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 $\alpha$-cadherin (CDH1) and induction of $\alpha$-smooth muscle actin ($\alpha$-SMA). TNF$\alpha$, in macrophage conditioned media inhibited VDR expression, whereas downregulation of VDR further mediated the promotion of TGF$\beta$-induced EMT by TNF$\alpha$. In addition, $\beta$-catenin expression was inhibited in VDR-overexpressing breast cancer cells and tumor xenografts. Finally, administration of calcitriol $[1,25-(OH)_2] D_3$, 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 $\beta$-catenin.

Implications: VDR suppression by TNF$\alpha$ mediates the prometastatic effect of TAMs through enhancement of the $\beta$-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 Wnt5a noncanonical pathway, and the induction of TNF$\alpha$ by the NF-$\kappa$B 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, exerts 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 × 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 weight 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 ATCC. Cells were grown in RPMI-1640 (GIBCO) supplemented with 10% FBS and grown in a 5% CO2 atmosphere at 37°C. Reconstituant human TGFβ1 and TNFα were purchased from R&D Systems. SPD304 and 1,25(OH)2D3 were purchased from Sigma and Bio Basic Inc.

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). 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, 2 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-ES10- puro vector (Biosettia). 4T1 cells were transfected with pLV-H1-ES10-shRNA-sc-puro or pLV-H1-ES10-shRNA-mVDR-puro expression plasmids. In brief, 1 × 10^5 4T1 cells were plated in the 6-well plate and then transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

IHC and immunofluorescence staining

For IHC, 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′-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 × 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). 4T1 cells (1.6 × 10^5) 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 × 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 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 fixed and 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 $1 \times 10^7$ 4T1 cells were seeded into the upper chambers and incubated for 24 hours at $37^\circ$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 $2 \times 10^5$ 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 TGFrβ1 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 χ² test, 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. 2D and 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 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

<table>
<thead>
<tr>
<th>Tissue classification</th>
<th>VDR IRS</th>
<th></th>
<th></th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–1</td>
<td>2–4</td>
<td>6–12</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tissue source</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/paracarcinoma</td>
<td>0</td>
<td>1 (10%)</td>
<td>9 (90%)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>12 (17.1%)</td>
<td>31 (44.3%)</td>
<td>27 (38.6%)</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1–2</td>
<td>8 (15.4%)</td>
<td>23 (44.2%)</td>
<td>21 (40.4%)</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>4 (22.2%)</td>
<td>8 (44.5%)</td>
<td>6 (33.3%)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3 (12.5%)</td>
<td>10 (41.7%)</td>
<td>11 (45.8%)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2 (16.6%)</td>
<td>5 (41.7%)</td>
<td>5 (41.7%)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>III–IV</td>
<td>7 (20.6%)</td>
<td>16 (47.1%)</td>
<td>11 (32.3%)</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

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), and (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, and 7 weeks after injection. Scale bar, 100 μm. Right, the quantification of the VDR expression shown in B. The data are shown as mean ± SEM. ***; P < 0.001.
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.
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 < 0.001; ****P < 0.0001. F, 4T1 cells were treated with 10 ng/mL TNFα, with or without 1 or 2 ng/mL TGFβ1 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 TGFβ1 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.

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 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 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 induced by macrophage CM. Moreover, TNFα can inhibit VDR expression while enhancing the ability of TGFβ1 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 TNFα on VDR expression in different cell lines and the synergistic effect of TNFα and TGFβ1 on EMT induction (14, 27, 28).

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

**Figure 6.** Administration of vitamin D decreases metastasis of breast cancer cells both in vitro and in vivo. WT 4T1 cells were treated with 10 ng/mL TNFα alone or TNFα plus pretreatment of 10⁻⁶, 10⁻⁵, 10⁻⁴, or 10⁻³ mol/L 1,25(OH)2D3. A, Western blot analysis was performed using antibody against VDR. Bottom, the quantification result of VDR expression. B, female Balb/c mice were injected into the fourth 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 mg/kg body weight 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 TNFα plus 2 ng/mL TGFβ1 with or without pretreatment of 10⁻⁷ mol/L 1,25(OH)2D3. Wound-healing assay compared the migration ability of 4T1 cells with different treatment. Bottom, quantification result of the interval distance. D and 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 immunohistologic 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, quantification result of β-catenin expression. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
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: X. Tan
Development of methodology: Z. Zhang
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. Tao
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

Grant Support
The study was supported by a grant from the National Basic Research Program of China 2011CB944003 (R. Xiang) and the National Natural Science Foundation of China 30900540 (X. Tan) and 81273331 (R. Xiang).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 20, 2014; revised April 28, 2014; accepted April 29, 2014; published OnlineFirst May 12, 2014.

References
3. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression by TNFα-facilitating EMT (32, 33).
5. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression by TNFα-facilitating EMT (32, 33).
8. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression by TNFα-facilitating EMT (32, 33).
VDR Status Arbitrates the Prometastatic Effects of Tumor-Associated Macrophages

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


Updated version Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-14-0036

Supplementary Material Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2014/05/12/1541-7786.MCR-14-0036.DC1

Cited articles This article cites 33 articles, 5 of which you can access for free at: http://mcr.aacrjournals.org/content/12/8/1181.full#ref-list-1

Citing articles This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/12/8/1181.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/12/8/1181. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.