VDR Status Arbitrates the Pro-Metastatic Effects of Tumor-Associated Macrophages

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Abstract

The relationship between tumor-associated macrophages (TAMs) and epithelial-to-mesenchymal transition (EMT) during the initiation and progression of metastasis is still unclear. Here, a role for the vitamin D receptor (VDR) in metastasis was identified, as well as a role in the relationship between TAMs and EMT. First, the expression level of VDR was examined in clinical tissue from human breast cancer patients or a mouse model of breast cancer with differential metastasis. These results revealed that VDR expression negatively correlates with metastasis in breast cancer. Second, co-culture of VDR-overexpressing breast cancer cells with a macrophage cell line demonstrated that overexpression of VDR alleviated the pro-metastatic effect of co-cultured macrophages on breast cancer cells. Furthermore, VDR overexpression abrogated the induction of EMT in breast cancer cells by co-cultured macrophage cells, as measured by a loss of E-cadherin (CDH1) and induction of α-smooth muscle actin (α-SMA). TNF-α in macrophage conditioned media inhibited VDR expression, while down-regulation of VDR further mediated the promotion of TGF-β-induced EMT by TNF-α. In addition, β-catenin expression was inhibited in VDR-overexpressing breast cancer cells and tumor xenografts. Finally, administration of calcitriol (1,25-(OH)2D3), an active vitamin D metabolite, exerted similar anti-metastatic effects in breast cancer cells in vitro and a mouse model of breast cancer in vivo with preservation of VDR and suppression of β-catenin.

Implications: VDR suppression by TNF-α mediates the pro-metastatic effect of TAMs through enhancement of the β-catenin pathway.

Keywords

Breast cancer; Metastasis; Tumor-associated macrophages; Vitamin D Receptor; TNF-α.
Introduction

Metastasis has emerged as the primary cause of poor prognosis for breast cancer patients, in part as a result of significant progress in the early diagnosis and therapy during recent years. Accumulating evidence suggests that the derangement of the tumor microenvironment is one of the critical factors in the malignant progression of tumor. The tumor microenvironment includes a wide variety of cells that are involved in the acquisition of malignant tumor hallmark traits (1). It is currently believed that macrophages are the most abundant cells in the tumor microenvironment, playing active roles in almost all aspects of tumor growth and development (2, 3). Anti-tumor strategies targeting tumor-associated macrophages (TAMs) have achieved encouraging results in impairing the metastasis of solid tumors (4, 5). Several factors have been found to be involved in macrophage-stimulated invasiveness, such as an epidermal growth factor (EGF) – colony stimulating factor 1 (CSF-1) paracrine interaction, the Wnt5a non-canonical pathway and the induction of tumor necrosis factor-alpha (TNF-α) by the NF-κB pathway (6-8). However, the precise mechanisms underlying the pro-metastatic role of macrophages remain to be fully elucidated.

The vitamin D receptor (VDR) belongs to the nuclear hormone receptor superfamily and mediates the major biological effects of vitamin D. Upon ligand binding, VDR recruits and forms complexes with cofactors such as the retinoid X receptor. The complex then binds to the vitamin D response element in the promoter region of target genes to regulate gene transcription. Previous studies have shown that VDR gene polymorphism alters the risk of breast cancer (9-11). Comparative genome analysis identified VDR as a direct transcriptional target of p53, and that VDR plays a role in p53-mediated suppression of tumor growth (12). More recently, a positive association between VDR expression level and a prolonged progression-free and overall survival of breast cancer patients has been reported (13). However, the mechanisms behind the loss of VDR and its subsequent influence on tumor metastasis remains poorly understood.

In a previous study, we reported that TNF-α in the extracellular matrix inhibited VDR expression in renal epithelial cells, potentially mediating the interaction between inflammation and fibrosis (14). Here we examine whether the tumor microenvironment, especially TAMs, exerts a
similar effect on tumor cells. We also examine the cytokines or chemokines responsible for this effect. Additionally, we investigate the involvement of VDR, TAMs and metastasis in breast cancer.

Materials and methods

Tissue microarray

VDR expression was detected in high-density tissue microarrays on samples from a cohort of 80 breast cancer patients (Cat#.BR801, Alenabio, Xi’an, China). For quantification, VDR expression in breast cancer tissues was assessed according to Remmele and Stegner as previously described (15). In brief, Category A documented the intensity of immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong); Category B documented the number of immunoreactive cells as 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). Values for category A and B were multiplied to construct an immunoreactivity score (IRS) ranging from 0 to 12.

Animals

Female BALB/c mice aged 6–8 weeks were purchased from the Laboratory Animal Center, the Academy of Military Medical Sciences (Beijing, China) and housed under standard laboratory conditions. All animal experiments were performed according to health guidelines of the Nankai University Institutional Animal Use and Care Committee. For establishing the syngeneic, orthotopic mouse models of breast cancer, mice were injected once with either $1 \times 10^5$ wild-type (WT), vector control or VDR-overexpressing 4T1 cells into the fourth mammary fat pads (16). For the active vitamin D administration experiments, 1,25-dihydroxyvitamin D3 ($1,25(OH)2D3$) or vehicle control was administered from the day before tumor cells injection by intraperitoneal injection at a dose of 0.3 µg/kg body wt once every other day, respectively. Tumor volumes were measured daily from the tenth day after injection. Mice were sacrificed at the eighth week after injection. Lung and tumor tissues were isolated under anesthesia for further analysis. Metastatic nodule count per lung was used as the quantitative indicator for the lung metastasis as described previously (17).

Cell lines and reagents
4T1 and RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 (GIBCO, Carlsbad, CA) supplemented with 10% FBS and grown in a 5% CO2 atmosphere at 37°C. Recombinant human tumor growth factor-beta 1 (TGF-β1) and TNF-α were purchased from R&D Systems (Minneapolis, MN). SPD304 and 1,25(OH)2D3 were purchased from Sigma (Sigma-Aldrich, St Louis, MO) and Bio Basic Inc. (Markham, ON).

Vector construction
To establish stable VDR-overexpressing cell line, a VDR expression plasmid was generated by inserting the mouse VDR gene into the pLV-EF1-MCS-IRES-Bsd (Biosettia, San Diego, CA). Lentivirus production and infection were performed according to the manufacturer’s protocol and positive cells were selected by Blasticidin S at a concentration of 4 μg/ml. For knockdown analysis, two shRNAs targeting mouse VDR were designed and chemically synthesized as shRNA-mVDR and a scrambled sequence was used as control. The palindromic DNA oligos were annealed to each other and ligated to the linearized pLV-H1-EF1α-puro vector (Biosettia). 4T1 cells were transfected with pLV-H1-EF1α- shRNA-sc-puro or pLV-H1-EF1α-shRNA-mVDR-puro expression plasmids. In brief, 1x10⁶ 4T1 cells were plated in the 6-well plate and then transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Immunohistochemistry and Immunofluorescence staining
For immunohistochemistry, paraffin sections were incubated at 4°C overnight with primary antibody after dewaxing and hydration. Then, slides were incubated with a biotinylated secondary antibody for 1.5 hours and then with avidin-peroxidase complex for 0.5 hour. The slides were visualized with 3, 3′-diaminobenzene (DAB) and counterstained with hematoxylin. For immunofluorescence staining, cells were fixed with cold methanol at −20°C for 20 min and blocked in 2% bovine serum albumin (BSA) for 1 hour. Cells were incubated with primary antibodies overnight at 4°C, following by incubation with FITC-labeled secondary antibody for 1 hour. For nuclear staining, cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA). Antibodies for E-cadherin (Cell Signaling, Danvers, MA), VDR (Santa Cruz Biotechnology, Santa Cruz, CA) and β-catenin (Cell Signaling) were used at a 1:100
dilution, while the anti-α-SMA (Sigma-Aldrich) antibody was used at 1:50.

Coculture assay

4T1 cells were cocultured with RAW 264.7 macrophage cells without cell-cell contact. RAW 264.7 cells (4 × 10^5) suspended in 1 ml RPMI-1640 were added to the hanging inserts of a 6-well Boyden chamber with a 0.4-μm pore membrane (Millipore, Billerica, MA). 4T1 cells (1.6 × 10^6) in 2 ml RPMI-1640 were seeded on the bottom of each well. Cells were cocultured for 48 hours and cell lysates were collected separately. For longer cocultures, the cell suspensions were further diluted with fresh media. Suspensions were diluted by four times for three day cocultures, sixteen times for five days, and sixty-four times for seven days.

Cell migration and invasion assay

4T1 cells (1 × 10^5) cocultured with or without RAW 264.7 cells were added to the hanging insert of a Boyden chamber with an 8-μm pore membrane and 1 ml RPMI-1640 medium supplemented with 10% FBS in the bottom well. After eight hours of incubation at 37 °C, cells on the upper side of the insert were removed with a cotton swab. The bottom side was then fixed and stained with DAPI. Viable cells were counted under a microscope (Olympus Co. Tokyo, Japan). Each assay was done in triplicate. For invasion assays, Matrigel (BD Biosciences, San Jose, CA) was diluted to 1 mg/ml with serum-free culture medium and immediately applied to each membrane insert to form the upper chambers of the multi-well invasion assay plate. 1 × 10^5 4T1 cells were seeded into the upper chambers and incubated for 24 hours at 37°C before analysis. The wound healing assay was carried out according to the established protocols (18).

Western blot analysis

Detection of protein expression by Western blot was carried out according to established protocols described previously (19). Anti-VDR (1:1000), α-SMA (1:500), E-cadherin (1:1000), β-catenin (1:1000) and β-actin (1:10000) primary antibodies were used. Secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Bio-Rad, Hercules, CA) were used at a 1:10000 dilution and detected using enhanced chemiluminescence (ECL, Millipore).

Immunoprecipitation assay

Cell lysates were immunoprecipitated with 4 μg of anti-VDR antibody conjugated to Dynabeads
for 1.5 hours at room temperature. Beads were then washed and the target antigen eluted and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. Immunoblotting with anti-β-catenin antibody was then performed. The proteins were detected using ECL reagent (Millipore).

Dual Luciferase Assay
Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to test the relative activity of Firefly luciferase (FL) versus that of Renilla luciferase (RL). Briefly, vector control or VDR-overexpressing 4T1 cells were cultured in 24-well plates at 2x10^5/wells and transfected with a DNA mix contained 700 ng pGM-Luciferase vector containing the TCF/LEF1 response element sequence and 70 ng pRL-TK plasmid. For vitamin D treated group, 1,25(OH)2D3 was added to the growth medium 6 hours after transfection. Cells were harvested after 48 hours of transfection and the activation of TCF/LEF1 response element was quantified as a ratio of FL/RL activity in each well following the manufacturer’s instructions.

Preparation of conditioned medium and treatment with cytokines and chemical inhibitors
Following coculture with 4T1 cells for 48 hours, the RAW 264.7 cell culture medium was changed to serum-free medium. RAW 264.7 conditioned medium was then harvested after 24 hours. 4T1 cells were seeded at approximately 50% confluence and cultured in complete medium for 24 hours. The culture medium was then replaced with a 2 ml mixture of the RAW 264.7 conditioned medium and basic medium at different ratios with or without 50 μm SPD 304. For the experiments of cytokine treatment, 4T1 cells were treated with 1 or 2 ng/ml TGF-β1 in the absence or presence of 10 ng/ml TNF-α and various concentration of 1,25(OH)2D3 for 48 hours.

Statistical analysis
All data are presented as the mean ± S.E.M. Statistical analysis of the data was performed using the GraphPad Prism software (GraphPad Software, San Diego, CA). Differences between individual groups were analyzed by paired t-test or χ², as appropriate. P-values less than 0.05 were considered statistically significant.

Results
Expression level of VDR negatively correlated with the metastatic progress of breast cancer.
In order to reveal the correlation between the VDR level and the metastatic status in breast cancer, we first performed immunohistological staining on the samples from human breast cancer patients, as well as samples from the orthotopic mouse model of breast cancer with 4T1 cells. In the human samples, our results identified a stratified expression pattern for VDR in tissue sections (Fig 1A). VDR level in the tumor site was significantly lower than that in the normal or paracarcinoma tissue. Moreover, the expression of VDR was negatively correlated with tumor grade (Table 1).

Similarly, immunohistological analysis of samples from the mouse model of breast cancer with 4T1 cells demonstrated that the suppression of VDR in the tumor correlated with disease progress (Fig 1B).

**VDR overexpression suppressed metastasis in a mouse model of breast cancer.**

To further explore the role of VDR in breast cancer metastasis, we established VDR-overexpressing and vector control 4T1 stable cell lines. Mouse models of breast cancer were generated by injecting VDR-overexpressing, vector control or wild type 4T1 cells into the mammary fat pad separately. Immunohistological staining and real-time PCR confirmed VDR overexpression at the tumor site in the VDR-overexpressing group (Figs 2A, B). In accordance with the results described above, VDR expression in tumors appeared almost undetectable by the eighth week after tumor cells implantation in the wild type and vector control group. Notably, there was no significant difference in the tumor size among the three groups ($P = 0.27$, Fig 2C). Evaluation of lung metastasis showed that the number of metastatic lung nodules in the VDR-overexpressing group was significantly less than that in the other two control groups (Figs 2D, E). Taken together, these results suggested that while VDR overexpression did not affect primary tumor size, it did suppress metastasis.

**VDR overexpression reduced the migration and invasion ability of breast cancer cells induced by macrophage coculture.**

Transwell assays were performed using cultured VDR-overexpressing, vector control or wild type 4T1 cells to explore the mechanism underlying the effect of VDR on breast cancer metastasis. No significant difference could be detected in migration and invasion among the three groups (Figs 3A, B). Given the critical role of TAMs and the tumor microenvironment, we next performed
coculture of breast cancer cells with macrophages. Cancer cell migration increased with increased coculture time. Moreover, this increased migration ability conferred by coculture with macrophages was suppressed in VDR-overexpressing 4T1 cells when compared with vector control (Fig 3C). Similar results were obtained from an invasion assay; macrophage coculture increased 4T1 cell invasiveness, which was also suppressed by VDR overexpression (Fig 3D).

**VDR overexpression suppressed the macrophage-induced inhibition of E-cadherin and induction of α-SMA in breast cancer cells.**

EMT is essential in breast cancer cell metastasis. Therefore, we measured expression of the epithelial marker E-cadherin and the mesenchymal marker α-SMA in 4T1 cell lines cocultured with RAW 264.7 cells by Western blot. Macrophage coculture inhibited E-cadherin expression and induced α-SMA expression in control, but not in VDR-overexpressing 4T1 cells (Fig 3E). There are two major characteristics of the EMT process: loss of the epithelial phenotype and acquisition of the mesenchymal phenotype. Previous studies have suggested that the loss of E-cadherin is the initial and essential step for EMT (20). Here, immunofluorescence staining showed a prominent downregulation of E-cadherin in vector control 4T1 cells from the third day of coculture with RAW 264.7 cells, becoming almost undetectable by the seventh day. However, E-cadherin levels remained stable in VDR-overexpressing cells even after seven days of coculture (Fig 3F).

**TNF-α secreted by macrophages inhibits VDR expression in breast cancer cells.**

We next explored the functional role of macrophage coculture on the metastatic ability and EMT potential of breast cancer cells. Conditioned media (CM) from a 48-hour coculture of RAW 264.7 and 4T1 cells was collected and mixed with fresh basal media (BM) at several ratios. CM/BM was then added to 4T1 cells. VDR expression was suppressed in these cells in a dose-dependent manner (Fig 4A). Furthermore, elevated TNF-α mRNA level in RAW264.7 cells could be detected after coculture of 4T1 cells (Fig 4B); at the same time, the inhibitory effect on VDR could be rescued by administration of SPD304, a specific TNF-α inhibitor (Fig 4C), which suggests a role for TNF-α in the inhibition of VDR expression. This effect does not appear to be cell-line or species specific as we found that TNF-α inhibited VDR expression in either 4T1 cells (Figs 4D, E) or MCF7 cells (Fig S1A, B) in both time- and dose-dependent manners.
VDR downregulation mediates the effect of TNF-α on promoting the EMT potential in breast cancer cells.

We next evaluated whether VDR inhibition by TNF-α increased the EMT potential in breast cancer cells. Pretreatment with TNF-α significantly enhanced the sensitivity of 4T1 cells to TGF-β1–induced EMT (Fig 4F). Consistent with our observations from above, knockdown of VDR expression by shRNA transfection in conjunction with a low dose of TGF-β1 decreased E-cadherin and increased α-SMA (Fig 4G). Taken together, these data indicated that the loss of VDR triggered by TNF-α sensitized 4T1 cells to EMT. Similar results were obtained in the experiments with MCF7 cells (Fig S1C, D).

VDR overexpression suppressed β-catenin in breast cancer cells in vitro and in vivo.

Accumulating evidences supports an important role for the β-catenin pathway in the EMT process. Therefore, we investigated whether β-catenin pathway was involved in mediating the effects of VDR on EMT. We detected a significant downregulation of β-catenin in VDR-overexpressing 4T1 cells when compared with vector control (Figs 5A, B). Immunofluorescence staining of β-catenin in these cells supported these results. Moreover, β-catenin expression was suppressed in both the nuclei and cytoplasm of VDR-overexpressing 4T1 cells (Fig 5C). Correspondingly, suppression of β-catenin was detected at primary tumor sites in the orthotopic mouse model of breast cancer generated by the VDR-overexpressing 4T1 cells (Fig 5D). We also demonstrated interaction between VDR and β-catenin in VDR-overexpressing 4T1 cells using immunoprecipitation (Fig 5E). Furthermore, luciferase reporter assay data suggested that the TCF/LEF1 transcriptional activity was repressed in VDR-overexpressing 4T1 cells (Fig 5F).

1,25(OH)2D3 protected against the loss of VDR and increased sensitivity to pro-EMT stimuli induced by TGF-β1 in 4T1 cells in vitro; 1,25(OH)2D3 administration suppressed EMT and metastasis in a mouse model of breast cancer.

In view of the critical role of vitamin D, ligand of VDR, in mediating the various effects of VDR, we further checked the effect of 1,25(OH)2D3 on the migration in 4T1 cells in vitro, as well as the EMT and metastasis in mouse model of breast cancer. Our data showed that 1,25(OH)2D3 prevented the inhibition of VDR induced by TNF-α in 4T1 cells as well as loss of VDR in the
tumor tissue of mouse model of breast cancer (Figs 6A, B). Migration assay revealed that 1,25(OH)2D3 rescued the increased migration ability of 4T1 on the stimulation of TNF-α and TGF-β1 (Fig 6C). Similar results were obtained from the experiments with MCF-7 cells (Fig S2). In vivo experiment of 1,25(OH)2D3 administration showed suppression of α-SMA and restore of E-cadherin in the group of 1,25(OH)2D3 (Fig 6D, E), as well as the suppression of the metastatic lung nodules when compared with vehicle control (Fig 6F), with no influences on the tumor volume (Fig 6G). Consistent with the results from the in vitro experiment, VDR expression was protected and β-catenin was inhibited in the 1,25(OH)2D3 administration group (Figs 6H, I).

Discussion

A correlation between VDR polymorphism, breast cancer susceptibility and tumor angiogenesis has been previously identified (10, 21). However, data investigating the relationship between VDR and tumor metastasis remains quite rare. Ditsch and colleagues previously reported that the VDR expression level correlates closely with the survival of breast cancer patients (13). In this study we demonstrated that decreased VDR expression correlated with increased levels of tumor metastasis in both a tissue microarray of breast cancer patient samples and a mouse model of breast cancer. While further evidence is required before the use of VDR as a prognostic indicator in breast cancer can be reliably adopted, our current data clearly demonstrate that VDR expression is correlated with the breast cancer metastatic potential.

VDR has a well-recognized role in impairing proliferation and inducing apoptosis of tumor cells (22). Unexpectedly, while metastasis of VDR-overexpressing tumors was inhibited in our mouse model, there was no reduction in primary tumor size. This might be explained by use of a routine diet without additional vitamin D supplementation, or the saturation of VDR at the tumor initiation stage. In vitro, no differences between the migration and invasive capacities of VDR-overexpressing and control cell lines were detected. However, the increased metastatic capacity conferred upon cells following coculture with macrophages was significantly inhibited by VDR overexpression. Furthermore, our findings suggested that overexpression of VDR impaired the decrease of E-cadherin and the increase of α-SMA normally observed in 4T1 cells following coculture with macrophages. This loss of epithelial markers with a concurrent increase in
mesenchymal markers is the key indicator for EMT (23, 24), which is regarded as a critical pathological event in the initiation and promotion of metastasis (1). Therefore, our findings suggest that the loss of VDR is likely required for the pro-metastatic effect of TAMs.

The contribution of TAMs to various aspects of tumor behavior has been extensively studied (25, 26). Therefore, we hypothesize that the inhibitory effect of VDR on tumor metastasis might depend on the interruption of the critical feedback loop between TAMs and tumor cells. Given that the coculture system used in this study does not involve direct cell-cell contact, we speculated that macrophage-derived cytokines in the conditioned media are involved in the effect on VDR expression. Our data showed that treatment with specific inhibitor of TNF-α, an important pro-inflammatory cytokine, suppressed the inhibition of VDR expression induced by macrophage-conditioned media. Moreover, TNF-α can inhibit VDR expression while enhancing the ability of TGF-β1 to stimulate EMT in breast cancer cells, as determined by the induction of α-SMA and the loss of E-cadherin. This is in accord with previous research from our group and others which has identified both the inhibitory effect of TNF-α on VDR expression in different cell lines, and the synergistic effect of TNF-α and TGF-β1 on EMT induction (14, 27, 28). Our current findings suggest that inhibition of VDR mediates TNF-α-mediated EMT, and that this may be a key underlying mechanism in the interaction between TAMs and tumor cells.

It is generally accepted that activation of VDR after binding with the ligand, active vitamin D, although a few researches have indicated the non-ligand depended effect of VDR (29, 30). Ellison and Engelhard et al have reported the ligand-independent regulatory effect of VDR on the vitamin D-responsive 24-hydroxylase promoter and the direct transcriptional regulation of hairless by VDR, respectively. Whether the ligand-independ transactivation also contributes to the effect of VDR on EMT, or the trace amount of active vitamin D presents in the standard medium intrigues the initial step of cascade activation of VDR is still an unsolved puzzle in the study. However, further evidence is undoubtedly worthy especially considering the “dependence receptors theory” which postulates that some receptors are active in the absence of their ligands (31). To further evaluate the role of active vitamin D in the pro-metastatic effect of inflammation on the metastasis of breast cancer cells, we performed a set of separated experiments.
1,25(OH)2D3 inhibited the migration induced by TNF-α combined with TGF-β1 and alleviated EMT as well as metastasis in the mouse model of breast cancer in vivo. Therefore, it is not hard to speculate that the protective effect on VDR contribute to the inhibitory role of 1,25(OH)2D3 in metastasis, at least partially.

Activation of VDR depended on several signaling pathways which have been implicated in inflammation-related activation of EMT during tumor metastasis, including TGF-β, Wnt, Notch, Hedgehog, and others. These pathways converge on a common set of transcription factors, including Snail, Slug, Twist, ZEB 1/2 and the SMADs, thereby facilitating EMT (32, 33). β-catenin is a downstream effector of the Wnt pathway. Upon activation of Wnt signaling, β-catenin accumulates in the cytoplasm and translocates to the nucleus, influencing gene transcription (34). A previous study demonstrated that VDR knockdown enhanced β-catenin activation in colon cancer cells (35). In this study, we found that β-catenin expression was decreased in both cultured 4T1 cells stably overexpressing VDR and in tumor tissues from our VDR-overexpressing mouse model. Our data further showed that ectopic expression of VDR promotes the formation of a complex with β-catenin and further inhibits the transcriptional activity of TCF/LEF1. This suggests that inhibition of the Wnt/β-catenin pathway may be the mechanism by which VDR suppresses tumor metastasis. However, the exact nature of the interaction between VDR and β-catenin remains unclear. A recent study has also revealed that VDR binds SMAD3 target sites and reduces SMAD3 occupancy, thus affecting activation of the TGF-β/SMAD pathway (23). This suggests that several mechanisms may mediate the effect of VDR on tumor metastasis. Clearly, further study on the broader range of pathways contributing to this effect is warranted.

In summary, our findings demonstrate that VDR suppression by TNF-α may mediate the promotion of breast cancer metastasis by TAMs. This effect is likely related to the relief of Wnt/β-catenin pathway inhibition, thus facilitating the EMT process. These results provide a new angle by which to view the relationship between EMT and the tumor microenvironment. Furthermore, these findings suggest VDR ligands may be a potential therapeutic target for breast cancer metastasis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure Legends

Figure 1. Expression level of VDR correlates negatively with the metastatic status of breast cancer. (A) High-density tissue microarrays of breast cancer containing samples from 80 patients were stained with antibody against VDR. Representative pictures show the stratified expression of VDR in tissue samples. (a) Normal breast tissue; (b) Malignant breast tumor (grade 1, well-differentiated); (c) Malignant breast tumor (grade 2, moderately-differentiated); (d) Malignant breast tumor (grade 3, poorly differentiated); (e) Malignant breast tumor (grade 4, undifferentiated). Scale bar, 100 μm. (B) Representative images show VDR expression in the tumor tissues of an orthotopic mouse model of breast cancer with 4T1 cells at 3, 5, 7 weeks after injection. Scale bar, 100 μm. Right panel shows the quantification of the VDR expression shown in B. The data are shown as mean ± S.E.M. *** P < 0.001.

Figure 2. VDR upregulation suppresses lung colonization in the orthotopic mouse model of breast cancer. Female BALB/c mice were injected into the forth fat pad with stable VDR-overexpressing 4T1 cells. Wild type or stable control vector 4T1 cells served as control (n=5). Mice were sacrificed at the 8th weeks after injection, and then isolated primary tumor and lung for analysis. (A) Representative images show VDR expression in primary tumors. Scale bar, 100 μm. (B) Real time-PCR shows the mRNA level of VDR expression in primary tumors. (C & D) Bar plots show the statistical results of the tumor sizes and metastatic nodules counts of lung in different groups. (E) Left panel shows the representative pictures of the metastatic lung nodules in different groups (black arrows). Right panel provide representative pictures of HE staining of malignant lung tissue under 100× or 200×. Scale bar, 1mm/200μm. The data are shown as mean ±
S.E.M. * $P < 0.05$, *** $P < 0.001$.

**Figure 3.** Ectopic overexpression of VDR suppresses metastatic ability and attenuates inhibition of E-cadherin induced by coculture of 4T1 breast cancer cells with macrophages.

We established stable control vector or VDR overexpressing 4T1 cell lines, respectively. Transwell assay were performed to detect metastatic ability of different cells. (A) Left panel shows representative images of fields in the transwell migration assay of wild type, stable vector control and stable VDR-overexpressing 4T1 cells, respectively. Scale bar, 100 μm. Bar plot in the right panel shows the quantification of the migrated cells per field shown in A. (B) Quantification of the amount of the cells invaded through the matrigel in the transwell invasion assay. (C) Stable vector control or VDR-overexpressing 4T1 cells were cocultured with RAW 264.7 cells. Then, 1x10⁵ 4T1 cells were transferred to the upper chamber of the plates for transwell assay. 8 hours later, the bottom chambers were fixed with methanol and stained with DAPI. The left panel shows the representative images of the fields (Scale bar, 100 μm) and the right panel indicates the statistical results. (D) Quantification of results of transwell invasion assay performed after the coculture of 4T1 and RAW 264.7 cells. (E) Stable vector control or VDR-overexpressing 4T1 cells were cocultured with RAW 264.7 cells for different time periods as indicated. Results of Western blot analysis show the expression of VDR, E-cadherin and α-SMA in 4T1 whole cell lysate after coculture of different periods. (F) Immuofluorescence staining using primary antibody against E-cadherin and VDR in different 4T1 cells cocultured with RAW 264.7. Three independent experiments were performed and 10 fields per well were counted for each experiment. Scale bar, 50 μm. The data are shown as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 4.** TNF-α inhibits VDR expression and promotes EMT initiated by TGF-β1 stimulation. Conditioned media of RAW 264.7 cells after 48 hours of coculturing with 4T1 cells was collected and mixed with elementary media with variable ratios. (A) Western blot analysis reveals the downregulation of VDR expression with conditioned media from RAW 264.7 cells in a dose-dependent pattern. (B) Real time PCR analysis reveals the upregulation of TNF-α mRNA expression in RAW264.7 cells after cocultured with 4T1 cells for 48 hours. (C) 4T1 cells were cultured in conditioned media from RAW 264.7 with pre-cocultured of 4T1 cells with or without
SPD304, a specific inhibitor of TNF-α. Western blot analysis shows that SPD304 rescues the VDR expression suppressed by conditioned media from RAW 264.7 cells. (D) 4T1 cells were treated with 10 ng/ml of TNF-α for different time course. VDR expression was detected by western blot analysis. Quantification of results is shown at the right. Data presented are shown as mean ± S.E.M and collected from three independent experiments. (E) 4T1 cells were treated with 1, 2, 5 or 10 ng/ml of TNF-α for 48 hours. Western blot analysis was used to detect VDR expression. Quantification of results is shown at the right. The data are shown as mean ± S.E.M. ** P < 0.01, *** P < 0.001. (F) 4T1 cells were treated with 10 ng/ml TNF-α, with or without 1 or 2 ng/ml TGF-β1 for 48 hours. Western blot analysis was performed using anti-VDR, E-cadherin and α-SMA. (G) 4T1 cells were treated with or without 1 or 2 ng/ml TGF-β1 for 48 hours after being transfected with VDR shRNA. Western blot was used to detect expression of VDR, E-cadherin and α-SMA. Our results shows that downregulation of VDR induced by TNF-α or VDR shRNA has similar effect on promoting the inhibition of E-cadherin and induction of α-SMA initiated by TGF-β1 in breast cancer cells.

**Figure 5. Upregulation of VDR suppresses β-catenin expression both in vitro and in vivo.**
mRNA and protein samples were isolated separately from wild type, vector control- or VDR-overexpressing stable cells. (A) Real-time PCR assay for the inhibition of β-catenin mRNA expression in the VDR-overexpression cells compared with vector control or wild type 4T1 cells. (B) Western blot analysis using the antibody against VDR and β-catenin shows the suppression of β-catenin in the VDR-overexpression cells compared with vector control or wild type 4T1 cells. For VDR expression, data of different exposure time were presented. (C) Results of immunofluorescence staining of wild type, vector control and VDR-overexpressing stable cells using antibody against VDR or β-catenin. Scale bar, 50 μm. (D) Orthotopic mouse model was established according to method described in Fig. 2. The left panel shows the representative images of immunohistological staining of β-catenin expression in the tumor tissues. Scale bar, 100 μm. Right panel is the quantification results of β-catenin expression. (E) Detection of interaction between VDR and β-catenin in wild type, vector control and VDR-overexpression 4T1 cells. Cell extracts were immunoprecipitated with VDR antibody-conjugated Dynabeads, followed by immunoblotting with antibody against anti-β-catenin. Immunoblotting of whole-cell
lysates without immunoprecipitation was used to detect protein expression throughout experiments. (F) Dual luciferase method was used to analyze the TCF/LEF1 transcriptional activity in vector control or VDR-overexpressing 4T1 cells treated with or without 10^{-7} M 1,25(OH)2D3. The data are shown as mean ± S.E.M. * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 6. Administration of Vitamin D decreases metastasis of breast cancer cells both in vitro and in vivo.** Wild type 4T1 cells were treated with 10 ng/ml TNF-α alone or TNF-α plus pre-treatment of 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M 1,25(OH)2D3. (A) Western blot analysis was performed using antibody against VDR. Lower panel is the quantification result of VDR expression. (B) Female Balb/c mice were injected into the forth fat pad with 4T1 cells to establish the orthotopic mouse model. 1,25(OH)2D3 or vehicle control was given via intraperitoneal injection at a dose of 0.3 μg/kg body wt per day (n=5). Western blot analysis results of VDR expression in the homogenate of tumor tissue. (C) 4T1 cells were treated with 10 ng/ml TNF-α plus 2 ng/ml TGF-β1 with or without pre-treatment of 10^{-7} M 1,25(OH)2D3. Wound healing assay compared the migration ability of 4T1 cells with different treatment. Lower panel is the quantification result of the interval distance. (D) & (E) Representative images and quantification of immunofluorescence staining using antibody against α-SMA and E-cadherin in the 1,25(OH)2D3 treatment and control groups. Scale bar, 100 μm. (F) Bar plots show the statistical results of the metastatic nodules counts of lung in different groups. (G) Bar plots show the statistical results of the tumor volume in different groups. (H) Representative images of immunohistological staining using antibody against β-catenin in the orthotopic mouse model treated with 1,25(OH)2D3 or vehicle control. Scale bar, 100 μm. (I) the quantification result of β-catenin expression. Data are shown as mean ± S.E.M. * P < 0.05, ** P < 0.01, *** P < 0.001.

**References**


Figure 1
Table 1. The correlation of VDR with clinical status of patients with breast cancer

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<th>Tissue classification</th>
<th>VDR Immunoreactivity score (IRS)</th>
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<th>P Value</th>
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<tr>
<td></td>
<td>0-1</td>
<td>2-4</td>
<td>6-12</td>
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<td>Tissue source</td>
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</table>
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Molecular Cancer Research

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Yan Zhang, Quanjun Guo, Zhujun Zhang, et al.

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