ORIGINAL RESEARCH

Oxymatrine reverses epithelial-mesenchymal transition in breast cancer cells by depressing αVβ3 integrin/FAK/PI3K/Akt signaling activation

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Purpose: Oxymatrine, an alkaloid extracted from the Chinese herb *Sophora flavescens* Aiton, possesses anti-inflammatory, anti-immune, anti-hepatic fibrosis, and anti-cancer properties. However, the effects of oxymatrine on epithelial-mesenchymal transition (EMT) of breast cancer cells are still unclear.

Aim: The present study was performed to investigate whether oxymatrine reverses EMT in breast cancer cells and to explore the underlying molecular mechanisms.

Materials and methods: MTT assay was performed to evaluate cell viability. Wound-healing assay and transwell chamber assay were used to assess cell migration and invasion, respectively. Immunofluorescence and Western blot were used to study the expression of EMT-related molecules and αVβ3 integrin/focal adhesion kinase (FAK)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling transduction. Fibronectin, a physiologic ligand of αVβ3 integrin, was used to stimulate αVβ3 integrin signaling.

Results: Our results demonstrated that oxymatrine effectively suppressed the viability of MDA-MB-231 and 4T1 breast cancer cells, and oxymatrine showed less cytotoxicity on normal breast mammary epithelial MCF-10A cells. In addition, oxymatrine reversed EMT in the MDA-MB-231 and 4T1 cells at nontoxic concentrations. Oxymatrine significantly inhibited cell migration and invasion, downregulated the expression of N-cadherin, vimentin, and Snail in MDA-MB-231 and 4T1 cells, but upregulated the expression of E-cadherin in 4T1 cells. The mechanism revealed that oxymatrine decreased the expression of αV and β3 integrin and their co-localization. It also inhibited αVβ3 integrin downstream activation by suppressing the phosphorylation of FAK, PI3K, and Akt. Furthermore, oxymatrine prevented fibronectin-induced EMT and αVβ3 integrin/FAK/PI3K/Akt signaling activation.

Conclusion: Our results revealed that oxymatrine effectively reversed EMT in breast cancer cells by depressing αVβ3 integrin/FAK/PI3K/Akt signaling. Thus, oxymatrine could be a potential therapeutic candidate with anti-metastatic potential for the treatment of breast cancer.

Keywords: oxymatrine, breast cancer, αVβ3 integrin, epithelial-mesenchymal transition

Introduction

Breast cancer is one of the most common malignancy occurring among women worldwide.[1] More than 90% of breast cancer-related deaths are not caused by the primary tumors, but by distant metastases.[2] In spite of great improvements in the early detection and diagnosis, no gold standard treatment has been defined or validated for metastatic breast cancer so far.[3] Once breast cancer progresses to the metastatic stage, the clinical prognosis is guarded and the five-year relative survival rate is poor.[4] Therefore, new therapeutic strategies constraining metastasis

in breast cancer are still warranted. Metastasis is accomplished by a shift of the polarized epithelial tumor cells to mesenchymal cells, which is known as epithelial to mesenchymal transition (EMT).[5] Aberrant activation of EMT program powers the cancer cells to lose cell-cell adhesion and acquire enhanced migratory and invasive behavior so that they can navigate through the natal sites and eventually lead to distant metastases.[6] Thus, reversal or suppression of EMT may prevent the metastatic spread of breast cancer cells.

Integrins are responsible for cellular adhesion to extracellular matrix proteins though forming a heterodimer of α and β subunits.[7] They allow the cells to interact with their environment and transmit bidirectional signaling as "outside-in" and "inside-out" thereby modulating cell proliferation, cytoskeletal organization, adhesion, migration, and differentiation.[8] In addition, integrins transmit signals through crosstalk with several growth factor receptors or directly recruit and activate focal adhesion kinase (FAK) and Src-family kinases, and subsequently stimulated downstream signaling cascades, including the phosphoinositide kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPK), and extracellular signal-regulated kinase (ERK).[9]

Abnormal high expression of $\alpha_V\beta_3$ integrin significantly correlates with tumor progression in several cancers.[10] In breast cancer, $\alpha_V\beta_3$ integrin is strongly expressed in the invasive tumors and distant metastatic sites within the tumors and is an indicator of poor prognosis.[11,12] It has been revealed that $\alpha_V\beta_3$ integrin promotes several steps of breast cancer metastasis. As an adhesion receptor, $\alpha_V\beta_3$ integrin supports the tumor cells to attach to the extracellular matrix, and interact with platelets to arrest within the capillaries during blood flow.[13] It also induces metastatic phenotype of breast cancer cells by intensifying their migratory and invasive behavior.[14] In addition, $\alpha_V\beta_3$ integrin is required for enhancing the EMT process in breast cancer cells.[15] Gene silencing of $\alpha_V\beta_3$ integrin leads to decreased fibroblast growth factor 1-enhanced N-cadherin expression and cell motility in breast cancer cells.[16] Knockout of $\alpha_V\beta_3$ integrin transcription factor HoxD3 was found to significantly suppress lipopolysaccharide-induced EMT in breast cancer cells.[17] Thus, these results suggest that $\alpha_V\beta_3$ integrin may be a potential therapeutic target for reversing EMT in breast cancer, thereby providing an alternative avenue for the treatment of patients having a metastatic risk.

Oxymatrine is an alkaloid derived from the traditional Chinese herb *Sophora flavescens* Aiton.[18] It exhibits multiple pharmacological activities, such as anti-viral, anti-fibrotic,

anti-inflammatory, anti-allergic, and cardiovascular protective effects.[19] In addition, oxymatrine also possesses anti-tumor effects by targeting multiple pathways through inhibiting cell growth and angiogenesis, inducing apoptosis and cell cycle arrest, reducing cancer metastasis and invasion, and reversing multidrug resistance in cancer cells.[20] It has also been considered as a promising anticancer agent in breast cancer. Oxymatrine significantly suppresses proliferation and promotes S-phase cell cycle arrest through mitochondria-mediated apoptosis in MDA-MB-231 and MCF-7 breast cancer cells.[21] Furthermore, oxymatrine inhibits proliferation of cancer stem-like cells in MCF-7 cells via suppression of Wnt/β-catenin signaling pathway.[22] However, it is still unclear whether oxymatrine reverses EMT in breast cancer cells.

In this study, we used two breast cancer cell lines, MDA-MA-231 and 4T1, exhibiting highly invasive and metastatic potentials,[23,24] to evaluate whether oxymatrine could reverse EMT in these two types of cells. We also explored the mechanisms related to the effects of oxymatrine on $\alpha_V\beta_3$ integrin/FAK/PI3K/Akt signaling pathway.

Materials and methods
Materials
Oxymatrine was purchased from Zelang Pharmaceutical Technology Co., Ltd (Nanjing, Jiangsu, China). Cyclo (Arg-Gly-Asp-d-Phe-Lys, -RGDfK, #A8164) was obtained from WeiHuan Biotech Co., Ltd (Shanghai, China). Fibronectin (FN, #F2006) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). Matrigel (#356237) was obtained from BD Biosciences (Bedford, MA, USA). 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, #804W059) was obtained from Solarbio Co., Ltd (Beijing, China). The primary antibodies against E-cadherin (#BS1098, 1:800), vimentin (#BS1491, 1:800), N-cadherin (#BS2224, 1:800), phosphorylated-PI3K (#AP0152, 1:800), PI3K (#BS3006, 1:1000), phosphorylated-FAK (#BS4684, 1:1000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #MB001, 1:5000) were obtained from Bio-World (Dublin, OH, USA). The primary antibody against Akt (#BA2318, 1:300) was obtained from Boster Biological Technology (Wuhan, Hubei, China). The primary antibodies against phosphorylated-Akt (#4058S, 1:800) and Snail (#3879, 1:800) were obtained from Cell Signaling Technology (Beverly, MA, USA). The primary antibodies against integrin β_3 (#sc-46655 1:1000) and FAK (#sc-557, 1:1000) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-α_V integrin antibody

(#ab179475, 1:1000) was purchased from Abcam, Inc. (Cambridge, MA, USA). Horseradish peroxidase-labeled goat anti-mouse (#BS12478, 1:3000) and goat anti-rabbit (#BS13278, 1:3000) secondary antibodies were purchased from Bio-World. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (#BS11502, 1:100) and goat anti-rabbit secondary antibody (#ab150077, 1:400) were purchased from Bio-World and Abcam, respectively.

Cell culture

MDA-MB-231 and 4T1 breast cancer cell lines were purchased from Kunming Cell Biological Institute of the Chinese Academy of Science (Kunming, Yunnan, China). The cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, Carlsbad, CA, USA). The medium was supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). The MCF-10A cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM/F-12 with 5% horse serum (HS; 1312396, Gibco), 20 ng/mL epidermal growth factor (AF-100-15, PeproTech, London, United Kingdom), 0.1 μg/mL cholera enterotoxin (C8052, Sigma), 10 μg/mL insulin (I6634, Sigma), 0.5 μg/mL hydrocortisone (H0888, Sigma). These cells were incubated in presence of 5% CO_2 at 37°C.

MTT assay

The cells were digested, collected, and seeded in 96-well plates for 24 h. The cells were treated with 0, 1.01, 1.68, 2.8, 4.67, 7.78, 12.96, 21.60, 36, 60, and 100 μmol/mL oxymatrine for 24, 48, or 72 h. MTT solution (5 mg/mL, 20 μl/well) was then added and the cells were continuously incubated for 4 h. The medium was subsequently replaced with DMSO (100 μl/well). The optical density (OD) was detected with a spectrophotometer at 570 nm by a microplate reader (ELx800, General Electric, Fairfield, CT, USA).

Wound healing assay

The cells were seeded into 12-well plates and grown to a monolayer until it reached 90% confluence. The cell monolayer was scratched by a 10 μl pipette tip to form a straight wound. The cells were then treated with oxymatrine in a medium containing 1% FBS for 48 h. The images were captured by a Leica DMI 1 microscope at 0 and 48 h (Leica, Wetzlar, Germany) at 50× magnification.

Invasion assay

The cell invasion was detected by Matrigel-coated transwell chamber (8-μm pore size, Millipore, Billerica, MA, USA) assay. After treatment, the cells were resuspended in a serum-free media (5×10^4 cells/well) and were added into the upper chambers. The medium containing 10% FBS was placed in the bottom chamber as a chemotaxin. After 24 h, the membrane was lightly washed with phosphate-buffered saline and fixed with 4% paraformaldehyde. The non-invasive cells on the upside of the membrane were removed and the invasive cells on the downside of the membrane were stained with Hematoxylin and Eosin (H&E, Beyotime). The images were captured by a Leica DMI 1 microscope (Leica, 100× magnification).

Western blotting

The collected cells were lysed using a lysis buffer (Beyotime). The lysates were centrifuged at 4 °C for 20 min at 12,000 g. The protein concentration was estimated by bicinchoninic acid protein assay kit (Thermo, Waltham, MA, USA). The total protein was separated by electrophoresis in sodium dodecyl sulfate-10% polyacrylamide gel and transferred on a polyvinylidene fluoride membrane (Millipore). After blocking with 5% non-fat milk for 1 h, the membrane was incubated with the appropriate primary antibodies overnight at 4 ºC and subsequently incubated with the secondary antibodies for 1.5 h at 37 ºC. The results were analyzed using the ChemiDoc XRS + system and Image Lab Software (Version 5.2, Bio-Rad).

Immunofluorescence microscopy

The coverslips were fixed with 4% paraformaldehyde (15 min), permeabilized with 0.2% Triton X-100 (15 min), and blocked with goat serum (1 h). Then coverslips were incubated with primary antibodies for α_V integrin (1:300), β_3 integrin (1:100), N-cadherin (1:100) and vimentin (1:400) overnight at 4 °C. The coverslips were subsequently incubated with FITC-conjugated secondary antibodies for 1 h and counterstained with 4,6'-diamidino-2- phenylindole dihydrochloride (DAPI, 1 μg/mL) for 40 min. The images were captured by using a DMi8 fluorescence microscope and Leica X software (Leica, 400×magnification).

Statistical analyses

All data are represented as the mean ± standard error of the mean (SEM), and the experiments were performed in

triplicates. One-way analysis of variance and Bonferroni post-hoc test was performed using GraphPad Prism 5. A *P*-value of <0.05 was considered statistically significant.

Results
Oxymatrine inhibited the viability of MDA-MB-231 and 4T1 cells

MTT assay was performed to evaluate the effects of oxymatrine on cell viability in breast cancer MDA-MB-231 and 4T1 cells, and normal breast mammary epithelial MCF-10A cells at different time points. Oxymatrine significantly suppressed the viability of MDA-MB-231 and 4T1 cells. The IC_{50} values of oxymatrine in MDA-MB-231 cells at 24, 48, and 72 h were 34.55 ± 1.48, 23.71 ± 0.31, and 12.45 ± 1.98 $\mu mol/mL$, respectively (Figure 1A). Additionally, the IC_{50} values of oxymatrine on 4T1 cells at 24, 48, and 72 h were 26.15 ± 0.99, 14.18 ± 1.93, and 10.88 ± 0.87 $\mu mol/mL$, respectively (Figure 1A). Oxymatrine showed less toxicity on MCF-10A cells with IC_{50} values for 24-, 48-, and 72-h treatments of 65.23 ± 1.22 $\mu mol/mL$, 58.20 ± 2.63 $\mu mol/mL$, and 50.96 ± 3.47 $\mu mol/mL$, respectively. In addition, the results demonstrated that treatment with oxymatrine at concentrations of 1.5, 3, and 6 $\mu mol/mL$ for 48 h had no obvious cytotoxicity in MDA-MB-231, 4T1 and MCF-10A cells (Figure 1B). Therefore, these non-cytotoxic concentrations of oxymatrine were used in the subsequent experiments.

Oxymatrine inhibited the migration and invasion of MDA-MB-231 and 4T1 cells

We next assessed the inhibitory activities of oxymatrine on cell migratory and invasive abilities by wound-healing assay and transwell chamber assay, respectively. The results showed that oxymatrine significantly inhibited the migratory abilities of MDA-MB-231 and 4T1 cells as compared to the control cells (Figure 2A). After treatment with oxymatrine at 1.5, 3, or 6 $\mu mol/mL$ for 48 h, the migration rate decreased to $91.8\pm5.6\%$, $74.6\pm10.6\%$, and $59.8\pm6.6\%$ in the MDA-MB-231 cells, and $70.9\pm8.1\%$, $58.4\pm6.2\%$ and $46.1\pm5.4\%$ in the 4T1 cells, respectively. As shown in Figure 2B, oxymatrine also dramatically decreased cell invasive ability. Treatment with 1.5, 3, and 6 $\mu mol/mL$ oxymatrine reduced the invasive cells to $82.9\pm6.8\%$, $47.4\pm4.5\%$, and $33.1\pm6.5\%$ in the MDA-MB-231 cells and $88.0\pm10.9\%$, $51.1\pm5.9\%$, and $19.7\pm4.9\%$ in the 4T1 cells, respectively.

Oxymatrine reversed the expression of EMT-related markers in MDA-MB-231 and 4T1 cells

We continued to examine whether oxymatrine affected the expression of EMT-related makers by Western blot and immunofluorescence microscopy. Oxymatrine significantly enhanced E-cadherin expression in 4T1 cells and reduced the expression of N-cadherin, vimentin, and Snail in MDA-MB-231 and 4T1 cells as compared to the control (Figure 3A). Similarly, immunofluorescence results also confirmed that N-cadherin and vimentin decreased in MDA-MB-231 cells after oxymatrine treatment (Figure 3B). In addition, the morphological changes of vimentin were also observed after oxymatrine treatment. The cells in the control group showed extended filamentous of vimentin, which was obviously decreased in the oxymatrine-treated group (Figure 3B).

Oxymatrine suppressed $\alpha_V\beta_3$ integrin expression and its downstream FAK/PI3k/Akt signaling activation

We explored the underlying molecular mechanisms of oxymatrine on EMT suppression. It was demonstrated that oxymatrine significantly inhibited the expression of α_V and β_3 integrin in MDA-MB-231 and 4T1 cells as compared to the control (Figure 4A). We also confirmed the influence of oxymatrine on the expression and co-localization of α_V (green) and β_3 (red) integrin in MDA-MB-231 cells using immunofluorescence staining. The yellow immunofluorescence represented co-localization of the two molecules. It was observed that oxymatrine reduced the α_V and β_3 integrin-related fluorescence intensity and decreased the co-localization of α_V and β_3 integrin in the cytoplasm (Figure 4B). In addition, increased $\alpha_V\beta_3$ integrin expression has been reported to stimulate FAK/PI3K/Akt signaling in breast cancer.[25] Thus, we continually estimated the effects of oxymatrine on the phosphorylation of FAK, PI3K, and Akt. It was demonstrated that oxymatrine significantly downregulated the phosphorylation of FAK (Tyr576) but did not change the total FAK expression in MDA-MB-231 cells (Figure 4C). Meanwhile, oxymatrine inhibited the phosphorylation of PI3K (p85α Tyr467) and Akt (Ser473) (Figure 4C). The total protein expression of PI3K and Akt also did not change after oxymatrine treatment.
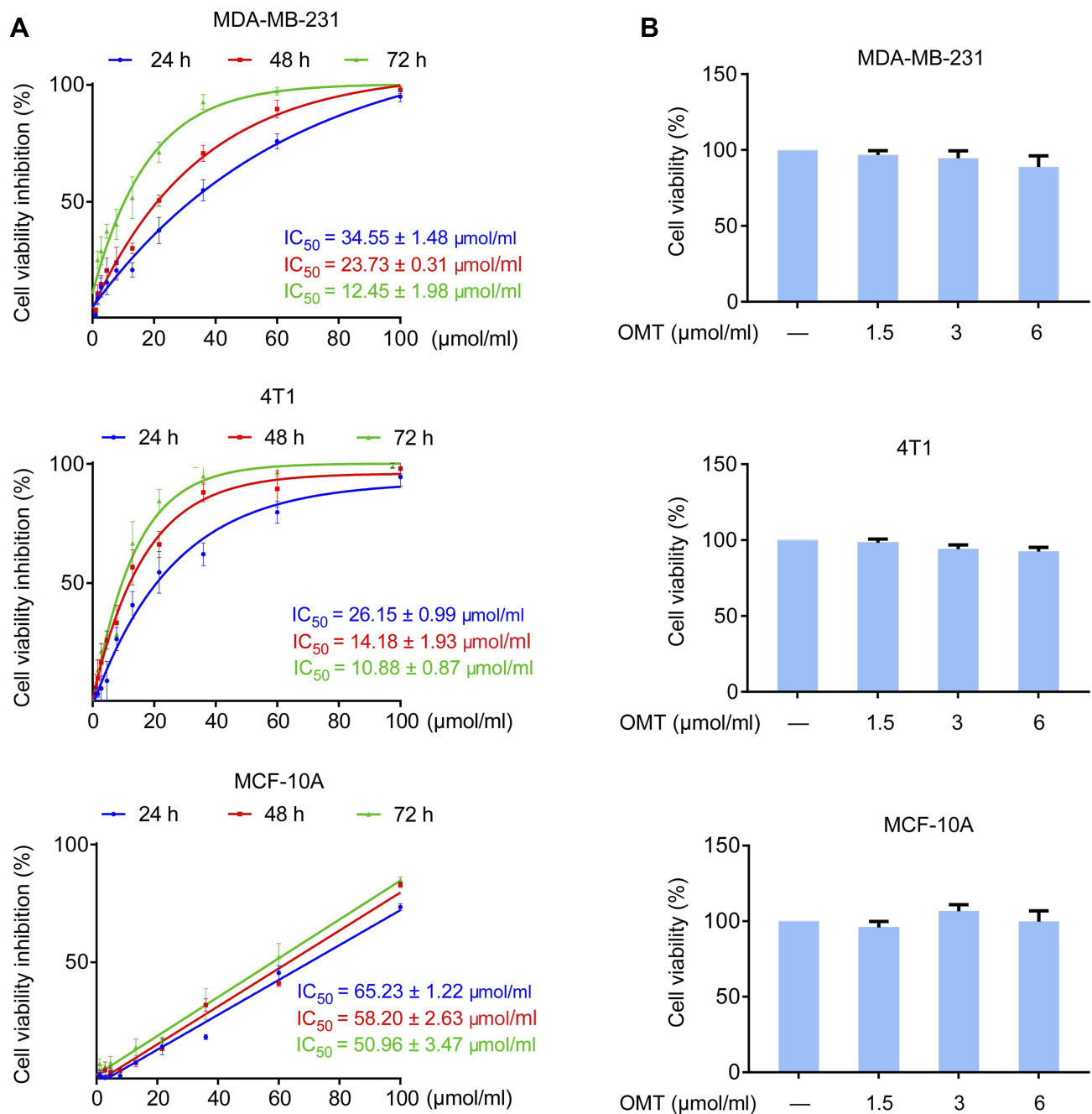
Figure 1 Effects of oxymatrine on the viability of MDA-MB-231, 4T1 and MCF-10A cells.
Notes: (**A**) Inhibition of cell viability and IC$_{50}$ values for oxymatrine in MDA-MB-231, 4T1 and MCF-10A cells. The cells were treated with various concentrations of oxymatrine (0–100 μmol/mL) for 24, 48, and 72 h. (**B**) The viability of MDA-MB-231, 4T1 and MCF-10A cells after 1.5, 3, and 6 μmol/mL oxymatrine treatment for 48 h. The values represent the mean ± SEM. The statistical data is provided from 3 independent experiments.
Abbreviation: OMT, oxymatrine.

Oxymatrine inhibited FN-enhanced α$_V$β$_3$ integrin/FAK/PI3K/Akt signaling activation

We used α$_V$β$_3$ integrin ligand FN and its specific antagonist cyclo-RGDfK to further investigate the inhibitory effects of oxymatrine on α$_V$β$_3$ integrin signaling. FN obviously augmented the expression of α$_V$ and β$_3$ integrin in MDA-MB-231 cells as compared to the control group (Figure 5A). However, oxymatrine significantly inhibited FN-promoted α$_V$ and β$_3$ integrin expression in a manner similar to cyclo-RGDfK. Meanwhile, FN
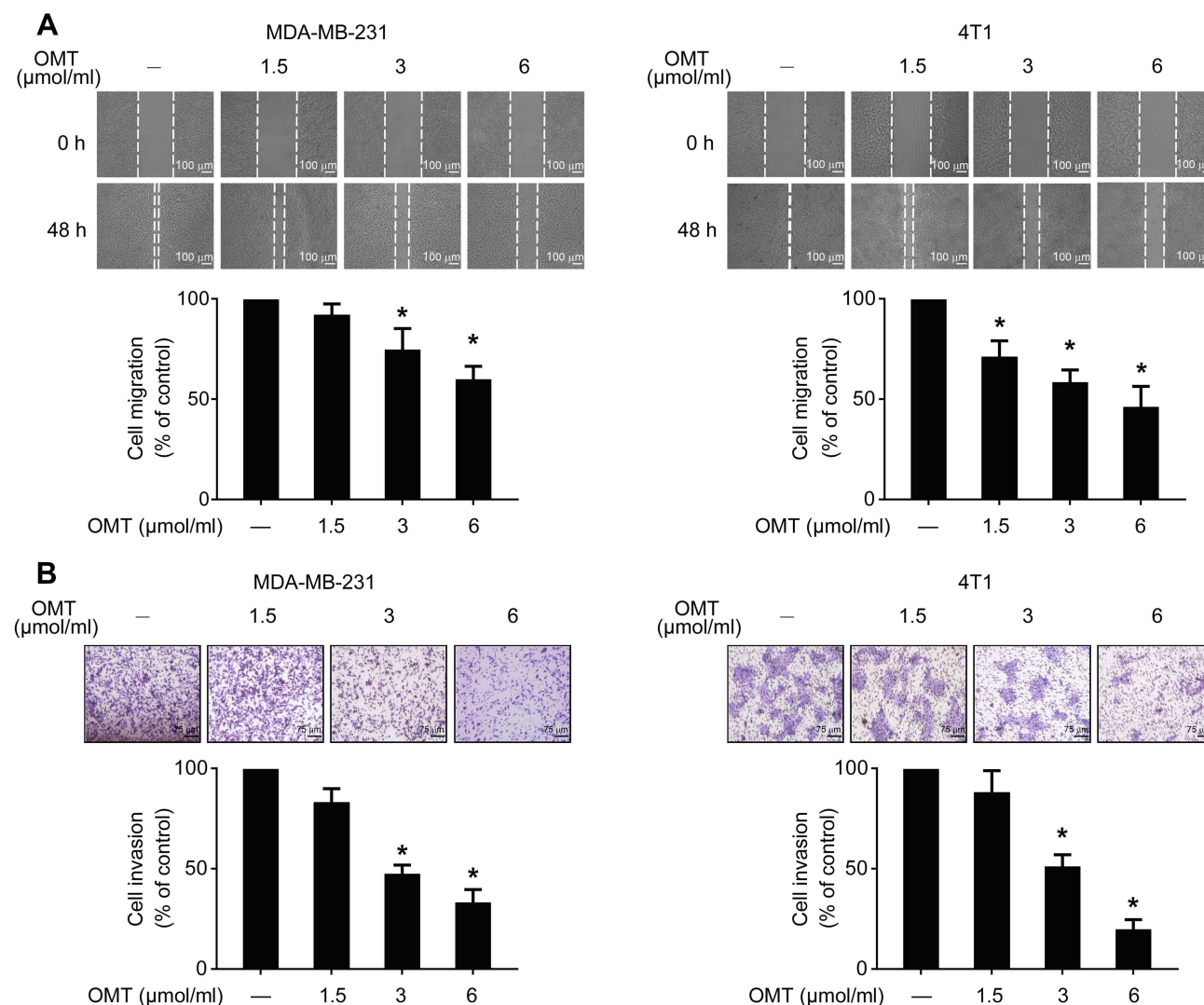
Figure 2 The effects of oxymatrine on cell migration and invasion.
Notes: (**A**) The cell migration was estimated using a wound healing assay. The images were captured at 0 and 48 h after wounding (magnification, 50×; scale bars, 100 μm). (**B**) The cell invasion was investigated using the Matrigel-coated transwell model (magnification, 100×; scale bars, 75 μm). The values represent the mean ± SEM, *P<0.05, as compared to the control group. The statistical data is provided from 3 independent experiments.
Abbreviation: OMT, oxymatrine.

evidently stimulated the downstream FAK/PI3K/Akt signaling activation accompanied by increased phosphory-lated FAK (Tyr576), PI3K p85α (Tyr467), and Akt (Ser473) (Figure 5B). Furthermore, pretreatment with oxymatrine and cyclo-RGDfK inhibited the phosphory-lation of FAK, PI3K, and Akt (Figure 5B). These results indicated that oxymatrine possesses inhibitory effects on $\alpha_V\beta_3$/FAK/PI3K/Akt signaling activation.

Oxymatrine inhibited FN-induced EMT in MDA-MB-231 cells

To further explore whether oxymatrine suppressed EMT via inhibition of $\alpha_V\beta_3$ integrin, we assessed the effects of

oxymatrine on FN-induced changes of EMT-related proteins, cellular migration, and invasion in MDA-MB-231 cells. Upon FN stimulation in MDA-MB-231 cells for 48 h, N-cadherin, vimentin and Snail were upregulated (Figure 6A). However, pretreatment with oxymatrine or cyclo-RGDfK significantly decreased the expression of N-cadherin, vimentin, and Snail as compared to the FN-treated group (Figure 6A). In addition, FN facilitated the migration and invasion of MDA-MB-231 cells, but oxy-matrine and cyclo-RGDfK pretreatment also depressed FN-enhanced cell migration and invasion (Figure 6B and C). It suggested that oxymatrine reversed EMT in breast cancer cells by suppressing $\alpha_V\beta_3$ integrin.
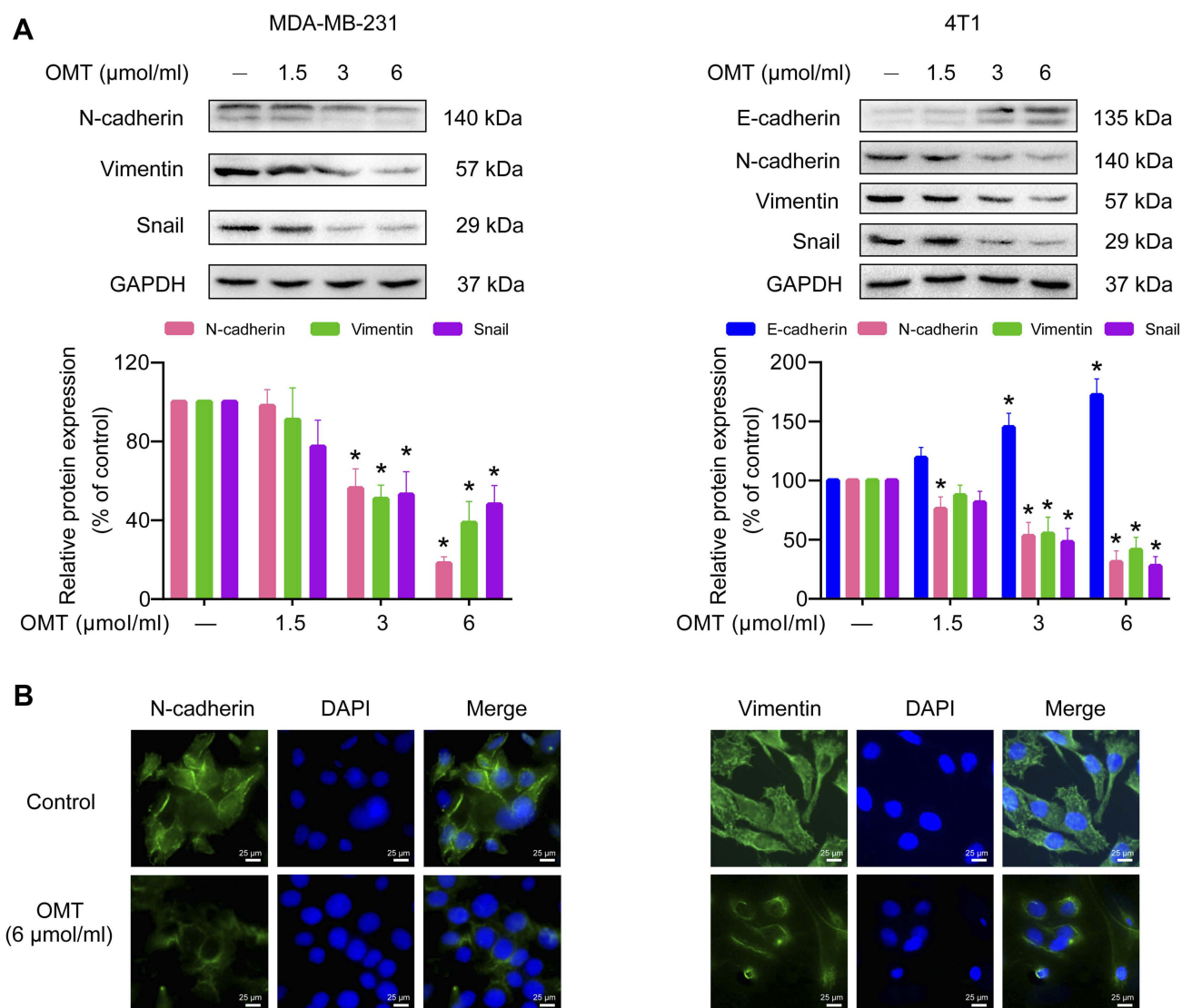
A

B

Figure 3 The effects of oxymatrine on the expression of EMT markers in MDA-MB-231 and 4T1 cells.

Notes: (**A**) The expression of E-cadherin, N-cadherin, vimentin, and Snail in MDA-MB-231 and 4T1 cells were detected by Western blot. Densitometric analysis of EMT-related protein expression normalized with GAPDH. The values represent the mean ± SEM. *$P<0.05$ as compared to the control group. (**B**) The expression and distribution of N-cadherin and vimentin in MDA-MB-231 cells were confirmed by immunofluorescence microscopy (magnification, 400×; scale bars, 25 μm). The statistical data is provided from 3 independent experiments.

Abbreviations: OMT, oxymatrine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

New generations of drugs targeting metastasis for the treatment of breast cancer are required.[26] Natural products are an important source of new drug.[27] Oxymatrine, a natural bioactive compound, has been approved as an anti-hepatitis B drug by the State Food and Drug Administration of China.[18] Oral administration of oxymatrine has demonstrated potential benefits and safety in chronic hepatitis B patients without causing serious adverse events.[28] Additionally, increasing pieces of evidence have confirmed that oxymatrine possesses anti-tumor efficacies against several types of cancer, including

breast cancer.[29] Drug repurposing, which deals with novel uses of existing drugs, is a practical and economical method to develop an anticancer drug.[30] Thus, evaluation of the anti-metastatic effects of oxymatrine may be an alternative promising strategy for inhibiting metastasis in breast cancer patients.

In our present study, we found that oxymatrine markedly reversed EMT in high invasive and metastatic breast cancer MDA-MB-231 and 4T1 cells. It significantly suppressed cell migration and invasion in the two cell lines. Beside increased migratory and invasive capacities, the EMT process is accomplished by loss of epithelial markers
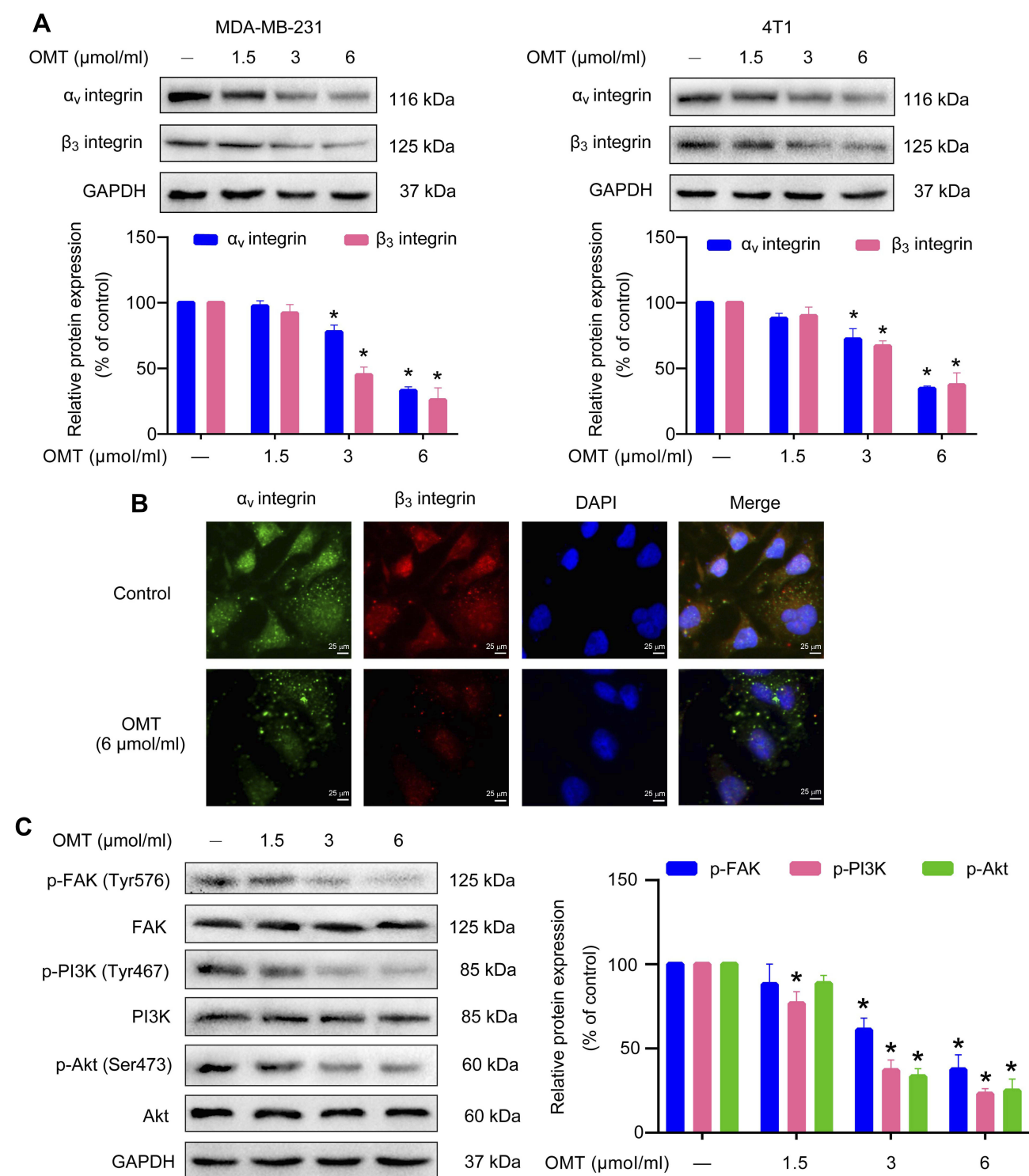
Figure 4 The inhibitory effects of oxymatrine on $\alpha_V\beta_3$ integrin/FAK/PI3K/Akt signaling.
Notes: MDA-MB-231 and 4T1 cells were treated with or without oxymatrine (1.5, 3 and 6 μmol/mL) for 48 h. (**A**) The expression of α_V and β_3 integrin was analyzed by Western blot. (**B**) The expression and co-localization of α_V and β integrin were evaluated by immunofluorescence microscopy (magnification, 400×; scale bars, 25 μm). (**C**) The expression of phosphorylated and total FAK, PI3K, and Akt were detected by Western blot. The values represent the mean ± SEM. *P<0.05 compared with the control group. The statistical data is provided from 3 independent experiments.
Abbreviations: OMT, oxymatrine; FAK, focal adhesion kinase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
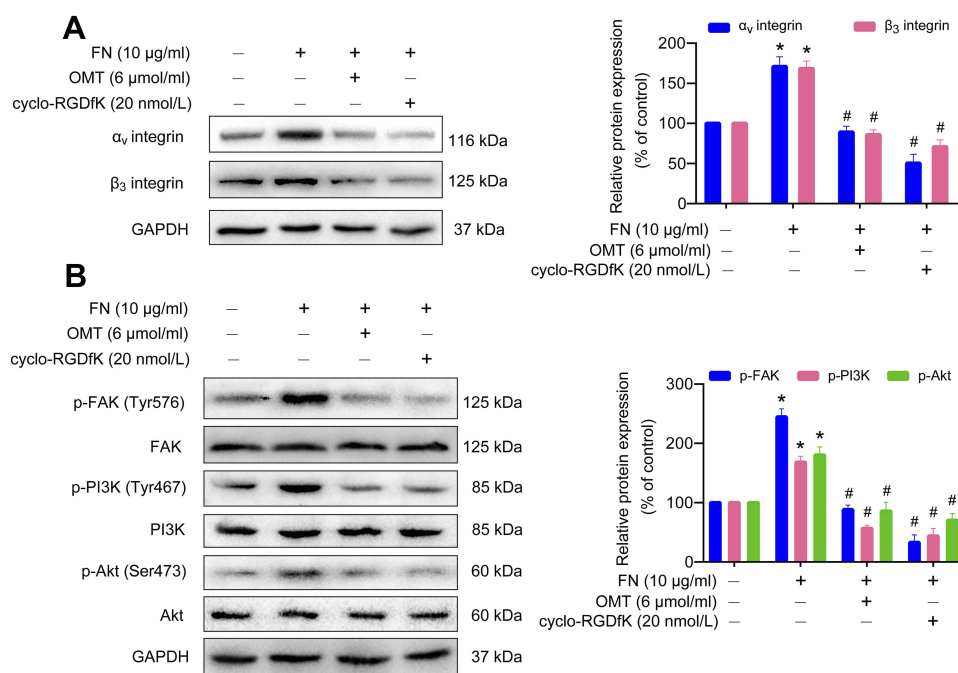
Figure 5 The effects of oxymatrine on FN-induced $\alpha_V\beta_3$ integrin up-regulation and FAK/PI3K/Akt signaling activation in MDA-MB-231 cells.
Notes: The cells were pretreated with oxymatrine and cyclo-RGDfK for 1 h, and then stimulated with FN for 48 h (serum-free condition). (**A**) The expression of α_V and β_3 integrin was analyzed by Western blot. (**B**) The expression of phosphorylated and total FAK, PI3K, and Akt was detected by Western blot. The data are shown as the mean ± SEM. *$P<0.05$ as compared to the control group, #$P<0.05$ as compared to the FN group. The statistical data is provided from 3 independent experiments.
Abbreviations: OMT, oxymatrine; FN, fibronectin; FAK, focal adhesion kinase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

as well as the elevation of mesenchymal markers and EMT-activating transcription factors.[31] The epithelial marker E-cadherin is a critical cell adhesion molecule that maintains the integrity of the epithelial cells and cell junction and inhibits cancer cell movement.[32] High expression of N-cadherin and vimentin contributes to directly enhancing the invasiveness and metastatic phenotype of cancer cells.[33] Snail, one of EMT-inducing transcription factor, is activated early in EMT,[34] and is known to activate the transcription of genes contributing to the mesenchymal phenotype.[35] Here, oxymatrine was found to reduce the expression of N-cadherin, vimentin, and Snail in MDA-MB-231 and 4T1 cells, and accelerate E-cadherin expression in 4T1 cells. These results suggested that oxymatrine might be a promising candidate to inhibit breast cancer metastasis by reversing EMT.

The integrins are presented as attractive therapeutic targets of drug designing for cancer metastasis. In recent decades, several integrin-based antibodies or antagonists are currently tested in clinical trials. Although some anti-integrin agents did not demonstrate any efficacy, others such as MK-0429 ($\alpha_V\beta_3$ antagonist) or GLPG0187 (multi-integrin antagonist) are still under clinical investigation and showed

promising results for the treatment of metastatic prostate cancer.[36] In addition, preclinical investigations have revealed that integrin $\alpha_V\beta_3$ antagonists (cilengitide) or antibody (CNTO 95) significantly reduced the metastatic tumor burden in the bone, brain, or lungs of rats bearing highly metastatic breast cancer cell xenografts.[37–39] It was indicated that breast cancer patients with metastatic risk might benefit from anti-integrin $\alpha_V\beta_3$ treatment. Our results demonstrated that oxymatrine significantly decreases the expression of α_V and β_3 integrin. It also suppressed the co-localization of α_V and β_3 integrin in the cytoplasm, which indicates that the frequency of their combination and formation of heterodimer were reduced. FN, a major extracellular matrix glycoprotein, stimulates biochemical and mechanical signaling though integrin.[40] It contains an arginine-glycine-aspartate (RGD) motif, which can be attached by $\alpha_V\beta_3$ integrin.[41] Thus, we used FN as a stimulator to confirm the role of $\alpha_V\beta3$ integrin on the oxymatrine-induced reversal of EMT. The results demonstrated that oxymatrine, similar to an $\alpha_V\beta_3$ integrin antagonist cyclo-RGDfK, suppressed FN-enhanced αv and β_3 integrin expression. Oxymatrine also decreased FN-induced cell migration, invasion, and EMT marker changes. Thus,
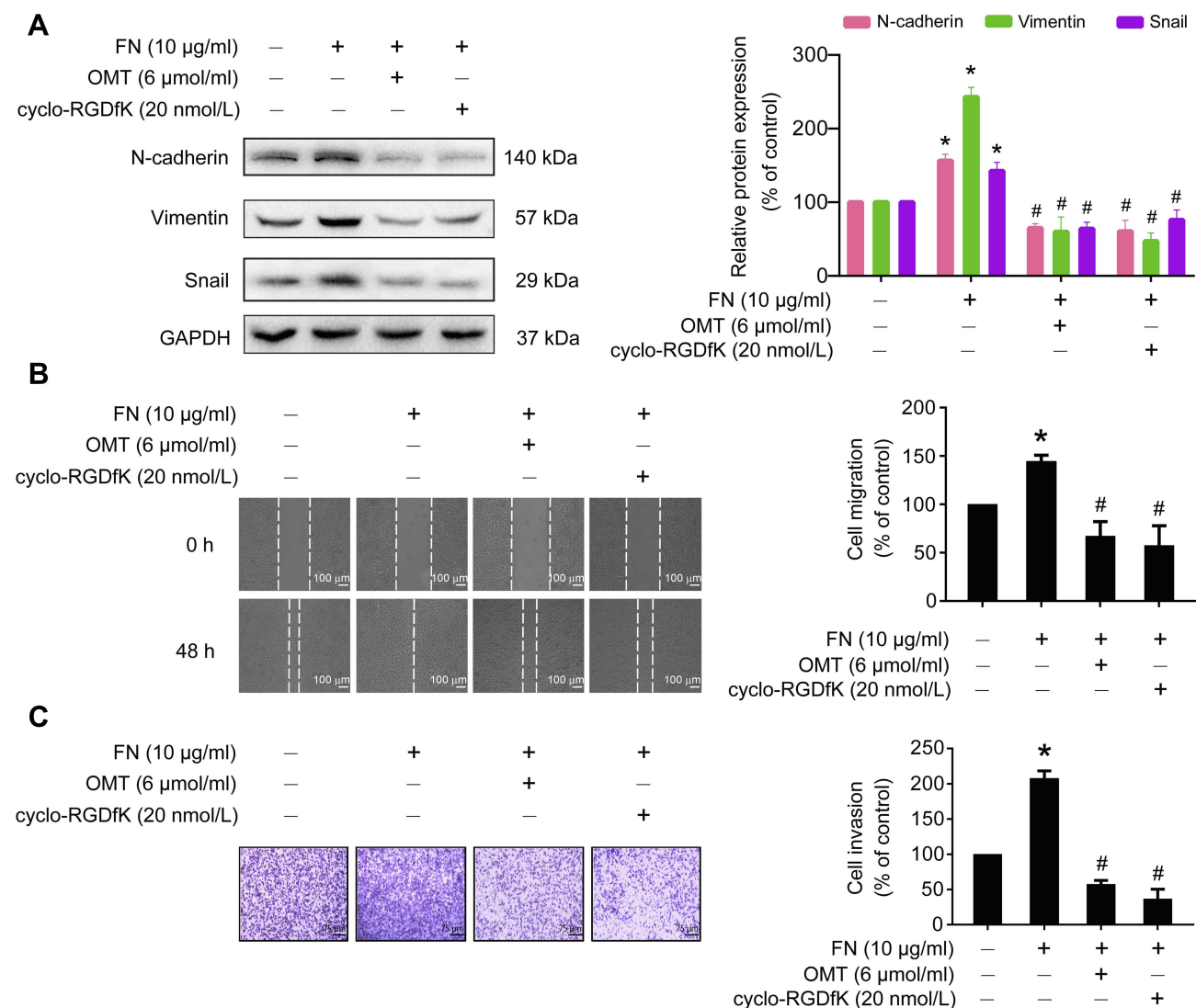
Figure 6 The effects of oxymatrine on FN-induced cell migration, invasion, and EMT-related protein expression.
Notes: The cells were pretreated with oxymatrine and cyclo-RGDfK for 1 h, and then stimulated with FN for 48 h (serum-free condition). (**A**) The effects of oxymatrine on FN-induced EMT-related protein expression. (**B**) The effects of oxymatrine on FN-induced cell migration by wound healing assay. The images were captured at 0 and 48 h after wounding (magnification, 50×, scale bars, 100 μm). (**C**) The effects of oxymatrine on FN-induced cell invasion by Matrigel-coated transwell assay. The images were captured by an inverted microscope (magnification, 100×, scale bars, 75 μm). The values represent the mean ± SEM. *$P<0.05$ compared with the control group; #$P<0.05$ compared with the FN group. The statistical data is provided from 3 independent experiments.
Abbreviations: OMT, oxymatrine; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

these results indicated that oxymatrine reversed EMT features of breast cancer via $\alpha_V\beta_3$ integrin suppression.

FAK is a main component of adhesion complex and acts as a central downstream effector of integrin signaling transduction which influences multiple cellular processes, such as survival, migration, invasion, and tissue remodeling.[42] Most integrins activate FAK through inducing its autophosphorylation at Tyr397 that produces a binding site for Src kinase. Following this, FAK is phosphorylated at Tyr576 and Tyr577 by Src, which displays the maximal catalytic activity.[9] As a non-receptor tyrosine kinase, activated FAK stimulates the downstream kinase PI3K by binding and phosphorylating the

p85α subunit of PI3K (regulatory subunit of PI3K) and subsequently activates Akt via phosphorylation.[43] Moreover, the FAK and PI3K/Akt are the common pathways involved in facilitating tumor cell EMT and metastasis.[44] Recent investigations have also confirmed that FAK/PI3K/Akt is the downstream signaling pathway for $\alpha_V\beta_3$ integrin-mediated breast cancer cell EMT, which is induced by 14,15-epoxyeicosatrienoic acid.[45] Here, we have found that oxymatrine markedly depressed the phosphorylation of FAK (Try576), PI3K p85α (Tyr467), and Akt (Ser473) in MDA-MB-231 breast cancer cells. Furthermore, oxymatrine also blocked FN-induced activation of FAK, PI3K, and Akt

similar to the actions of cyclo-RGDfK. These results demonstrated that oxymatrine exerts inhibitory effects on the $\alpha v\beta_3$ integrin/FAK/PI3K/Akt signaling activation.

Limitations

There are some further issues that need to be addressed by future investigations. It remains to be evaluated whether oxymatrine could suppress the metastases of breast cancer in vivo. It is also required to explore how oxymatrine affects $\alpha_V\beta_3$ integrin and whether oxymatrine interrupts its conformational rearrangements or binding with paxillin and talin. Moreover, the expression of $\alpha_V\beta_3$ integrin is normally found in the activated tumor endothelial cells, but not in the resting endothelial cells, and hence, it is required for forcing tumor cells to form new vessels under vascular endothelial growth factor stimulation.[46] Thus, $\alpha_V\beta_3$ integrin is considered an appropriate therapeutic target for the treatment of tumor angiogenesis, which is an essential step in cancer metastasis. It is possible that oxymatrine exerts inhibitory effects on angiogenesis of breast cancer, although further investigations are required in this regard.

Conclusion

In view of our results, oxymatrine effectively reversed EMT in breast cancer cells through inhibition of $\alpha_V\beta_3$ integrin signaling. This provides an important basis to further explore the potential mechanisms and beneficial effects of oxymatrine for the prevention of breast cancer metastasis.

Author contributions

All authors contributed to data analysis, drafting and revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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