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Valproic acid inhibits prostate cancer cell migration by up-regulating E-cadherin expression

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E-Cadherin plays important roles in cell-cell adhesion, epithelial-to-mesenchymal transition, cancer cell migration and invasion. Valproic acid (VPA), a well-known inhibitor of class I and class II histone deacetylases, has been considered a promising anticancer drug due to its capacity of inducing cancer cell proliferation arrest and death through different mechanisms. However, effects of VPA on E-cadherin mediated cell-cell adhesion and cancer cell migration remain unclear. In the present study, we found that VPA potently induced hyperacetylation of histone H3 and H4, increased the expression of E-cadherin and inhibited cell migration in prostate cancer cells. Furthermore, knock-down of E-cadherin significantly restored the effects of VPA on cell migration, while over-expression of E-cadherin in prostate cancer cells significantly inhibited cell migration to a similar level as VPA treatment. These results thus suggest that up-regulation of E-cadherin and inhibition of cell migration may represent a new anticancer mechanism of VPA.

1. Introduction

Prostate cancer is the second leading cause of cancer-related mortality in men in the United States (Keller et al. 2001). To identify and characterize new regulators and targets in prostate cancer is especially useful for the treatment of aggressive prostate cancer. Recent studies found that cells in the center of a prostate cancer tissue maintain an epithelial phenotype, whereas cells at the invasive front show a mesenchymal phenotype (Luo et al. 2006). This indicates the epithelial-to-mesenchymal transition (EMT) may play a critical role in the development and treatment of prostate cancer. Mounting evidence indicates that during carcinogenesis, developing prostate cancer cells acquire mesenchymal characteristics and migratory features such as increased expression of Snail, Slug, Twist, and N-Cadherin, concomitant with a loss of epithelial characteristics such as E-cadherin expression in response to cancer-related microenvironment (Richmond et al. 1997; Dunsmuir et al. 2000; Guaita et al. 2002; Vincent-Salomon and Theiry 2003; Kwok et al. 2005; Eger et al. 2005; Bindels et al. 2006).

The conversion of an epithelial cell into a mesenchymal cell accompanies a series of alterations in morphology, cellular architecture, adhesion, and migration (Vincent-Salomon and Theiry 2003; Theiry 2002). As a calcium-dependent intercellular adhesion molecule, E-cadherin (CDH1) plays a pivotal role in establishing cell polarity, maintaining epithelial integrity and cellular differentiation, and serves as a tumor invasion suppressor (Bussemakers et al. 1993; Wijnhoven and Pignatelli 1999). Low expression of E-cadherin may disrupt the E-cadherin-catenin complex and inactivate the E-cadherin-mediated invasion suppressor system, leading to a loss of cell adhesion, dedifferentiation and metastasis of cells during development of several invasive and metastatic carcinomas (Birchmeier and Gilles 1994; Hirohashi 1998; Christofori and Semb 1999). On the contrary, restored E-cadherin expression in cancer cells can reverse their invasive phenotype (Frixen et al. 1991; Vleminckx et al. 1991). Regulation of E-cadherin is then considered an important suppressor of invasion and metastasis in various carcinomas. However, how to regulate the expression of E-cadherin *in vivo* and its role in prostate cancer cells need to be further studied.

Acetylation and deacetylation of nucleosomal core histones are important for the modulation of chromatin structure and regulation of gene expression. Up-regulation of histone deacetylases (HDACs) may result in aberrantly low histone acetylation and abnormal gene expression critically involved in cancer development (Cress and Seto 2000; Johnstone 2002). HDAC inhibitors including VPA have been proven to efficiently induce proliferation arrest, differentiation, and/or apoptosis of several cancer cells through regulation of $p21^{Waf1}$, $p27^{Kip7}$, Bcl-2, cyclin-D1, cyclin-D2, etc (Johnstone 2002; Marks et al. 2001; Bolden et al. 2006; Takai et al. 2004a, b). Knock-down of HDAC3 expression significantly suppressed ovarian carcinoma cell migration via increasing E-cadherin expression, suggesting that histone acetylation plays important roles in E-cadherin expression and E-cadherin-mediated cell adhesion and migration (Hayashi et al. 2010).

In the present study, we found that the HDAC inhibitor VPA potently up-regulated E-cadherin expression and inhibited E-cadherin-mediated migration in invasive prostate cancer cells RM-1, suggesting that VPA may be useful to increase the expression of E-cadherin and inhibit prostate cancer cell migration.

2. Investigations and results

2.1. Effects of VPA on prostate cancer cell migration

To examine effects of VPA on the migration capability of prostate carcinoma cells, a wound-healing assay was performed

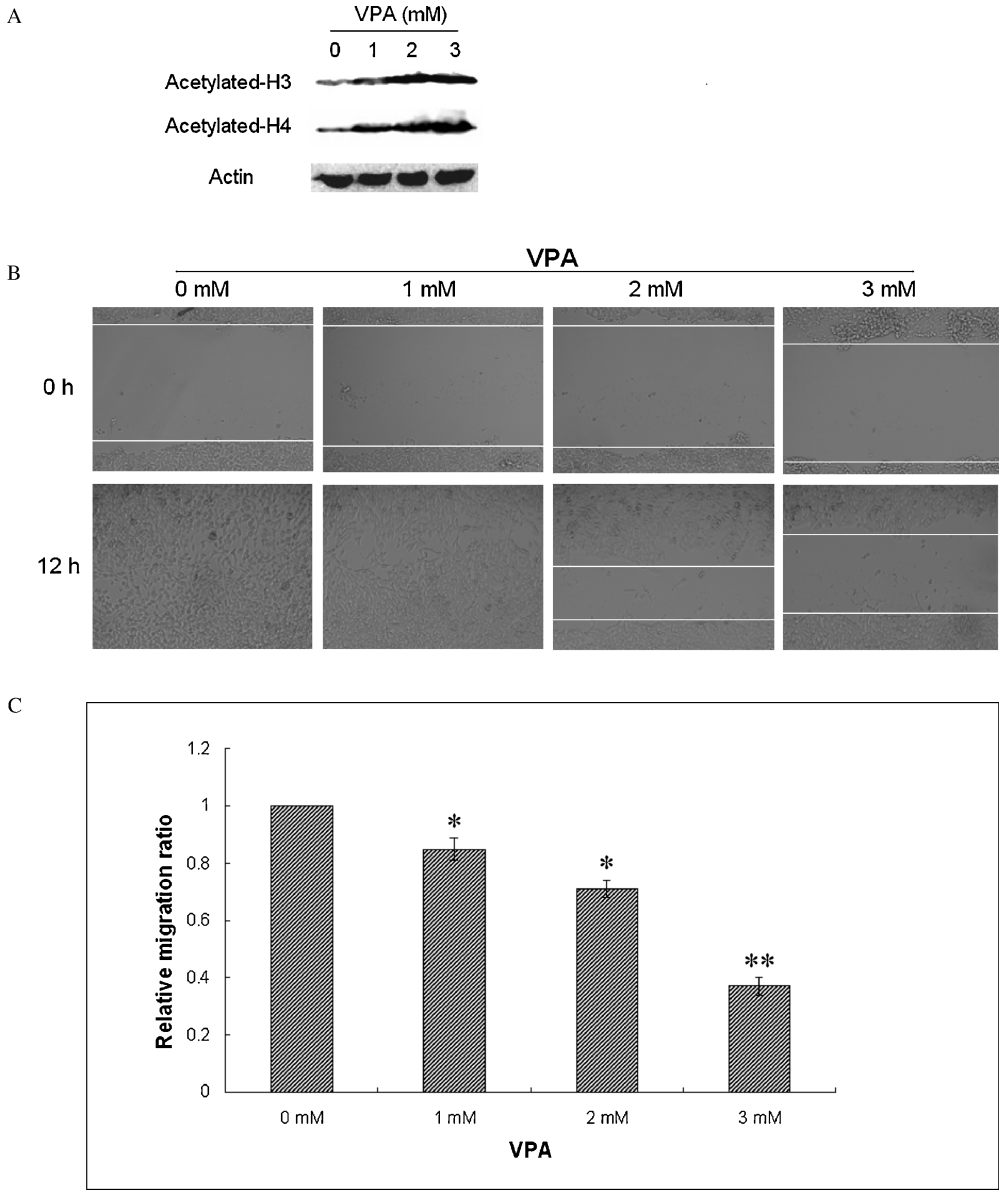

Fig. 1: Inhibition of VPA on prostate cancer cell migration. (A) Western blotting analysis was performed with the primary antibodies for mouse acetylated histone H3 and acetylated histone H4. The amount of protein was normalized by comparison with the level of beta-Actin. (B) After treated with 1 mM, 2 mM, and 3 mM of VPA for 24 h, monolayer of cells was wound-scratched. The width at 0 h and 12 h after scratching was measured by taking photos. (C) Statistically analyses of the migration ability of cells treated with different concentrations of VPA as shown in (B). $*P < 0.05$, $**P < 0.01$, vs the group of cells without VPA treatment

using RM-1 cells treated with 0 mM, 1 mM, 2 mM and 3 mM of VPA for 24 h. As shown in Fig. 1A, the global acetylation level of histone H3 and H4 was obviously increased after treatment with VPA, indicating that the used concentrations of VPA are efficient. Further results showed that VPA significantly suppressed the migration ability of RM-1 cells in a dose-dependent manner (Fig. 1B and 1C). Especially, when the cells were treated with 3 mM of VPA, their migration ability was suppressed by approximately 70%.

2.2. Effects of VPA on prostate cancer cell proliferation

To test whether the inhibition of VPA on prostate cancer cell migration resulted from its inhibition on cell proliferation, a MTT assay was performed in RM-1 cells. The results showed that exposure of RM-1 cells to VPA at concentrations from 1 mM to 3 mM for 24 h did not significantly inhibit RM-1 cell proliferation (Fig. 2), confirming the inhibitory effect of VPA on the migration of prostate cancer cells.

2.3. Induction of VPA on E-cadherin expression

To explore the inhibition mechanism of VPA on prostate cancer cell migration, expression of E-cadherin and nm23,a well-known cell-cell adhesion molecule and cell metastasis suppressor gene were investigated by using quantitative real-time RT-PCR. Results showed that VPA could significantly promote E-cadherin expression in cells treated with a concentration of 2 mM and 3 mM; no statistically significant effect on the expression of nm23 in prostate cancer cells was seen (Fig. 3).

2.4. Over-expression of E-cadherin inhibits prostate cancer cell migration, while knock-down of E-cadherin restores VPA-induced prostate cancer cell migration

To investigate the relationship of VPA-induced E-cadherin expression and cell migration, E-cadherin gene and the siRNA against E-cadherin were expressed in RM-1 cells, and the efficiency of the over-expression and knock-down vectors was confirmed by quantitative RT-PCR analysis (Fig. 4A).

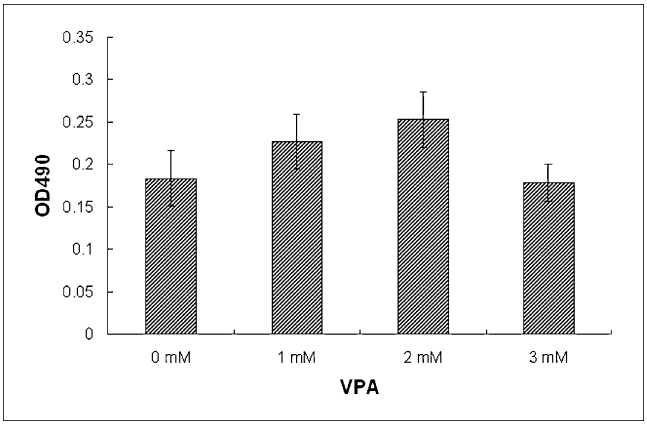

Fig. 2: Effects of VPA on prostate cancer cell proliferation. After treated with 1 mM, 2 mM, and 3 mM of VPA, cell proliferation ability was detected by the MTT assay

As shown in Fig. 4B, over-expression of E-cadherin obviously inhibited the wound-healing ability of RM-1 cells by about 90%. Although not significant, the inhibition effect of E-cadherin over-expression was slightly stronger than 3 mM of VPA treatment (Fig. 4C). Furthermore, knock-down of E-cadherin significantly attenuated the inhibitory effects of 3 mM of VPA on prostate cancer cell migration. Collectively, these data proved that the inhibition effects of VPA on prostate cancer cell migration at least partially resulted from its up-regulation on E-cadherin expression.

3. Discussion

Aberrant expression and/or activation of HDACs may result in histone hypoacetylation, which has been found to be involved in the development of various cancers including invasive prostate cancer. VPA, a well-known HDAC inhibitor, has been used in the treatment of epilepsy for almost 30 years and also shows promising anticancer effects in both solid and hematologic malignancies through inducing cancer cell proliferation arrest, differentiation, and apoptosis (Johnstone 2002; Marks et al. 2001; Bolden et al. 2006; Takai et al. 2004a, b). However, the effect and mechanism of VPA on E-cadherin mediated cell-cell adhesion, EMT transition, and cell migration in prostate cancer remain unclear. In the present study, we found that E-cadherin plays an important role in migration of prostate cancer cells, while VPA can inhibit migration of invasive prostate cancer RM-1 cells through up-regulation of E-cadherin expression.

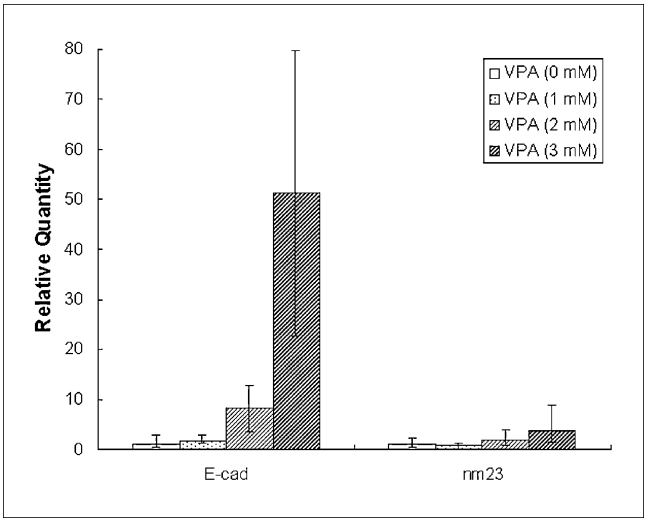

Fig. 3: Up-regulation of VPA on E-cadherin expression. After treatment with 1 mM, 2 mM, and 3 mM of VPA, the mRNA levels of E-cadherin (E-cad) and nm23 in each group of cells was examined by quantitative RT-PCR analysis, with the beta-actin gene as the internal control. $*P < 0.05$, $**P < 0.01$, vs the group of cells without VPA treatment

The process of cancer invasion and metastasis is controlled by epithelial-mesenchymal transition (EMT), through which epithelial cells lose their epithelial traits and acquire many of the attributes of mesenchymal cells, including the loss of association with epithelial cell sheets and the acquisition of cell motility and invasiveness (Scheel et al. 2007). During carcinogenesis, developing prostate cancer cells acquire mesenchymal characteristics and migratory features concomitant with a loss of epithelial characteristics such as E-cadherin expression in response to cancer-related microenvironment. E-Cadherin is the marker molecule of epithelial phenotype and down-regulation of E-cadherin is one of the most frequently reported phenomena in metastatic cancers. Moreover, loss of E-cadherin can initiate the EMT process (Christofori and Semb 1999). Thus, up-regulation of E-cadherin may effectively attenuate the first step of cancer cells metastasis. In our study, over-expression of E-cadherin itself significantly inhibited RM-1 cell migration, while knock-down of E-cadherin expression significantly attenuated VPA-caused prostate cell migration arrest. These data suggest that E-cadherin may serve as a useful target to treat invasive prostate cancer.

Previous studies showed that VPA treatment mainly exerts its anti-cancer activity through inducing cancer cell proliferation arrest, differentiation, and apoptosis (Johnstone 2002; Marks et al. 2001; Bolden et al. 2006). However, consistent with a previous study (Xia et al. 2006), VPA showed no inhibition effect on the proliferation of androgen-independent invasive prostate cancer cell line RM-1 in our study. Interestingly, VPA treatment significantly up-regulated E-cadherin expression and hence led to prostate cancer cell migration arrest in RM-1 cells. Although the detailed mechanism of VPA-induced E-cadherin expression remains to be further investigated, up-regulation of E-cadherin may represent an important mechanism of VPA to combat invasive cancer cells, such as androgen-independent invasive prostate cancer cells.

Metastatic prostate cancer, including bone metastasis and lymph node metastasis, refers to prostate cancer that has left the prostate gland and its neighboring organs and has bad prognosis. Therefore, up-regulation of E-cadherin to inhibit cancer cell metastasis at the initial step is a very important aspect for cancer prevention. Besides the properties of inhibiting proliferation and inducing apoptosis of cancer cells, our results prove that up-regulation of E-cadherin is one unrevealed mechanism of VPA in combating cancer, and suggest that VPA may be a potential agent for treatment of metastatic cancer.

In conclusion, VPA potently induced hyperacetylation of histone H3 and H4, increased the expression of E-cadherin and inhibited cell migration in prostate cancer cells. Furthermore, knock-down of E-cadherin significantly restored the effects of VPA on cell migration, while over-expression of E-cadherin in prostate cancer cells significantly inhibited cell migration to a similar level as VPA treatment. These results confirm the suppression effects of VPA on prostate cancer cell migration by up-regulating E-cadherin expression, representing a new anti-cancer mechanism of VPA in prostate cancer cells.

4. Experimental

4.1. Cell culture and plasmids

Murine RM-1 prostate cancer cells (C57Bl/6 background) (Baley and others 1995), purchased from Cell Bank Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, Shanghai, China), were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone,

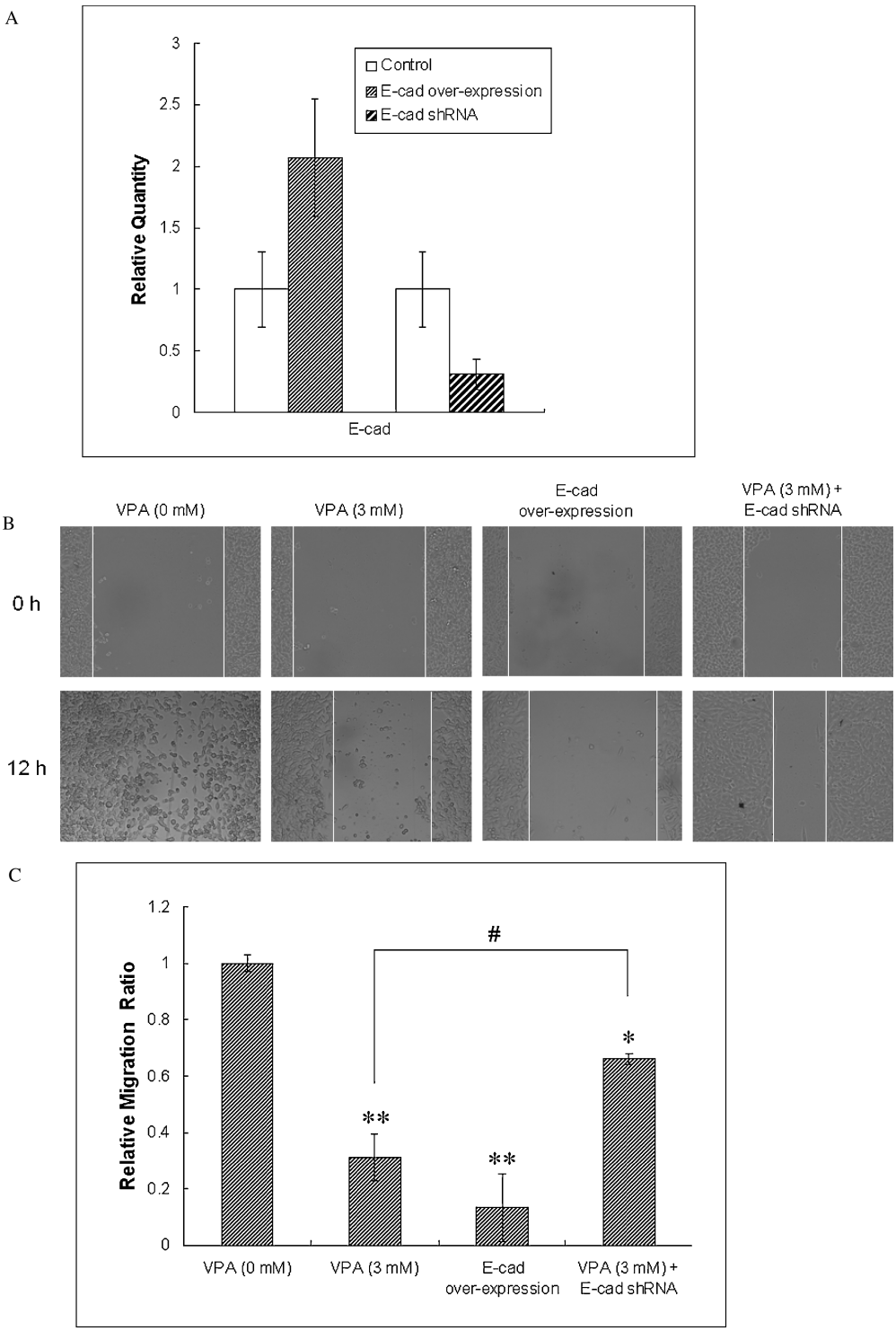

Fig. 4: Involvement of E-cadherin in VPA-induced cell migration inhibition. (A) The efficiency of the over-expression and knock-down vectors for E-cadherin (E-cad) was confirmed by the quantitative RT-PCR analysis. $^{*}P < 0.05$ vs the control group. (B) Cells transiently transfected with the over-expression and knock-down vectors of E-cadherin for 48 h were treated with 0 mM or 3 mM of VPA for 24 h, and wound-healing analysis was performed at 0 h and 12 h. (C) Statistically analyses of the migration ability of different groups of cells as shown in (B). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the group of cells without VPA treatment. $^{\#}P < 0.05$ vs cells treated with 3 mM of VPA

UT), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco), and grown in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2. The plasmid vectors for over-expression and knock-down of E-cadherin, pMx-Ecad and pMko.1-Ecad-shRNA were provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS.

4.2. Western blotting

Cells were lysed in 1 × lysis buffer that was diluted from 5× lysis buffer (0.5 mol/L Tris-HCl (pH6.8) 2.5 ml, DTT 0.39 g, SDS 0.5 g, bromophenol blue 0.025 g, glycerine 2.5 ml) and kept on ice for 10 min. An equal amount of protein was loaded onto 10% SDS-PAGE for electrophoresis and then transferred onto a nitrocellulose membrane. After blocking for 1 h at room temperature, the membrane was then incubated with primary antibodies overnight at 4 °C against mouse acetylated histone H3, acetylated histone H4, and the internal control beta-actin (Upstate, with dilution 1:3000). After incubation with appropriate secondary antibodies, signals were visualized by enhanced chemiluminescence (ECL).

4.3. Wound-healing assay

Cells (3×10^5) were seeded into 6-well culture plates and allowed to grow to 90–95% confluence. Similar sized wounds were introduced to monolayer cells using a sterile yellow pipette tip. Wounded monolayer cells were

washed three times by PBS to remove cell debris and then cultured with or without VPA. The speed of wound closure was monitored and photographed every 12 h using a phase contrast microscope until complete wound closure was observed in the untreated control.

4.4. Cell proliferation assay

Cell proliferation ability was assessed by a methyl-thiazol tetrazolium (MTT) assay. Exponentially growing cells were inoculated into 96-well plates with 1×10^4 cells per well, and after treatement with 0, 1, 2, 3 mM of VPA for 24 h, 20 µl of sterile MTT [5 mg/ml, Sigma-Aldrich Corp.] were added to each well. Following a further incubation at 37 °C for 4 h, the reaction was stopped by adding 150 µl of dimethyl sulfoxide (DMSO). After thoroughly mixed for 10 min, the formazan production was determined by measurement of the spectrometric absorbance at 490 nm on an enzyme immunoassay analyzer FlexStation 3^{TM} (Molecular Devices, Sunnyvale, CA, USA).

4.5. Quantitative real-time PCR

Total RNA from each group of cells was extracted with TRNzol-A$^+$ Reagent (TIANGEN, China). The first strand cDNA was synthesized using TIANScript Reverse Transcriptase Kit (TIANGEN) and Oligo(dT)$_{12–18}$ primer from 2 µg of total RNA, according to the manufacture's instruction. The primer sequences for the target *E-cadherin* gene were 5'-GCAGTCAGATCTCCCTGAGTTCGAG-3'(forward) and 5'-CTACATACAAAGGTCACTCTAGCAAC-3' (reverse); and for the internal gene *beta-actin* were 5'-GGCTGTATTCCCCTCCATCG-3' (forward) and 5'-CCAGTTGGTAACAATGCCATGT-3' (reverse). The PCR amplification were performed for 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 30 s for E-cadherin and actin. Real-time quantitative RT-PCR was performed on a Stratagene Mx3000P (Stratagene, La Jolla, CA) with 1.0 µl of cDNA and SYBR Green Real-time PCR Master Mix (TIANGEN). At the completion of cycling, melting curve analysis was performed to establish the specificity of the PCR product. Data was collected and stored in Excel format, and analyzed by use of the Mx3000P Software version 4.0 (Stratagene). The expression level of cDNA of each candidate gene was internally normalized using *beta-actin*. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), representing the amount of candidate gene expression with the same calibrators. Each experiment was performed in duplicates and repeated three times.

4.6. Transfection

Cells were transiently transfected with the pMx-Ecad and pMko.1-Ecad-shRNA vectors respectively using FuGENE® HD Transfection Reagent (Roche). After transfection for 48 h, cells were harvested, and the efficiency of gene silencing was checked by the quantitative real-time RT-PCR analysis as mentioned above. Also, after transfected with the pMx-Ecad and pMko.1-Ecad-shRNA vectors for 48 h, cell migration ability was investigated by the wound-healing assay, in parallel with treatment with 3 mM of VPA.

4.7. Statistical analysis

The results were analyzed by two-tailed student t-test SD using SPSS 11.0 (Aspire Software International, Leesburg, VA) and P-values were calculated. The difference was considered significant between two samples if $P < 0.05$.

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