negative in the control group, whereas 38% of cells were PCNA-negative in the BXL0124-treated group ($p < 0.01$) (Fig. 3C).

The Level of CD44 Protein Is Decreased by the Treatment with BXL0124 in MCF10DCIS.com Cells in a VDR-Dependent Manner. Next, we tested the effect of $1\alpha,25(OH)_2D_3$ or BXL0124 on the protein expression level of CD44 in MCF10DCIS.com cells in vitro. We found that $1\alpha,25(OH)_2D_3$ or BXL0124 down-regulated the protein expression of CD44s (85 kDa) and CD44v (100~250 kDa) in a dose-dependent manner. However, BXL0124 was more effective than $1\alpha,25(OH)_2D_3$ in MCF10DCIS.com cells (Fig. 4A). We have also tested the effect of BXL0124 on the CD44 marker in a different cell line, MCF10CA1a, which is known to be a highly aggressive cell line with metastatic capability among the MCF10 cell line series. BXL0124 also down-regulated the protein expression level of both CD44s and CD44v in MCF10CA1a cells (Fig. 4A). DAPI staining was used to recognize the nuclear morphology of cells. We found that the CD44 protein was localized specifically in the plasma membrane and the expression level was reduced by BXL0124 treatment without changes of any subcellular localization (Fig. 4B). MCF10DCIS.com cells were costained with CD44 and CD24 for flow cytometry. The fraction of $CD44^{+/high}/CD24^{-/low}$ cells, the subpopulation in which breast cancer stem cells are enriched, was significantly decreased by BXL0124 treatment ($p < 0.01$), whereas the fraction of $CD44^{+/high}/CD24^{+/high}$ cells were increased by BXL0124 treatment ($p < 0.01$) (Fig. 4C). Because the active form of vitamin D_3, $1\alpha,25(OH)_2D_3$, exerts the majority of its biological functions such as transcriptional activation or repression through binding to the VDR, we further tested whether the repression of CD44 is dependent on VDR. Knockdown of VDR by using two different VDR siRNAs blocked the repression of CD44 protein expression by BXL0124 in MCF10DCIS.com cells, indicating that the down-regulation of CD44 by BXL0124 is a VDR-dependent event (Fig. 4D).

BXL0124 Suppresses the Expression of CD44 mRNA and Induces the Expression of Osteopontin mRNA in MCF10DCIS.com Breast Cancer Cells. The transcriptional regulation of CD44 and osteopontin expression was investigated in MCF10DCIS.com cells. The down-regulation of CD44 mRNA by $1\alpha,25(OH)_2D_3$ (10 nM) or BXL0124 (10 nM) in MCF10DCIS.com cells was determined at different time points, and it showed maximum inhibition at 4 h with both compounds (Fig. 5A). When MCF10DCIS.com cells were treated with $1\alpha,25(OH)_2D_3$ or BXL0124 for 4 h, CD44 mRNA expression was down-regulated in a dose-dependent manner, and BXL0124 showed a stronger repressive effect than $1\alpha,25(OH)_2D_3$ at the same doses (Fig. 5B). Because osteopontin, which is one of the target genes regulated by vitamin D,

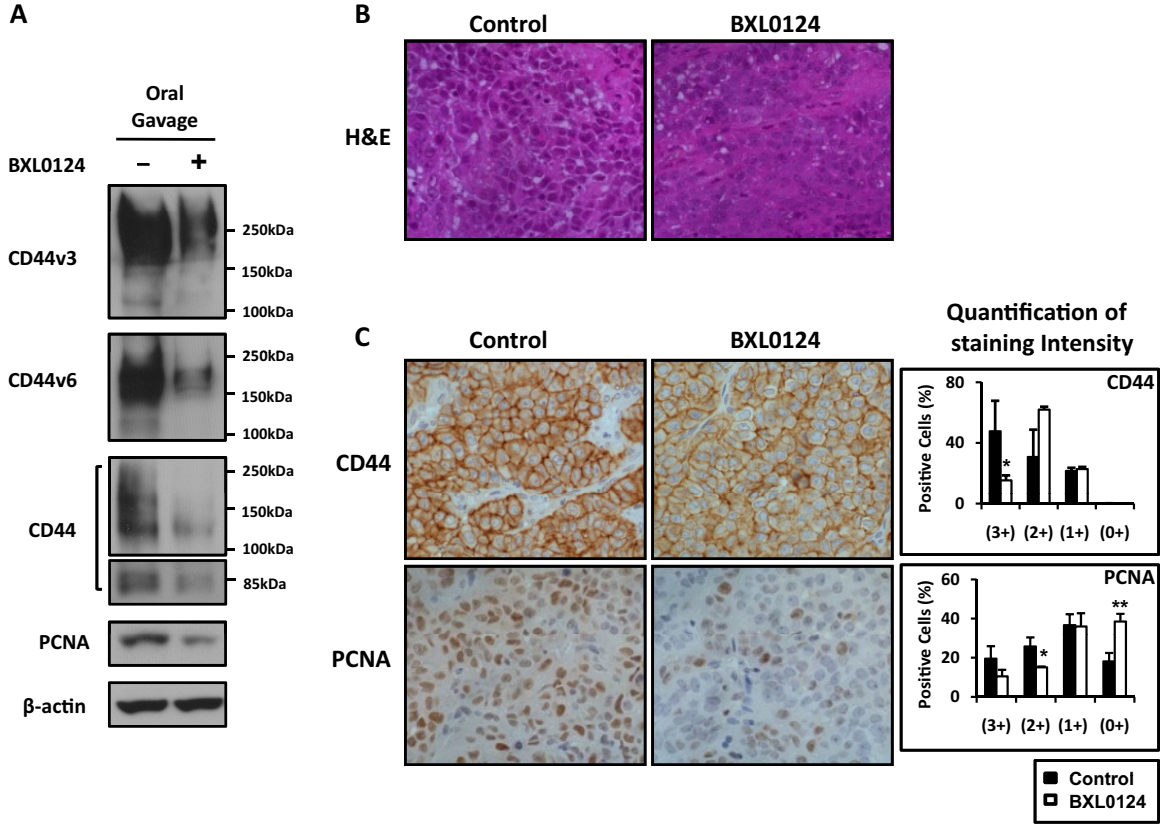
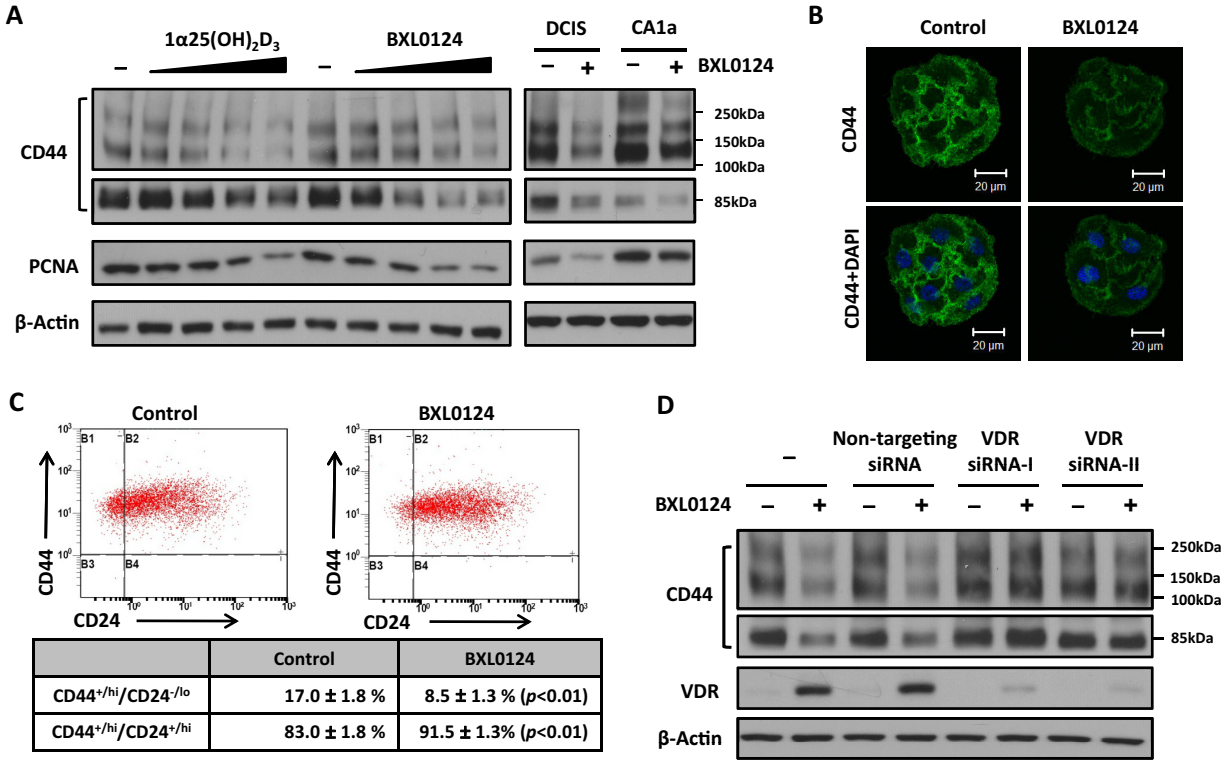


Fig. 3. Effects of BXL0124 on CD44 protein expression level in MCF10DCIS.com xenograft tumors in vivo. A, MCF10DCIS.com xenografted nu/nu mice were treated with DMSO or BXL0124 (0.1 μg/kg body weight) orally, and mammary tumors were collected at necropsy. Mammary tumors ($n = 5$) were pooled into either the control group or BXL0124-treated group for Western blot analysis against CD44, CD44v3, CD44v6, PCNA, and β-actin. B, a representative H&E staining in mammary tumors from MCF10DCIS.com xenografted nu/nu mice is shown (original magnification, 400×). C, a representative immunostaining against CD44 and PCNA in mammary tumors from MCF10DCIS.com xenografted nu/nu mice is shown (original magnification, 400×). Three mammary tumors from each group were selected and three representative areas from each tumor were analyzed for the expression of CD44 and PCNA. The mammary tumors immunostained against CD44 and PCNA were scored by four different levels of staining intensity and quantified by using Aperio Scan Scope. The data are presented as the mean ± S.D. (statistical analysis: *, $p < 0.05$; **, $p < 0.01$).

is known to interact with CD44, we also investigated the regulation of osteopontin mRNA by BXL0124. Both $1\alpha,25(OH)_2D_3$ (10 nM) and BXL0124 (10 nM) induced the expression of osteopontin mRNA starting at 4 h, and the induction of osteopontin mRNA by BXL0124 was stronger than by $1\alpha,25(OH)_2D_3$ at 24 h (Fig. 5C). When MCF10DCIS.com cells were treated with $1\alpha,25(OH)_2D_3$ or BXL0124 for 4 h, osteopontin mRNA expression was induced in a dose-dependent manner (Fig. 5D).

BXL0124 Repressed the Transactivation of CD44 Promoter in MCF10DCIS.com Cells in a p53-Dependent Manner. The transcriptional repression of CD44 by $1\alpha,25(OH)_2D_3$ or BXL0124 was shown by a CD44 promoter



	Control	BXL0124
$CD44^{+/hi}/CD24^{-/lo}$	17.0 ± 1.8 %	8.5 ± 1.3 % ($p<0.01$)
$CD44^{+/hi}/CD24^{+/hi}$	83.0 ± 1.8 %	91.5 ± 1.3% ($p<0.01$)

Fig. 4. Effects of BXL0124 on CD44 protein expression level in MCF10DCIS.com breast cancer cells in vitro. A, MCF10DCIS.com cells were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 24 h and analyzed for CD44 and PCNA protein expression levels by Western blot analysis. MCF10DCIS.com and MCF10CA1a cells were treated with BXL0124 (10 nM) for 24 h and analyzed for CD44 and PCNA protein expression levels by Western blot analysis. All splicing isoforms of CD44 were recognized by a CD44 antibody, which recognizes both CD44 standard and variants. B, MCF10DCIS.com cells were treated with DMSO or BXL0124 (10 nM) for 24 h and analyzed for CD44 expression level by confocal microscopy. C, MCF10DCIS.com cells were treated with DMSO control or BXL0124 (10 nM) for 24 h. The percentage of cells, which were categorized by the combination of CD44 and CD24 expression, was determined by flow cytometry. The experiment was repeated three times, and the data are presented as the mean ± S.D. D, MCF10DCIS.com cells were incubated without siRNA or with negative control siRNA or 1 μM concentration of each of two VDR siRNAs targeting different sequences in the VDR gene in Accell siRNA delivery medium for 72 h and followed by treatment with DMSO or BXL0124 (10 nM) for 24 h. The levels of CD44 and VDR protein were determined by Western blot analysis.

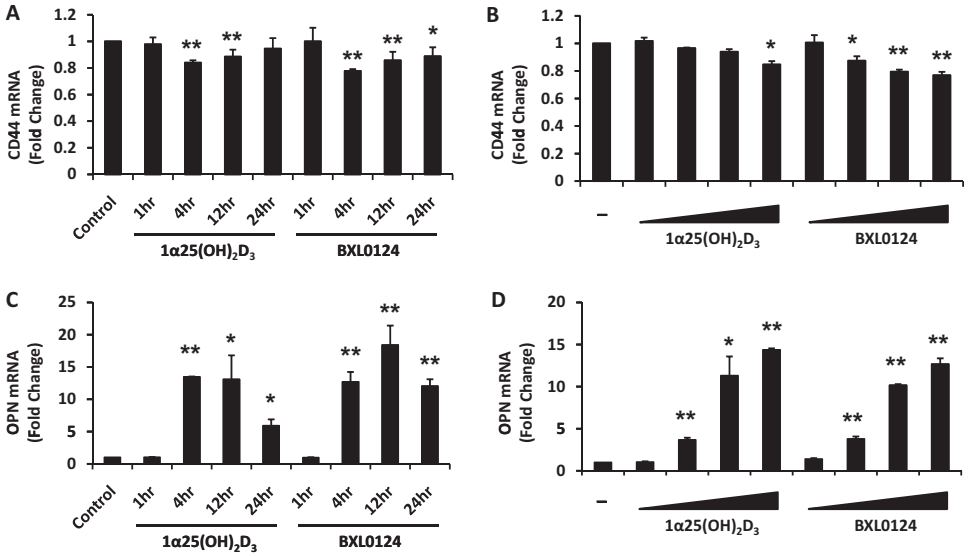


Fig. 5. $1\alpha,25(OH)_2D_3$ and BXL0124 repress CD44 expression and induce osteopontin expression at the transcriptional level. A, MCF10DCIS.com cells were treated with $1\alpha,25(OH)_2D_3$ or BXL0124 (10 nM) for 1, 4, 12, and 24 h and analyzed for CD44 mRNA expression level by quantitative PCR (qPCR). B, MCF10DCIS.com cells were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 4 h and analyzed for CD44 mRNA expression level by qPCR. C, MCF10DCIS.com cells were treated with $1\alpha,25(OH)_2D_3$ or BXL0124 (10 nM) for 1, 4, 12, and 24 h and analyzed for osteopontin mRNA expression level by qPCR. D, MCF10DCIS.com cells were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or BXL0124 (0.01, 0.1, 1.0, and 10 nM) for 4 h and analyzed for osteopontin mRNA expression level by qPCR. The data are presented as the mean ± S.D. (statistical analysis: *, $p < 0.05$; **, $p < 0.01$). OPN, osteopontin.

assay. Although both $1\alpha,25(OH)_2D_3$ and BXL0124 repressed the transactivation of the CD44 promoter in a dose-dependent manner, BXL0124 showed a more potent inhibitory effect than $1\alpha,25(OH)_2D_3$ (Fig. 6A). p53 has been reported to inhibit CD44 expression via binding to a p53-binding sequence on the CD44 promoter (Godar et al., 2008). Therefore, we determined the involvement of p53 for the repression of CD44 by BXL0124. The repression of CD44 promoter transactivation by BXL0124 was abolished when the p53-binding site of the CD44 promoter was mutated, indicating that p53 is necessary for the CD44 repression by BXL0124 (Fig. 6B).

Discussion

Accumulating evidence indicates that cancer stem cells are responsible for tumor-initiation, recurrence, metastasis, and the resistance to conventional chemotherapies (Dick, 2008; Visvader and Lindeman, 2008). Therefore, these cancer stem cells are becoming a critical target for cancer therapeutics. CD44 is a key cell-surface marker for cancer stem cells in pancreatic, prostate, and breast cancer (Al-Hajj et al., 2003; Collins et al., 2005; Li et al., 2007). Godar et al. (2008) demonstrated that CD44 suppression by CD44-specific short hairpin RNA infection not only inhibited tumor growth but also reduced the tumor-initiating ability of a human breast cancer cell xenograft. In addition, CD44 targeting by specific antibody treatment inhibited tumor recurrence after chemotherapy-induced remission of tumors in human breast cancer xenografts (Marangoni et al., 2009). These findings indicate that CD44 has a direct role in tumor initiation and recurrence in addition to serving as a useful marker for breast cancer stem cells.

CD44, a transmembrane glycoprotein encoded by a single gene with at least 19 exons, has multiple variants produced by alternative splicing (Ponta et al., 2003). In breast cancer, CD44 variants (CD44v, 100~250 kDa) rather than a CD44s (85 kDa) have been strongly associated with cancer metastasis and poor disease-free rate (Götte and Yip, 2006). Among many different CD44 variants, CD44v3, which is typically modified by heparin-sulfate side chains, is known to recruit matrix metalloproteinases and induce cell invasion and survival signaling (Yu et al., 2002; Ponta et al., 2003). In several cell lines, CD44v6 forms complex with Met receptor and acts as coreceptor to promote cell growth (Orian-Rousseau et al., 2002; Ponta et al., 2003). In addition, CD44v6 has been shown to interact with osteopontin, one of the target genes known to be regulated by vitamin D and its analogs (Ponta et al., 2003; Lee et al., 2007). The overexpression of endogenous osteopontin enhanced the expression of CD44v6 protein, which may be essential for mediating osteopontin-induced tumor cell metastasis (Khan et al., 2005). Because of the biological importance of CD44 variants, we investigated whether BXL0124 regulates the protein expression of CD44 variants, CD44v3 and CD44v6. MCF10DCIS.com xenograft mammary tumors expressed high level of CD44v3 and CD44v6, and the expression of these variants was markedly reduced by BXL0124 treatment, suggesting that repression of CD44 variants may contribute to the growth-inhibitory effect of BXL0124.

In our previous study, we reported that a Gemini vitamin D analog significantly inhibited the growth of MCF10DCIS.com xenografted tumors in immunodeficient mice (Lee et al., 2008). However, mechanistic studies including the upstream mediator regulated by the Gemini vitamin D analogs have not been determined in this model. Because CD44 is a main receptor for several key extracellular matrix proteins such as osteopontin and hyaluronan (Ponta et al., 2003), the repression of CD44 by $1\alpha,25(OH)_2D_3$ and its analogs may contribute to their anticancer activities. A recent colon cancer study demonstrated that $1\alpha,25(OH)_2D_3$ or its analogs increased the expression of osteopontin, but they suppressed the expression of CD44 and enhanced the expression of E-cadherin, which may contribute to their inhibitory effect on adenoma formation in $APC^{Min/+}$ mice (Xu et al., 2010). In the present study, BXL0124 decreased the expression of CD44 mRNA and protein (Figs. 4A and 5, A and B) and induced the expression of osteopontin mRNA (Fig. 5, C and D) in cultured MCF10DCIS.com cells. Although BXL0124 induced the expression of osteopontin mRNA, the repression of its receptor, CD44 may be the primary contributor to the growth-inhibitory effect of BXL0124 on MCF10DCIS.com cells.

Godar et al. (2008) found that the p53 tumor suppressor inhibits the expression of CD44 via binding to a p53-binding sequence in the CD44 promoter in transformed human mammary epithelial cells, suggesting that CD44 repression by p53 is critical for the tumor-suppressive action of p53 (Godar et al., 2008). In the present study, repression of CD44 promoter transactivation by BXL0124 treatment was abolished when the p53-binding site in CD44 promoter was mutated. This observation indicates that p53 is crucially involved in the repression of CD44 by BXL0124, although the exact molecular mechanisms of action involving p53 need to be further investigated. To determine how vitamin D and a Gemini vitamin D analog regulate the expression of CD44, we also investigated the involvement of the VDR. It is well known that the majority of the biological functions of vitamin D is

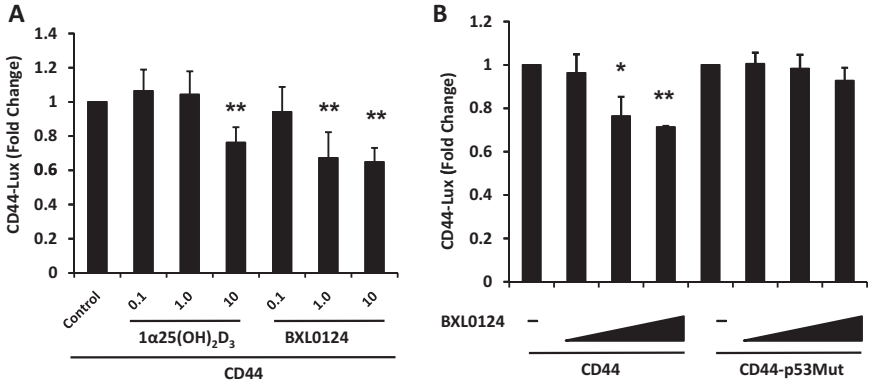


Fig. 6. $1\alpha,25(OH)_2D_3$ and BXL0124 repress transactivation of CD44 promoter in a p53-dependent manner. A, MCF10DCIS.com cells were transfected with full-length CD44-Luc DNA vector (CD44) for 6 h and were treated with increasing doses of $1\alpha,25(OH)_2D_3$ or BXL0124 (0.1, 1.0, and 10 nM) for an additional 24 h. B, MCF10DCIS.com cells were transfected with full-length CD44-Luc DNA vector (CD44) or p53 binding site mutated-CD44-Luc DNA vector (CD44-p53Mut) for 6 h and followed by treatment with BXL0124 (0.1, 1.0, and 10 nM) for 24 h. Luciferase activity was measured with a luminometer and normalized by β-galactosidase activity. The experiments were repeated at least twice, with each experiment done in duplicate. The data are presented as the mean ± S.D. (statistical analysis: *, $p < 0.05$; **, $p < 0.01$).

exerted through binding to VDR (Deeb et al., 2007). We demonstrated that knock-down of VDR using siRNA resulted in reversing the inhibitory effect of BXL0124 on CD44 repression (Fig. 4D). Furthermore, we found that putative VDR element sequences are present in the CD44 promoter region (data not shown), suggesting that the VDR liganded by vitamin D or its analog may directly bind to CD44 promoter region to repress its expression.

In conclusion, we demonstrated the repression of CD44 expression by the Gemini vitamin D analog BXL0124 in vivo and in vitro, which is probably via VDR- and p53-dependent mechanisms. Our study suggests novel Gemini vitamin D analogs as potentially useful agents for repressing CD44-expressing cancer stem cells in breast cancer.

Acknowledgments

We thank Lamberto R. Navoa of the Animal Facility in the department of Chemical Biology for technical assistance in taking care of the animals. We also thank Dr. Robert A. Weinberg for providing the CD44 promoter luciferase reporter constructs.

Authorship Contributions

Participated in research design: So, Lee, Cai, Liu, and Suh.

Conducted experiments: So, Lee, Smolarek, and Paul.

Contributed new reagents or analytic tools: Wang, Maehr, Uskokovic, Zheng, and Conney.

Performed data analysis: So, Lee, and Suh

Wrote or contributed to the writing of the manuscript: So, Lee, Conney, and Suh.

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