

# Anticancer activity of 1,25-(OH)<sub>2</sub>D<sub>3</sub> against human breast cancer cell lines by targeting Ras/MEK/ERK pathway

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**Purpose:** Breast cancer is the most common cancer among women with ~1.67 million cases diagnosed annually worldwide, and ~1 in 37 women succumbed to breast cancer. Over the past decades, new therapeutic strategy has substantially improved the curative effect for women with breast cancer. However, the currently available ER-targeted and HER-2-based therapies are not effective for triple-negative breast cancer patients, which account for ~15% of total breast cancer cases.

**Materials and methods:** We reported that 1,25-(OH)<sub>2</sub>D<sub>3</sub>, a biologically active form of vitamin D<sub>3</sub>, exhibited a strong anticancer effects on the proliferation, migration, invasion, cell cycle arrest, and apoptosis of both ER-positive (MCF-7) and ER-negative breast cancer cells (MDA-MB-453).

**Results:** The anticancer effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was more potent compared to the classical chemotherapeutics tamoxifen in MDA-MB-453 cells. Furthermore, we also found that 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased the expression of Ras and resulted in decrease of the phosphorylation of downstream proteins MEK and ERK1/2, indicating that 1,25-(OH)<sub>2</sub>D<sub>3</sub> plays its anticancer roles through targeting the Ras/MEK/ERK signaling pathway. In addition, Ras overexpression abrogated 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced G0/G1 cell cycle arrest and apoptosis of breast cancer cells, as well as the suppression of proliferation, migration, and invasion. Our study suggested that 1,25-(OH)<sub>2</sub>D<sub>3</sub> suppressed breast cancer tumorigenesis by targeting the Ras/MEK/ERK signaling pathway.

**Conclusion:** 1,25-(OH)<sub>2</sub>D<sub>3</sub> might serve as a promising supplement for breast cancer drug therapy, especially for the ER-negative breast cancer and drug-resistant breast cancer.

**Keywords:** breast cancer, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, ER-negative, cell apoptosis, cell proliferation

## Introduction

Breast cancer is an severe public health problem and one of the leading causes of cancer-related death in women.<sup>1</sup> There has been a 20% increase in the number of reported breast cancer patients worldwide, which resulted in 522,000 deaths since 2008 according to recent epidemiologic studies.<sup>2,3</sup> Initiation and progression of breast cancer occurrence have been attributed to both genetic and environmental factors.<sup>4</sup> These known prominent risk factors for the onset of breast cancer include age, life-style, fertility status, exposure to radiation, administration of estrogen medications, and diet, as well as certain hereditary mutations, which also play important roles in breast cancer.<sup>3,5</sup> Breast cancer is a heterogeneous disease that could be divided into multiple clinical subtypes based on expression of tumor histological markers, including the estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor-2 (HER-2).<sup>6,7</sup> Approximately >60% of total breast cancer cases were

reported to be ER, PR, and HER-2 positive.<sup>8</sup> Dysregulation of ER expression has been observed in 60%–70% of breast cancer patients.<sup>9,10</sup> Tamoxifen (TAM) is a nonsteroidal triphenylethylene derivative and known to compete with estrogen to inhibit ER activity associated with tumor cell growth. In clinics, TAM has been widely administrated to patients with early localized breast cancer, which could be removed by surgical resection and those with metastatic breast cancer as a mainstay hormone therapy to prevent the relapse of ER-positive cancers.<sup>11,12</sup> However, ER-negative breast cancer is usually associated with worse short-term outcomes than ER-positive cancer and accounts for 20%–30% of all patients with breast cancers, which is especially common in women with breast cancer diagnosed at young age and those in African ancestry.<sup>13</sup> In consideration of limitations in current therapeutic modalities for ER-negative tumors, it has been pressing needed to search for new prognostic tools and therapy targets.

Targeted therapy, also known as molecularly targeted therapy, refers to the modalities of cancer treatments featured by the suppression of malignant cell activity and cancer progression by targeting specific bioactive macromolecules especially functional proteins, but not simply killing all actively dividing cell as traditional chemotherapy.<sup>14,15</sup> A number of target therapy modalities such as monoclonal antibodies, tyrosine kinase inhibitors, cyclin-dependent kinase inhibitors, mammalian target of rapamycin inhibitors, and poly ADP-ribose polymerase inhibitors have been successfully applied or showed great potential as novel effective treatments for patients with breast cancer.<sup>16,17</sup> The roles of growth factors and mitogens in regulating the gene expression and cell apoptosis and differentiation were proved to be mediated by the Ras/MEK/ERK signaling cascade.<sup>18</sup> This pathway has been reported to be frequently activated in various human cancer types such as breast and prostate cancers. Thus, these major components of the Ras/MEK/ERK pathway are ideal and important candidate targets for therapeutic intervention of breast cancer development. As expected, specific inhibitors targeting Ras, Raf, MEK, and other downstream proteins of the Ras/MEK/ERK pathway have been developed for antibreast cancer drugs, and some of them are currently being testing by clinical trials.<sup>19</sup>

1,25-Dihydroxy vitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), a biologically active form of vitamin D<sub>3</sub>, is one of the major controlling hormones of intestinal calcium absorption.<sup>20</sup> In recent investigations, multiple new biological effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> has also been disclosed, including the inhibition of cell proliferation and induction of cell apoptosis, which are

closely associated with cancer initiation and development.<sup>21,22</sup> However, little is known about the potential roles of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on malignant cell functions.

In this study, we investigate the effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on the proliferation, apoptosis, invasion, and migration of human breast cancer cell lines MCF-7 (ER+ vitamin D receptor [VDR+]) and MDA-MB-453 (ER– VDR+). Also, the underlying molecular mechanisms were further explored with a focus on the Ras/MEK/ERK pathway. Collectively, our results proved the inhibitory roles of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on the proliferation, invasion, and migration of both the ER-positive and the ER-negative human breast cancer cell lines, which suggested that 1,25-(OH)<sub>2</sub>D<sub>3</sub> could serve as a promising supplement for breast cancer therapy and provided a theoretical basis for the extensive application of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in clinical practice.

## Materials and methods

### Cell culture and treatment

Breast cancer cell lines MCF-7 (ATCC<sup>®</sup> HTB-22) and MDA-MB-453 (ATCC<sup>®</sup> HTB-131), purchased from the American Type Culture Collection, were grown in a monolayer culture in the high glucose DMEM (#11995065; Thermo Fisher Scientific) with 10% (v/v) FBS (Gibco<sup>®</sup>) and 1% (v/v) Pen-Strep (10<sup>4</sup> U/mL penicillin G sodium, 10 mg/mL streptomycin sulfate) at 37°C with supply of 5% CO<sub>2</sub>. Bovine insulin was added for MCF-7 cultivation at concentration of 2×10<sup>-6</sup> mol/L. Both breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> (#NIST2972A; Sigma) at different concentrations according to designated experimental requirements. Plasmids were transfected into cells using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions.

### Cell proliferation assay

Cultured MCF-7 and MDA-MB-453 cells were seeded into 96-well plates, which were then treated with 0.1 μm TAM or 1,25-(OH)<sub>2</sub>D<sub>3</sub> at different concentrations (10<sup>-7</sup>, 10<sup>-8</sup>, and 10<sup>-9</sup> mol/L) for 48 hours. The proliferation rates of breast cancer cells were finally analyzed using cell counting kit-8 (Dojindo, Japan) according to the manufacturer's instructions. The optical densities were measured at 450 nm for evaluation of breast cancer proliferation. This assay was biologically repeated for more than three times.

### Scratch wound assay

MCF-7 and MDA-MB-453 cells were grown in 35 mm dishes to 100% confluence, which were then scratched using a sterile pipette tip to form a wound. The cells were then

treated with 0.1  $\mu\text{M}$  TAM or 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration gradient with absence or presence of combined treatment with ERK agonist (U46619) for 24 hours. Cell images were finally recorded using a photomicroscope for cell migration assessments. At least three biological repeats were performed for statistical analysis.

## Cell invasion assay

Cell invasion assay was performed using a polyethylene terephthalate membrane (8  $\mu\text{m}$  pore size) with Millicell hanging cell culture inserts and BD Matrigel (Millipore). The MCF-7 and MDA-MB-453 cells loaded into the upper chamber were treated with 0.1  $\mu\text{M}$  TAM or with 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the absence or presence of the ERK agonist (U46619). Conditioned medium containing 10% (v/v) FBS was added to each well, and breast cancer cells were allowed to invade by penetrating through the membrane during 24-hour incubation at 37°C. Cells that had migrated through the filter pores were fixed with methanol, stained with crystal violet, and the invasive potential of breast cancer cells was evaluated microscopically by counting the number of invading cells. More than three biological replicates were done for cell invasion evaluation.

## Cell cycle analysis

Cell cycle progression of breast cancer cells after designated treatments was analyzed by quantification of DNA content. Briefly, cultured breast cancer cells were harvested and fixed in 70% ethanol for 24–48 hours at 4°C, treated with ribonuclease to remove RNA contamination, and DNA was stained with propidium iodide (PI). Fluorescence of PI-stained cells was measured by flow cytometry. At least three biological repeats were performed for cell cycle progression analysis.

## Apoptosis analysis

Cultured breast cancer cells were harvested and stained with Annexin V FITC and PI (#V13242; Thermo Fisher Scientific) according to the manufacturer's protocol. Percentages of apoptotic cells in each group were detected by flow cytometry for calculation of the breast cancer cell apoptosis rates for three biological repeats.

## Western blotting

Breast cancer cells were collected after specified treatments and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) for 30 minutes on ice. The protein concentration was determined via the BCA protein assay. Approximately 30  $\mu\text{g}$  proteins of each group were boiled

at 100°C for 5 minutes, separated by SDS-PAGE electrophoresis, and transferred into the PVDF membrane. These membranes were then blocked with 5% skim milk, incubated with first antibody at 4°C overnight, washed with tris-HCl buffer solution and tween-80 (TBST), incubated with the secondary antibody at room temperature for 1 hour, followed by washing by TBST. ECL luminescent reagent was added and the resultant signals were recorded on X-ray film in darkroom. The glyceraldehyde-3-phosphate dehydrogenase antibody was used as loading control.

## Plasmid construction

For the overexpression of Ras gene in breast cancer cells, full-length *Homo sapiens* Ras genomic sequences based in NCBI were cloned and ligated onto pcDNA3.0 (Addgene, Watertown, MA, USA). The recombinant constructs were sequence verified through DNA sequencing by the Thermo Fisher Scientific.

## Statistical analysis

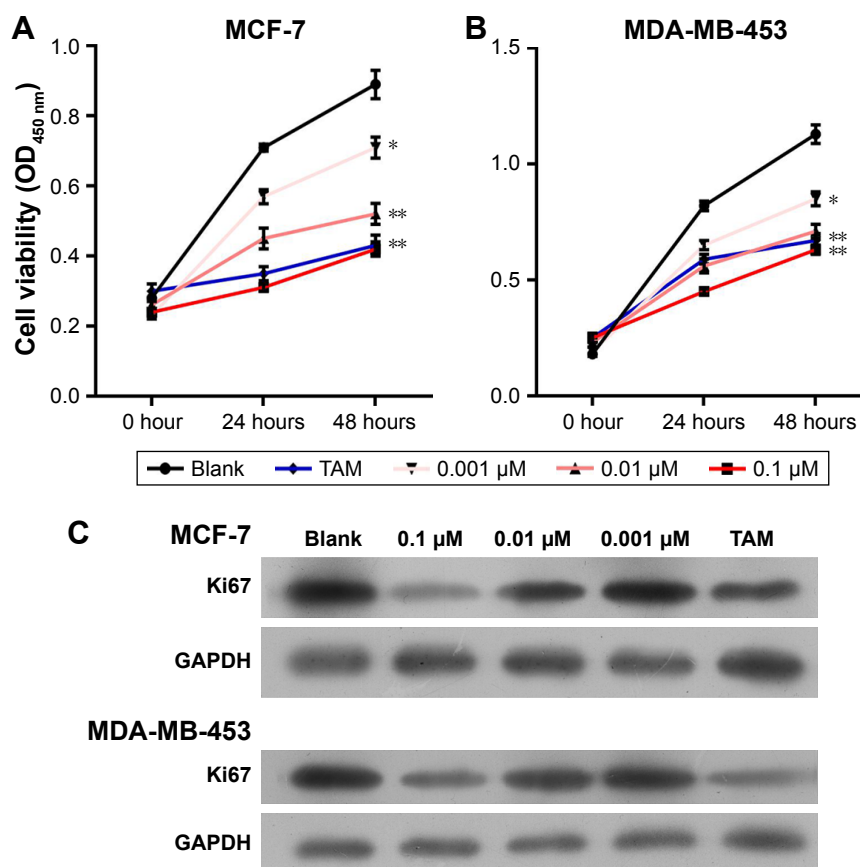
Statistical analysis in this study was performed using the GraphPad Prism software. Data were expressed as mean  $\pm$  SD and subjected to Student's *t*-test for evaluation of the significance of differences between two groups. Each assay was biologically repeated for at least three times. Significant differences were defined by a *P*-value of <0.05, <0.01, or <0.001.

## Results

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited breast cancer cell proliferation, migration, and invasion

To investigate the influence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on breast cancer cell growth and motility, we first used ER-positive breast cancer MCF-7 cells and ER-negative breast cancer MDA-MB-453 cells to perform cell growth assay. Cells were treated with different concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 48 hours, and the 0.1  $\mu\text{M}$  TAM group was used as positive control. We showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> could significantly inhibit the proliferation of both MCF-7 cells and MDA-MB-231 cells (Figure 1A and B). At concentrations of  $\geq 10^{-7}$  mol/L, 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited proliferation of MDA-MB-453 cells more potently than the classical antiestrogen TAM (Figure 1B). The IC<sub>50</sub> concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on MCF-7 and MDA-MB-231 cells were found to be 0.008 and 0.102, respectively. By Western blotting, we found that the expression level of Ki67, the proliferation marker, was greatly suppressed by 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in both breast cancer cell lines (Figure 1C).

To evaluate the potential antimetastatic effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, we analyzed its ability of inhibiting the migration



**Figure 1** 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited the proliferation of breast cancer cells.

**Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> in different concentrations or 0.1 μm tamoxifen (TAM). (A and B) The CCK-8 assay was used to determine the cell proliferation of MCF-7 cells and MDA-MB-453 cells, respectively, every 24 hours for 2 days. Error bars represent the SD of cell proliferation rates. (C) The expression of Ki67 protein was determined by Western blotting after the cells were treated for 48 hours. \*P<0.05, \*\*P<0.01 vs Blank.

**Abbreviations:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>; CCK-8, cell counting kit-8.

and invasion of both MCF-7 cells and MDA-MB-453 cells. It was demonstrated that 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited the migration (Figure 2A and B) and invasion (Figure 2C and D) of MCF-7 cells, while its inhibitory effect against MDA-MB-453 cells was much more potent than TAM (Figure 2A–F). Also, we found that the effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on breast cancer cell proliferation, migration, and invasion were dose dependent.

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced cell cycle arrest and apoptosis of breast cancer cells

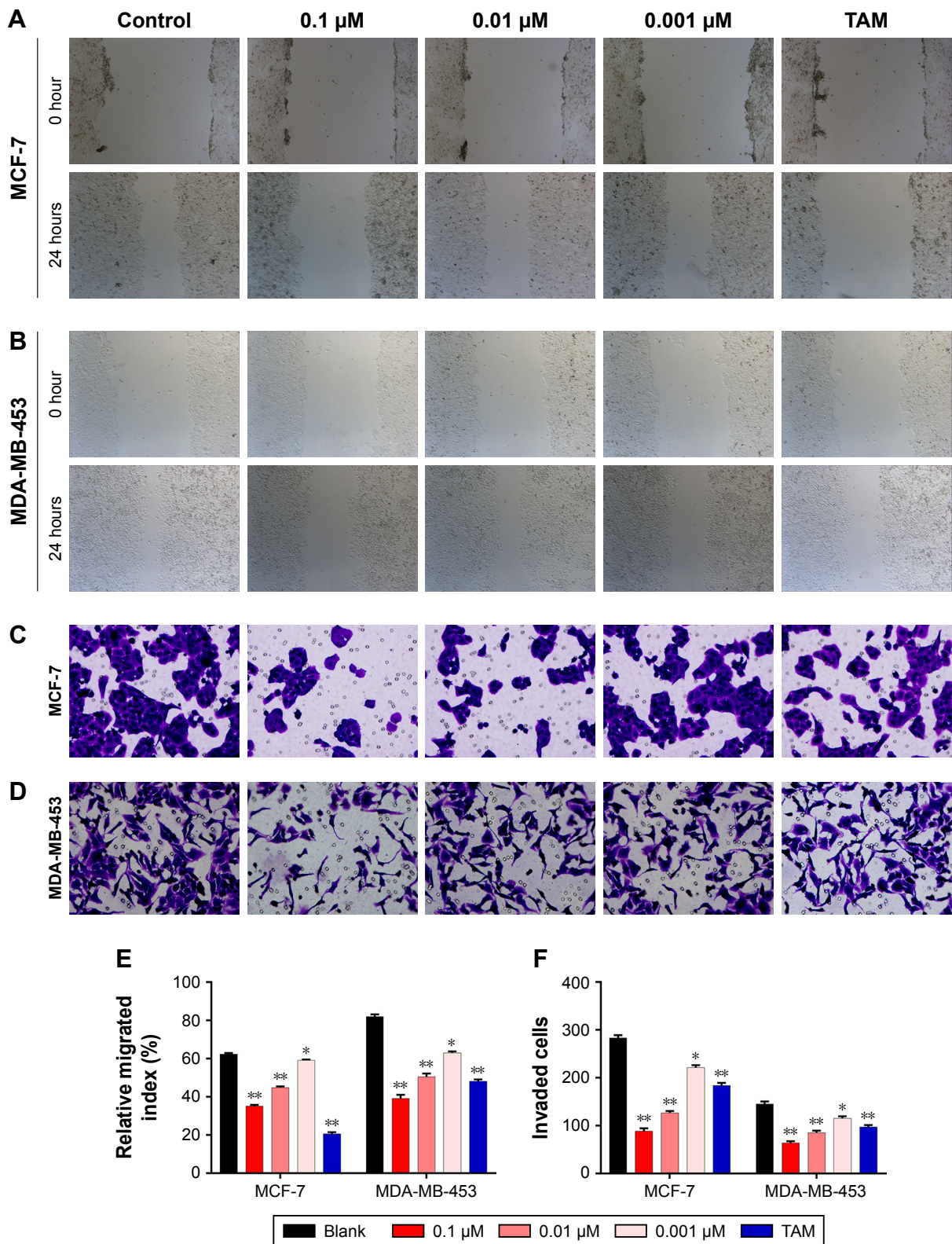
To further investigate the inhibition of breast cancer cells proliferation by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, we analyzed its effect on the cell cycle progression of breast cancer cell lines. Cell population in each phase of cell cycle was determined with PI staining, followed by flow cytometry after treatments with 1,25-(OH)<sub>2</sub>D<sub>3</sub> at different concentration for 48 and 72 hours. We showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> increased the population of both MCF-7 cells and MDA-MB-453 cells

in G0/G1 phase, which was accompanied by proportional decrease of breast cancer cells in S and G2/M phases (Figure 3A–C). Moreover, we demonstrated that 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced the apoptosis of both MCF-7 cells and MDA-MB-453 cells (Figure 3D–F). The effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on cell cycle arrest and apoptosis of both MCF-7 and MDA-MB-453 cells were more remarkable than cells treated with TAM (Figure 3A–F). The effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on breast cancer cell cycle progression and apoptosis were also dose and time dependent in MDA-MB-453 cells.

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited Ras expression and MEK and ERK phosphorylation in both ER-positive and ER-negative breast cancer cells

To illuminate the molecular mechanisms involved in the potential roles of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on breast cancer cells, we tested Ras protein abundance, and phosphorylation of MEK





**Figure 2** 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited the motility and invasive potential of breast cancer cells.

**Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> in different concentrations or 0.1  $\mu\text{M}$  tamoxifen (TAM). (A) Cell migration was determined using wound healing migration assay ( $\times 40$ ). (B) The width of wound was measured using a microscope. (C) Numbers of invading cells were determined by counting using a microscope. (D) MCF-7 and MDA-MB-453 cells were plated in upper compartments of Matrigel invasion chambers and exposed to 1,25-(OH)<sub>2</sub>D<sub>3</sub> or TAM. Then, invasive potential of treated cells was evaluated microscopically. Data are mean  $\pm$  SD of three independent experiments. Statistical analysis of the relative migration index (E) and invaded cell numbers (F) of MCF-7 and MDA-MB-453 cells. \* $P < 0.05$  vs Blank, \*\* $P < 0.01$ .

**Abbreviation:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>.

and ERK1/2 proteins in both MCF-7 cells and MDA-MB-453 cells after 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment for 48 hours. Our Western blot assay revealed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> strongly decreased Ras protein abundance and the phosphorylation of MEK

and ERK1/2 in both breast cancer cells (Figure 4). These data indicated that the anticancer effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was likely mediated by suppressing the activation of the Ras/MEK/ERK signal pathway.

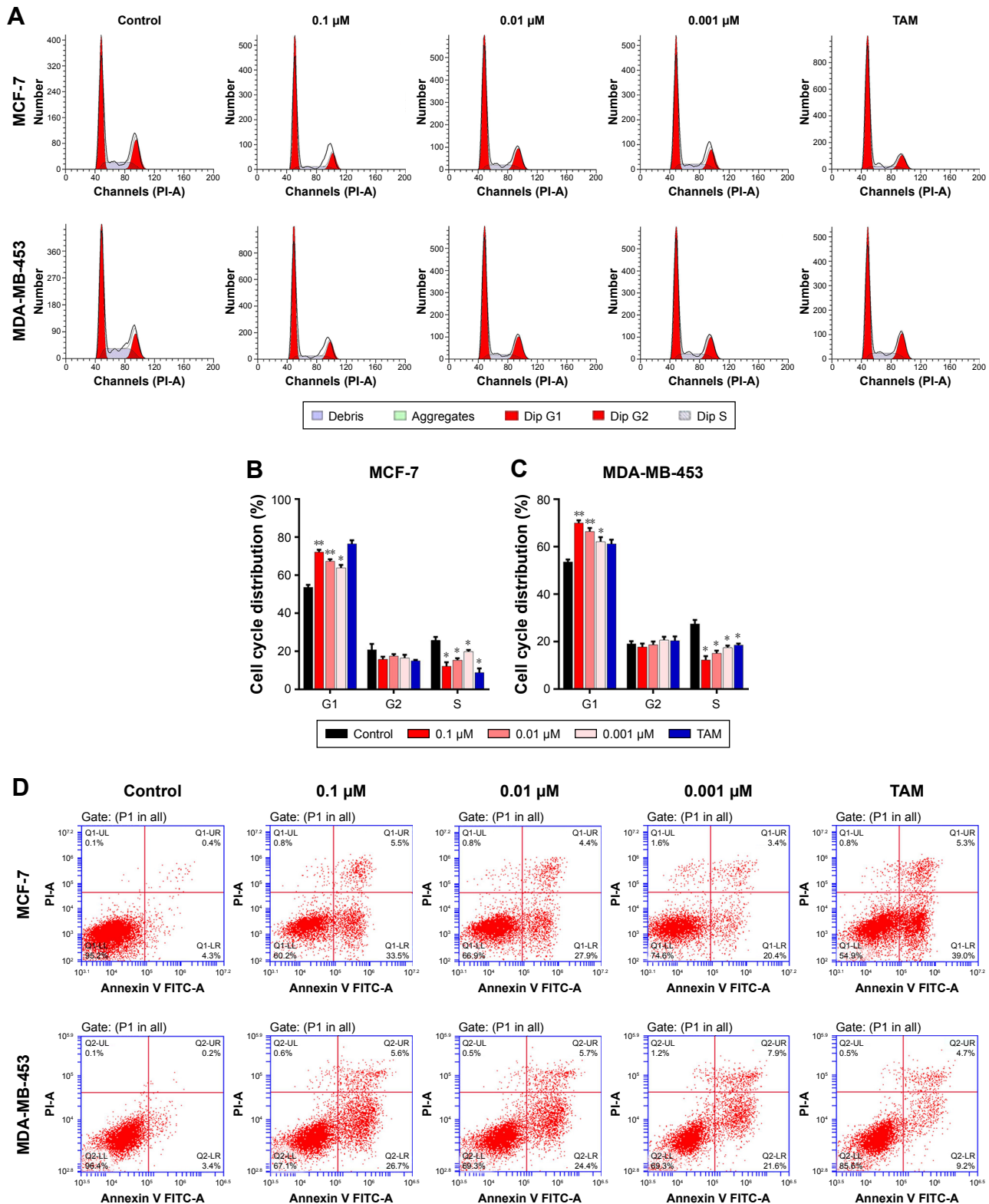
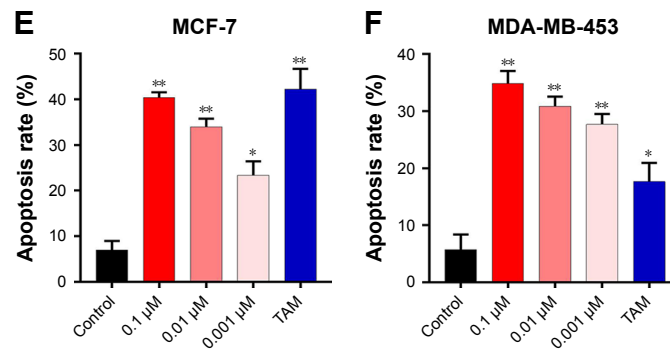


Figure 3 (Continued)



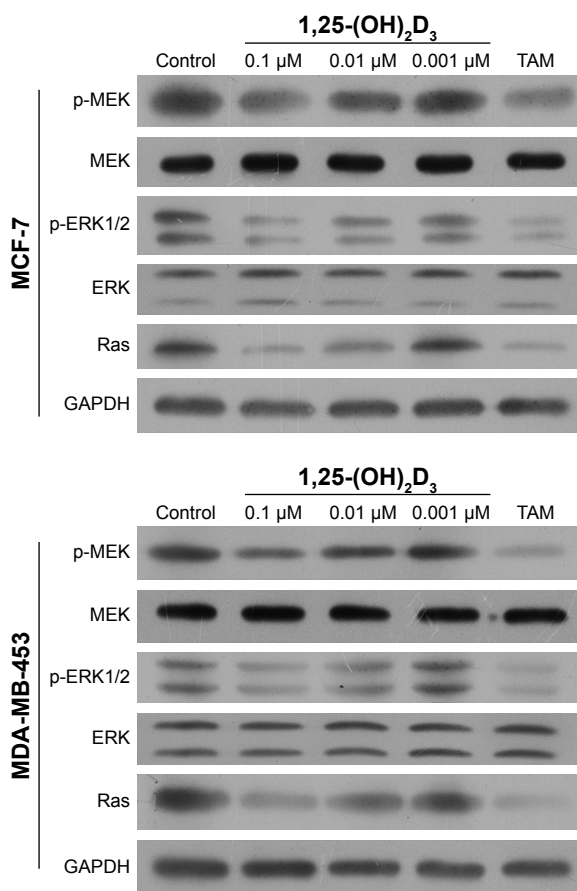
**Figure 3** 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced G0/G1 cell cycle arrest and apoptosis of breast cancer cells.

**Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> in different concentrations or 0.1 μM tamoxifen (TAM) for 48 hours. (A) The cell cycle of treated MCF-7 cells and MDA-MB-453 cells were measured by flow cytometry assay. The percentage of each cell cycle phase was calculated in (B and C). (D) The apoptosis rates of treated MCF-7 cells and MDA-MB-453 cells were measured by flow cytometry assay. The statistics of the apoptosis rate of MCF-7 cells and MDA-MB-453 cells were shown in (E and F), respectively. Error bars represent the SD. \*P<0.05, \*\*P<0.05 vs Control.

**Abbreviation:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>.

To explore the potential functions of the Ras/MEK/ERK signal pathway in breast cancer cell function regulation by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, both MCF-7 cells and MD-MB-453 cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> alone or in combination

with overexpression of Ras gene, followed by determination of breast cancer cell proliferation, migration, invasion, cell cycle progression, and apoptosis. We revealed that the alterations of these cellular processes in both MCF-7 cells and MDA-MB-453 cells induced by treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> were recovered to a certain extent by overexpression of Ras gene (Figures 5 and 6). The downregulation of Ki67 protein expression in breast cancer cells by 1,25-(OH)<sub>2</sub>D<sub>3</sub> was also repressed by Ras gene overexpression (Figure 5D and E). Also, the phosphorylation of MEK and ERK in breast cancer cells treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> was significantly upregulated by Ras gene overexpression (Figure 7). Thus, our data strongly indicated that the activity of Ras/MEK/ERK signal pathway was a major mechanism underlying 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced anticancer effect in both ER-positive and the ER-negative breast cancer cells.



**Figure 4** 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased the protein expression of Ras and the phosphorylation of MEK and ERK1/2 in breast cancer cells.

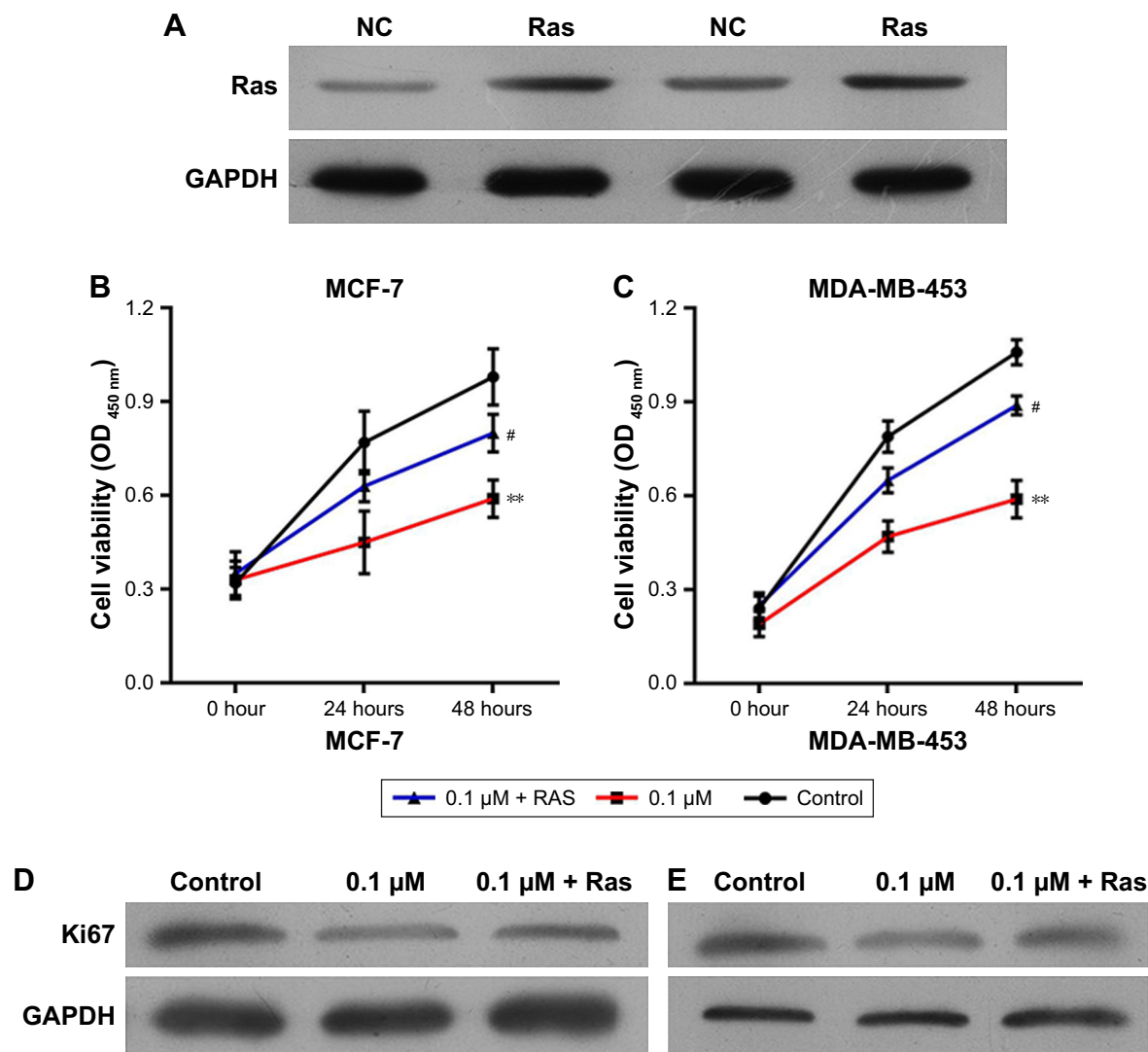
**Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> in different concentrations or tamoxifen (TAM) for 48 hours. The protein levels of Ras and the phosphorylation of MEK and ERK1/2 in both MCF-7 cells and MD-MB-453 cells were shown by Western blotting. The expression of GAPDH was used as an internal control.

**Abbreviation:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>.

## Discussion

Breast cancer remains a leading cause of cancer morbidity and mortality among women all over the world.<sup>23</sup> Previous statistics showed that each year ~2,400 deaths of women with breast cancer occurred at ages below 45 years, and the majority of breast cancer-related deaths happened in women older than 45 years.<sup>24</sup> The survival rate for women with nonmetastatic breast cancer was reported to be 98.5%, but there is a significant drop of survival rate for patients with metastatic breast cancer, which ranges from 84% to 24%.<sup>25</sup> TAM is known to compete with estrogen to inhibit the ER activity associated with tumor cell activity and has been approved for treatment of postmenopausal women suffering from advanced breast cancer by the US Food and Drug Administration in 1977 and later for postsurgery adjuvant treatment to eradicate micrometastasis derived from primary breast cancer.<sup>26</sup> The antiestrogenic activity of TAM mediated





**Figure 5** The proliferation of 1,25-(OH)<sub>2</sub>D<sub>3</sub>-treated breast cancer cells was abrogated by overexpression of Ras. **Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> alone or together with the overexpression of Ras by using plasmid for 48 hours. **(A)** The expression of Ras protein in MCF-7 and MDA-MB-453 cells transfected with Ras-overexpressing plasmids was determined by Western blotting after the cells were treated for 48 hours. **(B)** The CCK-8 assay was used to determine the cell proliferation of MCF-7 cells every 24 hours for 2 days. **(C)** The CCK-8 assay was used to determine the cell proliferation of MDA-MB-453 cells every 24 hours for 2 days. **(D)** The expression of Ki67 protein in MCF-7 cells was determined by Western blotting after the cells were treated for 48 hours. **(E)** The expression of Ki67 protein in MDA-MB-453 cells was determined by Western blotting after the cells were treated for 48 hours. Error bars represent the SD. \*\**P*<0.01 vs Control, #*P*<0.05 vs 0.1 μm. **Abbreviations:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>; CCK-8, cell counting kit-8.

by ER has been well established and was the main reason for TAM treatment in ER-positive breast cancer; however, recent clinical studies applying TAM in treating patients with ER-negative breast cancers failed to produce significant benefits in terms of mortality reduction or inhibition of breast cancer recurrence.<sup>27,28</sup> Thus, it is pressingly needed to develop novel effective therapeutic strategies for female patients with ER-negative and triple-negative breast cancers, the latter being an aggressive disease with worse clinical outcomes.

In this study, we found that TAM exhibited significant anticancer effects on both ER-positive and ER-negative breast cancer cells by inhibiting cell viability, proliferation,

migration, invasion, and cell cycle progression, while promoting breast cancer apoptosis. However, the therapeutic efficacy of TAM on ER-negative breast cancer cells was found weaker than in the ER-positive ones, indicating that TAM alone was not an ideal chemotherapeutic strategy for treating ER-negative breast cancer. Thus, we investigated the functions of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, one biologically active form of vitamin D<sub>3</sub>, in regulating breast cancer cell activities associated with tumorigenesis and metastasis. Hopefully, we showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> effectively inhibited the growth, proliferation, migration, and invasion capabilities of both MCF-7 cells and MDA-MB-453 cells in the dose-dependent



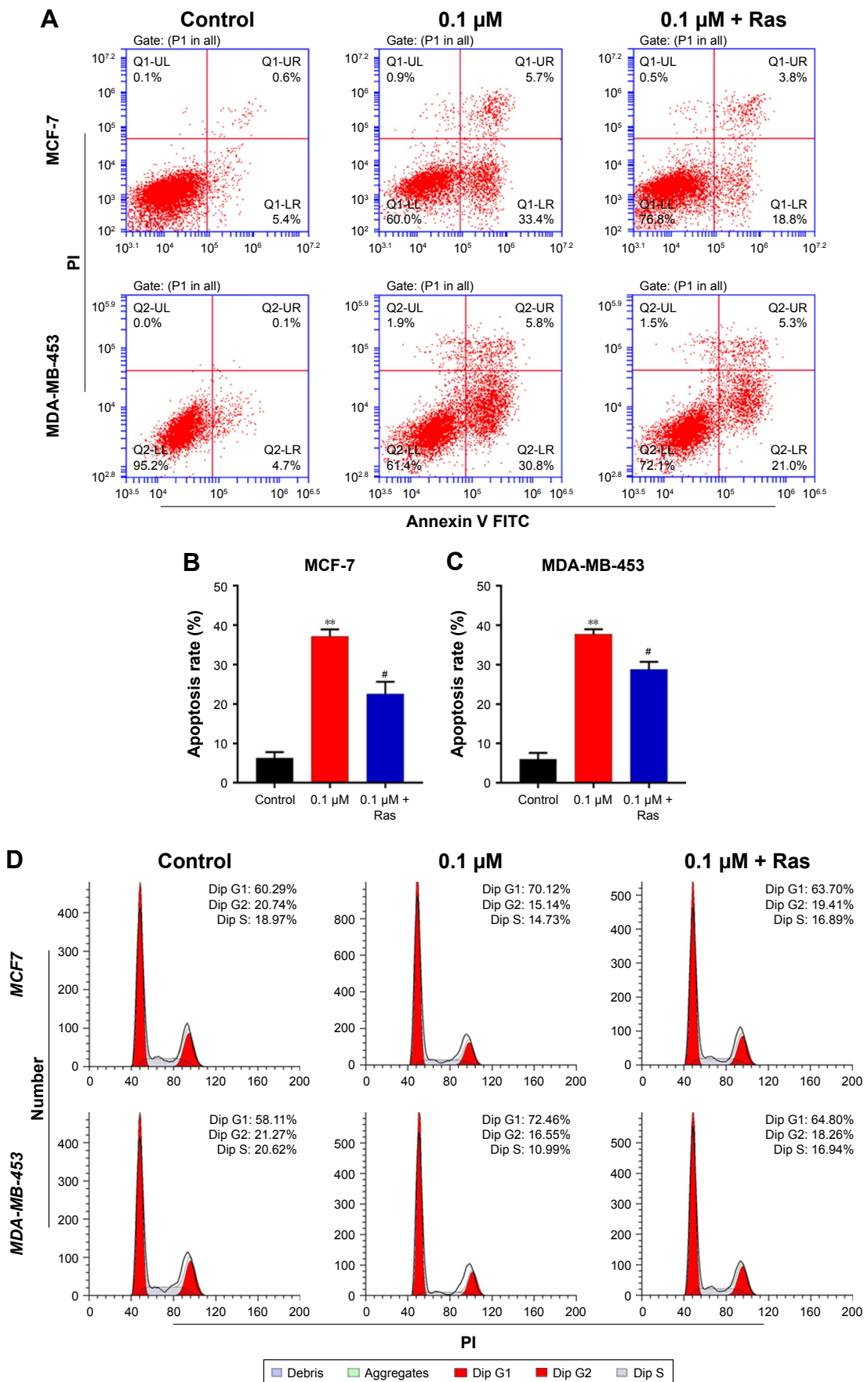
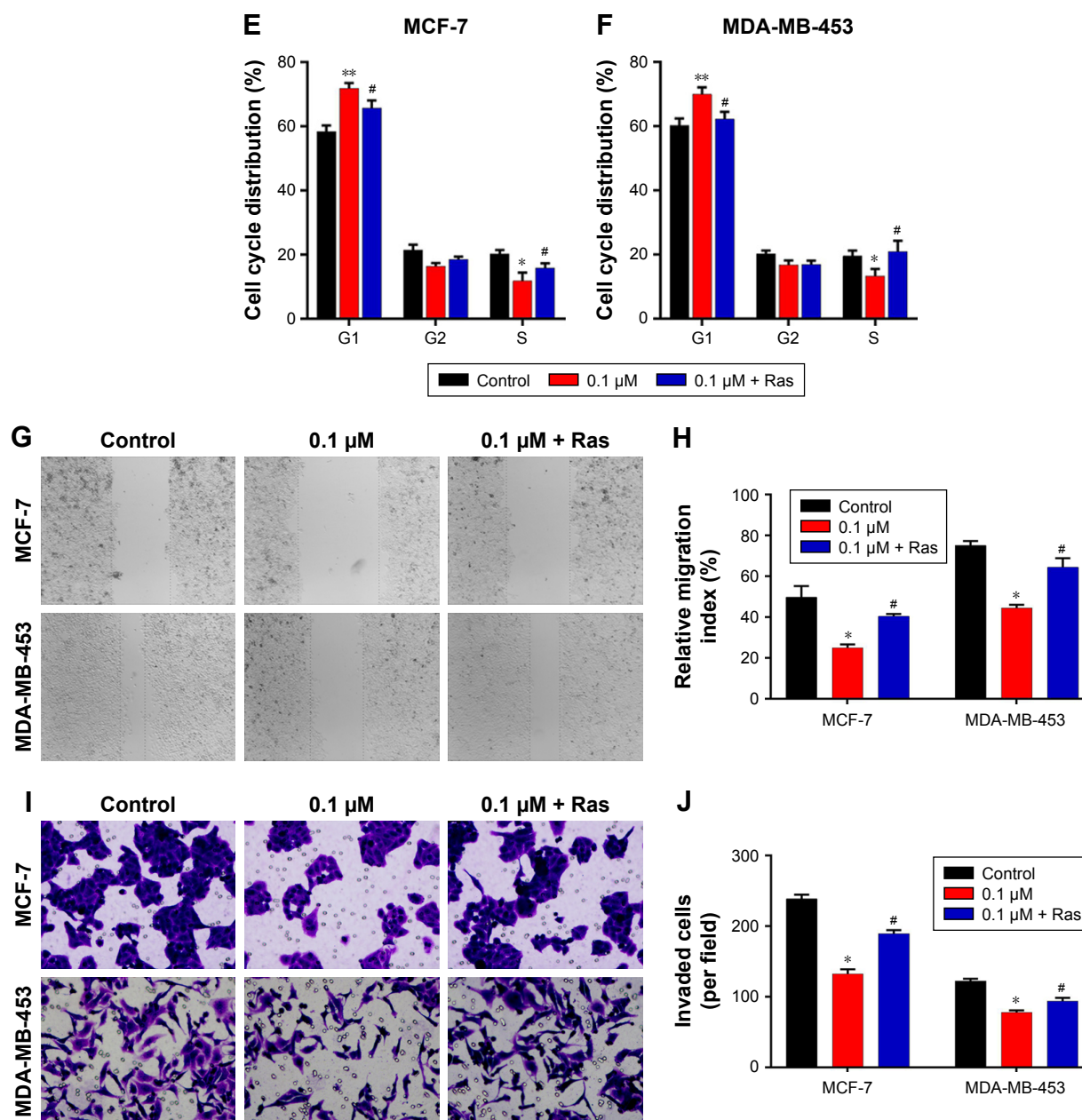


Figure 6 (Continued)



**Figure 6** The  $1,25\text{-(OH)}_2\text{D}_3$ -induced cell cycle arrest and apoptosis of breast cancer cells and the motility and invasive potential of  $1,25\text{-(OH)}_2\text{D}_3$ -treated breast cancer were abrogated by Ras overexpression.

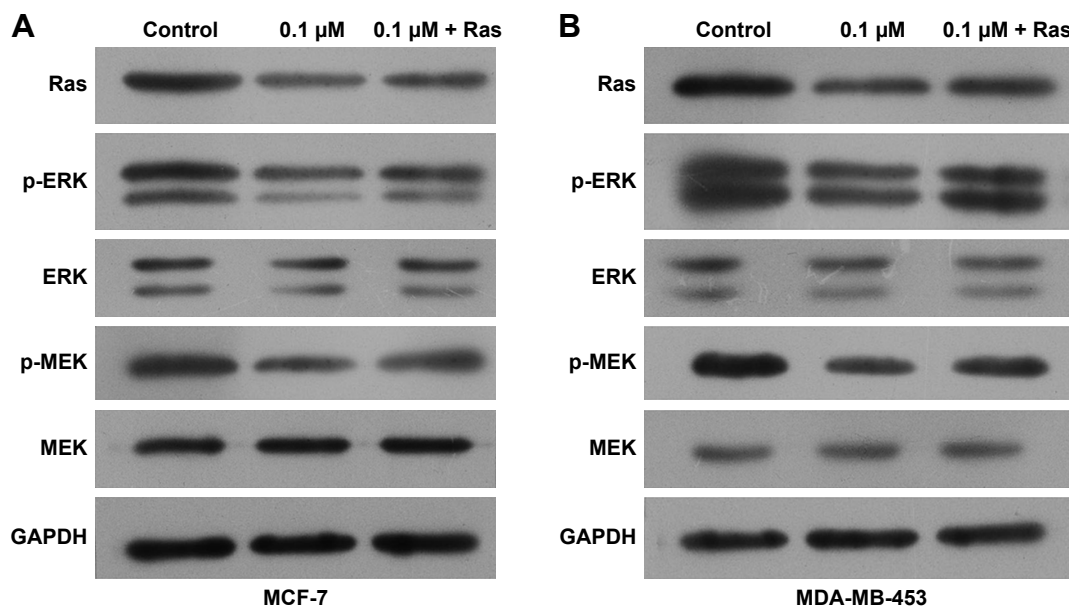
**Notes:** Breast cancer cells were treated with  $1,25\text{-(OH)}_2\text{D}_3$  alone or together with the overexpression of Ras by using plasmid for 48 hours. (A) The apoptosis rates of treated MCF-7 cells and MDA-MB-453 cells were measured by flow cytometry assay. Statistical analysis of apoptosis rates of MCF-7 (B) and MDA-MB-453 (C) cells. (D) The cell cycle of treated MCF-7 cells and MDA-MB-453 cells were measured by flow cytometry assay. Statistical analysis of cell cycles of MCF-7 (E) and MDA-MB-453 (F) cells. (G) Cell migration was determined using wound healing migration assay ( $\times 40$ ). The width of wound was measured using a microscope. (H) Statistical analysis of relative migration indexes of MCF-7 and MDA-MB-453 cells during wound healing migration assay. (I) MCF-7 and MDA-MB-453 cells were plated in upper compartments of Matrigel invasion chambers and exposed to  $1,25\text{-(OH)}_2\text{D}_3$  alone or together with the overexpression of Ras. Then, invasive potential of treated cells was evaluated microscopically. (J) Numbers of invading cells were determined by counting using a microscope. Data are mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs Control, # $P < 0.05$  vs  $0.1 \mu\text{M}$ .

**Abbreviations:**  $1,25\text{-(OH)}_2\text{D}_3$ ,  $1,25\text{-dihydroxy vitamin D}_3$ ; PI, propidium iodide.

manner, accompanied by induced G0/G1 arrest and apoptosis. These cellular assays showed that  $1,25\text{-(OH)}_2\text{D}_3$  possesses significant inhibitory effects on breast cancer cell activity.

The Ras/MEK/ERK mitogen-activated protein kinase cascade has been established as a key intracellular signaling

pathway responsible for regulating diverse cellular functions including cell proliferation, survival, apoptosis, motility, transcription, metabolism, and differentiation, which are all closely associated with tumorigenesis.<sup>29</sup> Multiple components of this pathway were reported to be mutated or



**Figure 7** Activation of the Ras/MEK/ERK signaling was abrogated by Ras overexpression.

**Notes:** Breast cancer cells were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> alone or together with the overexpression of Ras by using plasmid for 48 hours. The protein levels of Ras and the phosphorylation of MEK and ERK1/2 in MCF-7 cells (**A**) and MD-MB-453 (**B**) cells were shown by Western blotting. The expression of GAPDH was used as an internal control.

**Abbreviation:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>.

aberrantly expressed in human breast cancer cells and linked with poor prognosis of breast cancer patients. Moreover, the Ras/MEK/ERK pathway has also been associated with chemotherapeutic drug resistance in breast cancer.<sup>30</sup> Thus, it is reasonable to speculate that the Ras/MEK/ERK signaling pathway would be applied as an ideal target for therapeutic intervention of breast cancer progression.<sup>19</sup> In the present study, we found that 1,25-(OH)<sub>2</sub>D<sub>3</sub> depressed Ras expression level and the phosphorylation of MEK and ERK1/2 in both ER-positive and ER-negative breast cancer cells, suggesting the role of Ras/MEK/ERK signaling during breast cancer inhibition by 1,25-(OH)<sub>2</sub>D<sub>3</sub>. More importantly, we proved that the expression of Ki67 gene and the phosphorylation of MEK and ERK in both breast cancer cell lines treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> were significantly recovered by overexpression of Ras gene. Consistent with these molecular alterations, the alterations of breast cancer cell functions including growth, proliferation, migration, invasion, cell cycle progression, and apoptosis caused by 1,25-(OH)<sub>2</sub>D<sub>3</sub> were also remarkable suppression by Ras gene overexpression, which further validated that 1,25-(OH)<sub>2</sub>D<sub>3</sub> performs its inhibitory roles on breast cancer progression through modulation of the Ras/MEK/ERK signaling pathway.

## Conclusion

We have shown that 1,25-(OH)<sub>2</sub>D<sub>3</sub> possesses remarkable anticancer effects on both ER-positive and ER-negative

breast cancer cells. Furthermore, our study identified the depressed activation of the Ras/MEK/ERK pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub> as one key underlying mechanism. Our findings provided a rationale for preclinical and clinical evaluation of 1,25-(OH)<sub>2</sub>D<sub>3</sub> alone or in combination with other chemotherapeutic drugs for breast cancer therapy, especially for the ER-negative breast cancer.

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## Disclosure

The authors report no conflicts of interest in this work.

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