VDR Status Arbitrates the Prometastatic Effects of Tumor-Associated Macrophages

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Abstract
The relationship between tumor-associated macrophages (TAM) 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 patients with breast cancer or a mouse model of breast cancer with differential metastasis. These results revealed that VDR expression negatively correlates with metastasis in breast cancer. Second, coculture of VDR-overexpressing breast cancer cells with a macrophage cell line demonstrated that overexpression of VDR alleviated the prometastatic effect of cocultured macrophages on breast cancer cells. Furthermore, VDR overexpression abrogated the induction of EMT in breast cancer cells by cocultured macrophage cells, as measured by a loss of E-cadherin (CDH1) and induction of α-smooth muscle actin (α-SMA). TNFα in macrophage-conditioned media inhibited VDR expression, whereas downregulation of VDR further mediated the promotion of TGFβ-induced EMT by TNFα. 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 antimetastatic 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 TNFα mediates the prometastatic effect of TAMs through enhancement of the β-catenin pathway. Mol Cancer Res; 12(8); 1181–91. ©2014 AACR.

Introduction
Metastasis has emerged as the primary cause of poor prognosis for patients with breast cancer, 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). Antitumor strategies targeting tumor-associated macrophages (TAM) 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 EGF—colony-stimulating factor 1 (CSF1) paracrine interaction, the Wnt5α noncanonical pathway, and the induction of TNFα by the NF-κB pathway (6–8). However, the precise mechanisms underlying the prometastatic role of macrophages remain to be fully elucidated.

The vitamin D receptor (VDR) belongs to the nuclear hormone receptor superfamily and mediates the major biologic 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 VDR 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 patients with breast cancer have been reported (13). However, the mechanisms behind the loss of VDR and its subsequent influence on tumor metastasis remain poorly understood.
In a previous study, we reported that TNFα 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, elicits a similar effect on tumor cells. We also examine the cytokines or chemokines responsible for this effect. In addition, 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 patients with breast cancer (catalog no. BR801, Alenabio). 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 to 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 × 10⁵ 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 for 1.5 hours and then with avidin–peroxidase complex for 0.5 hour. The slides were visualized with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. For immunofluorescence staining, cells were fixed with cold methanol at −20°C for 20 minutes and blocked in 2% BSA for 1 hour. Cells were incubated with primary antibodies overnight at 4°C, followed 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). Antibodies for E-cadherin (Cell Signaling), VDR (Santa Cruz Biotechnology), and β-catenin (Cell Signaling) were used at a 1:100 dilution, whereas 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 × 10⁵) 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). 4T1 cells (1.6 × 10⁶) 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 4 times for 3-day cocultures, 16 times for 5 days, and 64 times for 7 days.

Cell migration and invasion assays

4T1 cells (1 × 10⁵) 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 8 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 stained with DAPI. Viable cells were counted under a microscope (Olympus Co.). Each assay was done in triplicate. For invasion assays, Matrigel (BD Biosciences) 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 multiwell invasion assay plate. About 1 × 10^3 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 blotting was carried out according to established protocols described previously (19). Anti-VDR (1:1,000), α-smooth muscle actin (α-SMA; 1:500), E-cadherin (1:1,000), β-catenin (1:1,000), and β-actin (1:10,000) primary antibodies were used. Secondary horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse antibodies (Bio-Rad) were used at a 1:10,000 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 SDS-PAGE 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) 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 2 × 10^5 per well and transfected with a DNA mix of 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 (CM) 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 CM and basic medium at different ratios with or without 50 μmol/L SPD 304. For the experiments of cytokine treatment, 4T1 cells were treated with 1 or 2 ng/mL TGFβ1 in the absence or presence of 10 ng/mL TNFα and various concentration of 1,25(OH)2D3 for 48 hours.

**Statistical analysis**

All data are presented as the mean ± SEM. Statistical analysis of the data was performed using the GraphPad Prism software (GraphPad Software). Differences between individual groups were analyzed by paired t test or χ^2, as appropriate. *P* < 0.05 was considered statistically significant.

**Results**

 Expression level of VDR negatively correlated with the metastatic progress of breast cancer

To reveal the correlation between the VDR level and the metastatic status in breast cancer, we first performed immunohistologic staining on the samples from human patients with breast cancer 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, immunohistologic 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 progression (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 WT 4T1 cells into the mammary fat pad separately. Immunohistologic staining and real-time PCR confirmed VDR overexpression at the tumor site in the VDR-overexpressing group (Fig. 2A and 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 WT and vector control group. Notably, there was no significant difference in the tumor size among the 3 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 2 control groups (Fig. 2A and B). 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 WT 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 3 groups (Fig. 3A and 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

Epithelial-to-mesenchymal transition (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 blotting. Macrophage coculture inhibited E-cadherin expression and induced α-SMA expression in control but not in VDR-overexpressing 4T1 cells (Fig. 3E). There are 2 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 7 days of coculture (Fig. 3F).

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

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TNFα 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. CM from a 48-hour coculture of RAW 264.7 and 4T1 cells were 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 TNFα 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 TNFα inhibitor (Fig. 4C), which suggests a role for TNFα in the inhibition of VDR expression. This effect does not appear to be cell line- or species-specific as we found that TNFα inhibited VDR expression in either 4T1 cells (Fig. 4D and E) or MCF7 cells (Supplementary Fig. S1A and S1B) in both time- and dose-dependent manners.

VDR downregulation mediates the effect of TNFα on promoting the EMT potential in breast cancer cells

We next evaluated whether VDR inhibition by TNFα increased the EMT potential in breast cancer cells. Pretreatment with TNFα significantly enhanced the sensitivity of 4T1 cells to TGFβ1-induced EMT (Fig. 4F). Consistent with our observations from above, knockdown of VDR expression by shRNA transfection in conjunction with a low dose of TGFβ1 decreased E-cadherin and increased α-SMA (Fig. 4G). Taken together, these data indicated that the loss of VDR triggered by TNFα sensitized 4T1 cells to EMT. Similar results were obtained in the experiments with MCF7 cells (Supplementary Fig. S1C and S1D).

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

Accumulating evidence 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 (Fig. 5A and 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 TGFβ1 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.
consistent with the results from the lung nodules when compared with vehicle control (Fig. 6F), as well as the suppression of the metastatic and restoration of E-cadherin in the group of 1,25(OH)2D3 administration group (Fig. 6H and 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 remain quite rare. Ditsch and colleagues previously reported that the VDR expression level correlates closely with the survival of patients with breast cancer (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 pathologic event in the initiation and promotion of metastasis (1). Therefore, our findings suggest that the loss of VDR is likely required for the prometastatic 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

Figure 4. TNFα inhibits VDR expression and promotes EMT initiated by TGFβ1 stimulation. CM of RAW 264.7 cells after 48 hours of coculturing with 4T1 cells were collected and mixed with elementary media with variable ratios. A, Western blot analysis reveals the downregulation of VDR expression with CM from RAW 264.7 cells in a dose-dependent pattern. B, real-time PCR analysis reveals the upregulation of TNFα mRNA expression in RAW264.7 cells after cocultured with 4T1 cells for 48 hours. C, 4T1 cells were cultured in CM from RAW 264.7 with pre-cocultured of 4T1 cells with or without SPD304, a specific inhibitor of TNFα. Western blot analysis shows that SPD304 rescues the VDR expression suppressed by CM from RAW 264.7 cells. D, 4T1 cells were treated with 10 ng/mL of TNFα 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 ± SEM and collected from 3 independent experiments. E, 4T1 cells were treated with 1, 2, 5, or 10 ng/mL of TNFα 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 ± SEM. **, 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 blotting was used to detect expression of VDR, E-cadherin, and α-SMA. Our results shows that downregulation of VDR induced by TNFα or VDR shRNA has similar effect on promoting the inhibition of E-cadherin and induction of α-SMA initiated by TGFβ1 in breast cancer cells.
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 CM are involved in the effect on VDR expression. Our data showed that treatment with specific inhibitor of TNFα, an important proinflammatory cytokine, suppressed the inhibition of VDR expression in the VDR-overexpressing cells compared with vector control or WT 4T1 cells. For VDR expression, data of different exposure time were presented. C, results of immunofluorescence staining of WT, 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. Left, the representative images of immunohistologic staining of β-catenin expression in the tumor tissues. Scale bar, 100 μm. Right, the quantification results of β-catenin expression. E, detection of interaction between VDR and β-catenin in WT, vector control, and VDR-overexpressing 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 μmol/L 1,25(OH)2D3. The data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Our current findings suggest that inhibition of VDR mediates TNFα-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 researchers have indicated the non–ligand-dependent effect of VDR (29, 30). Ellison and Engelhard and their colleagues 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-independent 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 prometastatic effect of inflammation on the metastasis of breast cancer cells, we performed a set of separated experiments. In vitro, treatment of 1,25(OH)2D3 inhibited the migration induced by TNFα combined with TGFβ1 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 contributes to the inhibitory role of 1,25(OH)2D3 in metastasis, at least partially. Activation of VDR was dependent on several signaling pathways, which have been implicated in inflammation-related activation of EMT during tumor metastasis, including TGFβ, Wnt, Notch, and Hedgehog. These pathways...
converge on a common set of transcription factors, including Snail, Slug, Twist, ZEB1/2, 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 TGFβ/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 TNFα 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 that 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.

**Authors’ Contributions**

Conception and design: Y. Zhang, Q. Guo, Z. Zhang, N. Bai, Z. Liu, M. Xiong, R. Xiang, X. Tan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhang, Q. Guo, Z. Zhang, N. Bai, Z. Liu, M. Xiong, R. Xiang, X. Tan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, X. Tan

Writing, review, and/or revision of the manuscript: Y. Zhang, Y. Wei, X. Tan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhang, X. Tan

Study supervision: R. Xiang

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