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A novel anticancer effect of butein: Inhibition of invasion through the ERK1/2 and NF-κB signaling pathways in bladder cancer cells

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Abstract There is increasing evidence that epithelial–mesenchymal transition (EMT) plays a critical role in cancer metastasis. Butein is a polyphenolic compound, which has been found to exhibit anti-proliferation effects on cancer cells. Here, we report that in addition to its function as an anti-proliferation agent, butein can inhibit migration and invasion through the ERK1/2 and NF-κB signaling pathways in human bladder cancer cells, and this inhibitory effect may be associated with reversal of EMT. These results were further confirmed by RNAi-mediated suppression of NF-κB, which partly reverses EMT and inhibits cell invasive ability in vitro. These results suggest a novel function of butein as an invasion inhibitor in bladder cancer.
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Keywords: Butein; NF-κB; Invasion; Epithelial–mesenchymal transition (EMT); Bladder cancer

1. Introduction

Cancer metastasis, rather than primary tumors, is responsible for most cancer deaths [1]. Epithelial–mesenchymal transition (EMT) was originally recognized as an important event of embryogenesis and was found to participate in mesoderm and neural crest formation. Recently, accumulating evidence suggested that EMT is also reactivated and plays a critical role in the metastasis process of human cancers [2,3]. Therefore, strategies that regulate EMT attract increasing attention.

3,4,2′,4′-Tetrahydroxychalcone (butein) is a polyphenolic compound, which can be extracted from stembark of cashews and *Rhus verniciflua* Stokes, and used as a food additive and a traditional herbal medicine. Previous studies suggested that butein exhibit anticancer activity; butein could induce apoptosis in human promyelocytic leukemia cells [4] and B16 melanoma cells [5]. In vitro, butein can suppress the proliferation of many human cancers, including breast carcinoma, colon carcinoma, osteosarcoma, and hepatic stellate cells [6–10]. But the anticancer mechanisms of butein are not fully understood.

BLS-211 and BLX-211 cell lines were previously established by our laboratory and derived from the same surgical sample of a 55-year-old male patient with gradeII transitional cell carcinoma (TCC) of the bladder. These two cell lines had similar chromosomal alterations [11], but showed remarkable differences in cell morphology and biological characteristics. BLS-211 cells showed mesenchymal-like phenotype, while BLX-211 cells displayed epithelial phenotype. Furthermore, BLS-211 cells were more motile and invasive than BLX-211 cells. Using suppression subtractive hybridization (SSH), we previously found BLS-211 cells express mesenchymal proteins, such as vimentin, N-cadherin, but BLX-211 cells express epithelial marker, such as E-cadherin [12]. Here, we used these two cell lines to assess the anticancer effects of butein, and to provide possible mechanisms.

2. Materials and methods

2.1. Cell culture

BLS-211 and BLX-211 cells were cultured with RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% bovine calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO2.

2.2. Western blot analysis

Briefly, cells were lysed with RIPA buffer containing protease inhibitor. Equal amounts of protein (10–30μg) were separated by 10% SDS–PAGE, electrophoretically transferred to nitrocellulose membrane, the membrane was then incubated with the relevant primary antibodies against p44/42 MAPK, phospho-p44/42 MAPK (Cell Signaling Technology, Danvers, MA, USA), NF-κB, Vimentin, E-cadherin (Santa Cruz, CA) or β-actin (Biosynthesis Biotechnology, Beijing, China) and appropriate secondary antibodies (Pierce Biotech, USA), signals were visualized by ECL reagent.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the protocol of Nuclear Extraction kit (KEYGEN Biotechnology). The sequence of the URE NF-κB-containing oligonucleotide as follows: 5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′. As the loading control, the octomer-1 oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. Electrophoretic mobility shift assay (EMSA) was carried out according to the protocol accompanying EMSA/Gel-Shift kit (Beyotime Biotechnology). The specificity of binding was examined by competition with the unlabeled probes. For supershift assays, the nuclear extract (5 μg) was incubated at room temperature for 20 min with ^{32}P-labeled probe and antibodies against p65 (Santa Cruz) (3 μl). DNA/nuclear protein complexes were separated by electrophoresis on a native 6% acrylamide gel, and the gel was subjected to autoradiography.

2.4. Immunofluorescent assay

Cells were grown on glass coverslips for 24 h and fixed with methanol for 10 min at −20 °C. For permeabilization, cells were incubated with 0.1% Triton X-100 for 5 min at room temperature and then washed three times in PBS. Cells were blocked with goat serum for

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30 min at room temperature, incubated with primary antibody for vimentin, E-cadherin and β-catenin (Santa Cruz) (1:100) diluted in PBS overnight, and then incubated with fluorescent secondary antibody for 30 min at room temperature. The specimens were observed under fluorescent microscope.

2.5. Migration and invasion assays

Cell migration and invasion were tested using a transwell chamber migration assay (8 μM pore size membrane, millipore) or invasion assay (matrigel-coated membrane). cells were seeded in serum-free medium into the upper chamber and allowed to migrate or invade toward media containing 20% FCS as a chemoattractant in the lower chamber for 8 h (migration assay) or 22 h (invasion assay), cells in the upper chamber were carefully removed using cotton buds, and cells at the bottom of the membrane were fixed and stained with Giemsa. Quantification was done by counting the stained cells.

2.6. Scratch assay

Cells were grown to 90% confluence in six-well culture plates. Cell monolayers were wounded by manually scraping the cells with a 200 μl plastic pipet tip. Debris was removed from the culture by washing with phosphate-buffered saline (PBS) twice, and the cells were then maintained at 37 °C in an incubator. Images were captured immediately after wounding and 24 h post wounding, and wound closure was monitored with microscopy. Wound sizes were verified with an ocular ruler to ensure that all wounds were the same width at the beginning of the experiment.

2.7. RNAi

Two shRNA insert sequences for NF-κBp65 (GenBank accession number, NM_021975) were as follows: 5′-gattcGGACATATGAGACCTT-CAAttcaagagaTTGAAGGTCTCATATGTCCtttttggaaa- 3′ and 5′-gattcCCCCTTCCAAGTTCCTATAttcaagagaTATAGG-AACTTG-GAAGGGGt tttttggaaa-3′. As a negative control, the empty vector of the pRNAT-U6.1/Neo (GenScript Corp, Piscataway, NJ, USA) was used. Sense and antisense oligonucleotides were annealed and subcloned into the shRNA expression vector pRNAT-U6.1/Neo. BLS-211 cells were transfected using Lipofectamine 2000 reagent(Invitrogen) according to the manufacturer's instruction, and then selected with 150 μg/ml G418(Gibco). Individual clones were picked, based on GFP expression and neomycin resistance, and maintained in 1640 medium containing 50 μg/ml G418.

2.8. RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies) and quantitated by absorbance at 260 nm. cDNA synthesis was conducted using ImProm-II™ Reverse Transcription System (promega) according to manufacturer's instructions. All primers were designed using Oligo6.0 software. The primer sequences are listed as follows (Table 1): F, forward primer; R, reverse primer.

2.9. Statistical analysis

The results were presented as means ± S.D. of three replicate assays. All statistical analyses were performed using the SPSS13.0 software. Differences between different groups were assessed using ANOVA or Dunnett *t*-test. $P < 0.05$ was considered to indicate statistical significance.

3. Result

3.1. BLS-211 cells show mesenchymal-like phenotype but BLX-211 cells epithelial phenotype

BLX-211 cells are polyhedral and grow in a tightly connected manner; while BLS-211 cells are spindle-shaped and lose intercellular junctions (Fig. 1A). We further examined the EMT-associated molecular by Western blot analysis. As shown in Fig. 1B, BLX-211 cells (called BLX(E) hereafter) express an epithelial marker E-cadherin; in contrast, BLS-211 cells (called BLS(M) hereafter) overexpress a mesenchymal marker vimentin and lose E-cadherin. Furthermore, we found higher level of NF-κB activity in BLS(M) compared to BLX(E) cells by EMSA (Fig. 1C).

3.2. Butein reduced phosphorylation of ERK1/2

Our data indicated that butein inhibited cell proliferation and clonogenecity of BLS(M) and BLX(E) in dose-dependent manner, but when butein <20 μM, there was no significant

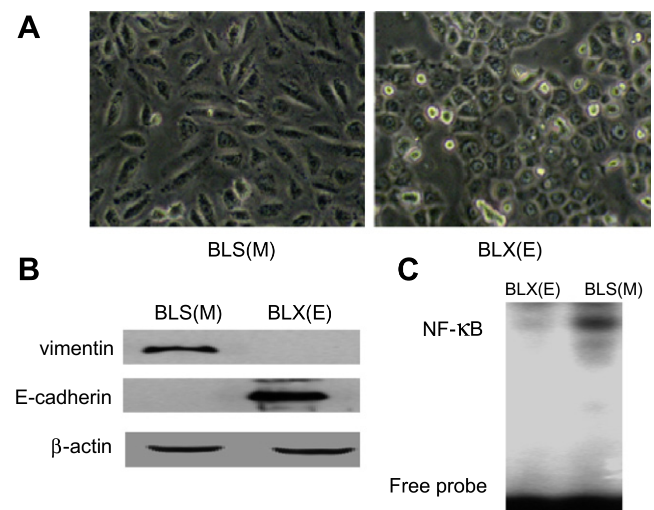

Fig. 1. (A) Morphology of BLX(E) and BLS(M) cells by light microscope. (B) Western blot assays show that BLX(E) cells express E-cadherin (an epithelial marker), while BLS(M) cells overexpress vimentin (a mesenchymal marker). (C) The significant difference of NF-κB activity between BLX(E) and BLS(M) cells by EMSA.

Table 1
The primer sequences for RT-PCR

Gene length	Primer	Position	Product (bp)
Snail (GenBank accession number NM_005985)	F5′-TCTAGGCCCTGGCTGCTACAA-3′ R5′-ACATCTGAGTGGGTCTGGAGGTG-3′	638–768	131
Slug (GenBank accession number NM_003068)	F5′-ATGCATATTCGGACCCACACATTAC-3′ R5′-AGATTTGACCTGTCTGCAAATGCTC-3′	690–847	158
Zeb1 (GenBank accession number NM_030751)	F5′-TGGCGATAGATGGTAATG-3′ R5′-AACTTGAAGTTGGCTAGGC-3′	1275–1465	209
Zeb2 (GenBank accession number NM_014795)	F5′-TGCGACATAAATACGAACA-3′ R5′-TGTGCTGCGAGTACGAG-3′	3524–3684	177
Twist (GenBank accession number NM_000474)	F5-GGCGGCCAGGTACATCGACTT-3′ R5′-GCTAGTGGGACGCGGACAT-3′	804–943	157
18SrRNA (GenBank accession number M-10098)	F5′-CAGCCACCCGAGATTGAGCA-3′ R5′-TAGTAGCGACGGGCGGTGTG-3′	1565–1816	253

difference in cell viability between the treated and control cells up to 48 h post-exposure time(data not shown). Thus, we chose butein at concentrations of 1–20 μM for subsequent experiments. To analyze the anti-proliferation mechanisms of butein, BLS(M) and BLX(E) cells were treated with butein (20 μM) for various time periods. Phosphorylation levels of ERK1/2 were obviously decreased in time-dependent manner (Fig. 2A; $^{*}P < 0.01$ compared with control group), but the expression of ERK1/2 had no obvious changes.

3.3. Butein inhibited NF-κB activity

Whether butein alone could inhibit constitutive NF-κB activity in BLS(M) cells was investigated. BLS(M) cells were treated with butein (20 μM) for various time periods and then analyzed for NF-κB activity. As shown in Fig. 2B, butein inhibited the constitutive NF-κB activity of BLS(M) cells in time-dependent manner. We next assessed the effects of butein on the NF-κB activity induced by TNF-α in BLX(E) cells. BLX(E) cells were first pretreated with butein for various times, then exposed to 5 ng/ml TNF-α for 1 h. The results indicated that butein could suppress not only constitutively active but also inducible NF-κB activity in bladder cancer cells (Fig. 2C).

3.4. Butein reversed mesenchymal-like phenotype of BLS(M) cells

It has been demonstrated that both ERK1/2 and NF-κB pathways were involved in EMT [2,3]. Based on the above observations that butein could inhibit both phosphorylation of ERK1/2 and constitutive NF-κB activity, we then asked whether butein could reverse mesenchymal-like phenotype of BLS(M) cells. After 48 h of butein (20 μM) treatment, we observed morphologic changes of BLS(M) cells from elongated morphology to rounded epithelial-like cells. Using immunofluorescence and western blot assay, we tested whether the morphologic changes correlated with EMT-associated proteins. Results showed downregulation of vimentin, but gain of E-cadherin in butein-treated BLS(M) cells compared to untreated control cells (Fig. 3A and B). Moreover, we found butein promote β-catenin membrane localization (Fig. 3A).

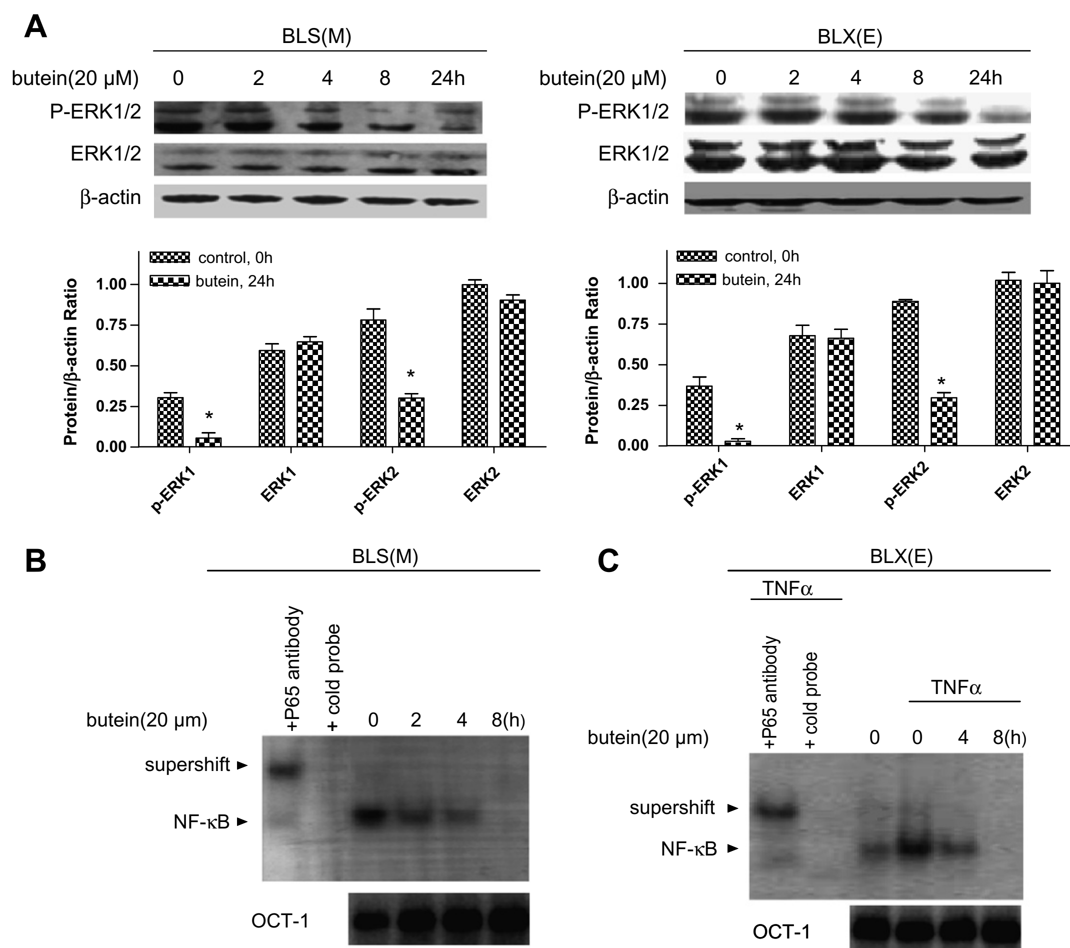

Fig. 2. (A) Effects of butein on ERK1/2 activation. BLS(M) and BLX(E) cells were treated by butein (20 μM) for the indicated times. Western blot analysis was conducted. Results were quantified by densitometry analysis of bands and then normalization to β-actin protein. $^{*}P < 0.01$ compare with control group. (B) Suppression effect of butein on constitutive NF-κB activity of BLS(M) cells by EMSA. (C) Suppression effect of butein on TNF-α-induced NF-κB activation of BLX(E) cells by EMSA. Oct-1 as loading control. The specificity of binding was examined by competition with the unlabeled probes (cold probes) and supershift assays.

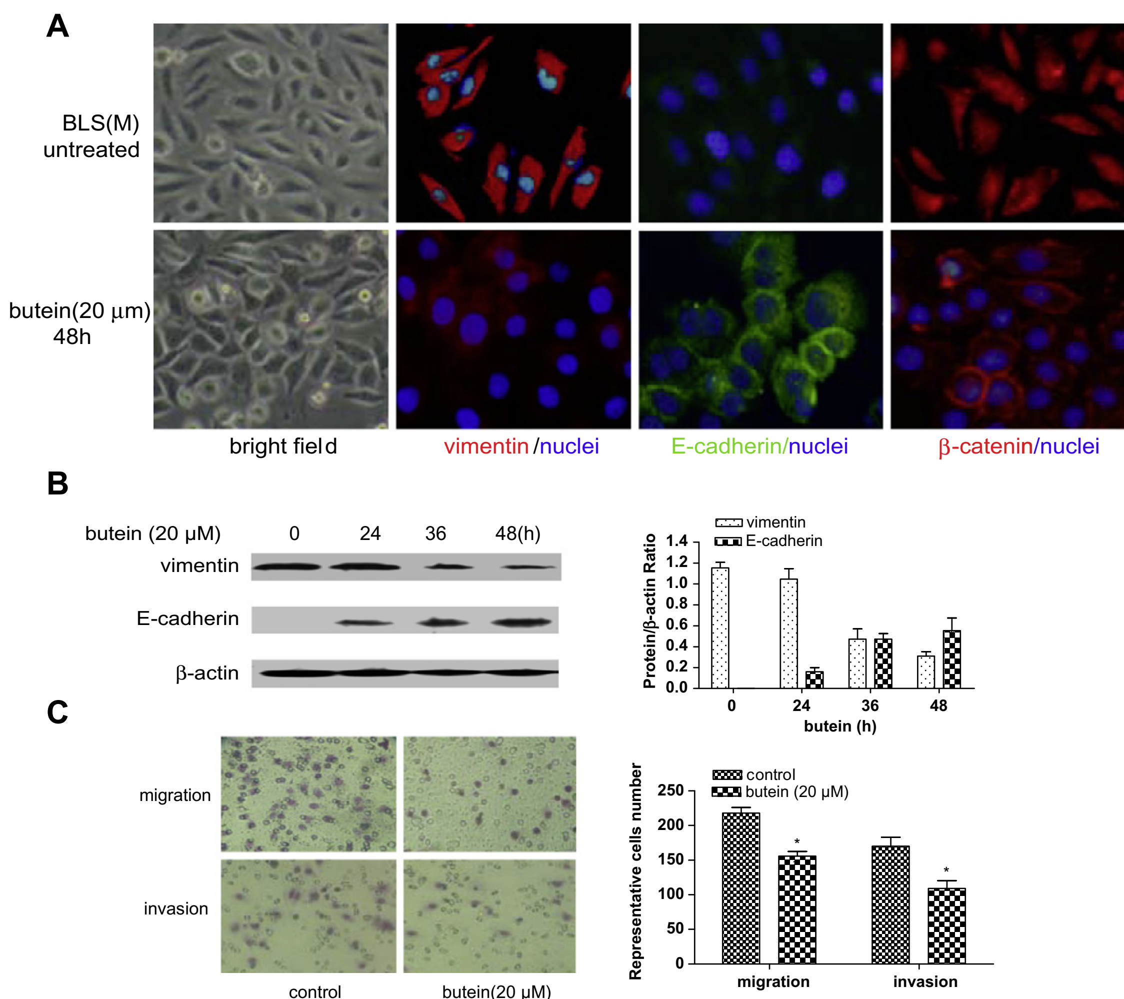

Fig. 3. Butein reversed mesenchymal-like phenotype of BLS(M) cells. BLS(M) cells were treated with butein (20 µM) for the indicate times. (A) Immunofluorescence analysis. (B) Western blot analysis showed reduced expression of vimentin and gain of E-cadherin in butein-treated cells compared to untreated control cells. Moreover, immunofluorescence analysis showed butein promote β-catenin membrane localization. (C) Butein inhibited the motility and invasion capacity of BLS(M) cells. Representative number of motility or invading cells was counted under the microscope in five random fields at 200×. The difference was significant between butein-treated cells and control cells ($^{*}P < 0.05$). Results are representative of three separate experiments. Representative visual field of butein-treated cells (right well) compared to Control cells (left well) is shown.

3.5. Butein suppressed motility and invasion capacity of BLS(M) cells

In order to study the effect of butein on motility and invasion activity of BLS(M) cells, BLS(M) cells were pretreated with different concentration butein (5,10,20 µM) for 48 h and seeded on Millicell chambers with uncoated or matrigel-coated filters. After 8 h or 22 h of incubation, we investigated both motility and invasive potential of BLS(M) cells pretreated with butein (20 µM) were significantly inhibited (Fig. 3C; $^{*}P < 0.05$), but when butein was 5 µM and 10 µM, there had no significant effect on motility and invasion potential of BLS(M) cells.

3.6. Butein reverted EMT-like phenotype induced by TNF-α in BLX(E) cells

Tumor necrosis factor-α (TNF-α), an activator of NF-κB, has been found to promote EMT in vitro [13]. BLX(E) cells were continuously cultured in medium containing TNF-α (5 ng/ml) for 3 and 6 days. TNF-α-induced BLX(E) cells exhibited more elongated morphology, which resembled that of cells undergoing EMT. Molecular hallmarks of EMT include downregulation of E-cadherin responsible for the loss of cell–cell adhesion and upregulation of mesenchymal-related proteins such as vimentin and N-cadherin [2,3]. To test this possibility, the expression and localization of E-cadherin and vimentin were determined by Western blot and immunofluorescence analysis, demonstrating reduced expression level and membrane localization loss of E-cadherin, and nuclear localization of β-catenin, but increased level of vimentin at 6 days posttreatment with TNF-α. These data suggested that TNF-α could induce EMT in BLX(E) cells. We further investigated whether butein can revert EMT-like phenotype induced by TNF-α. TNF-α-induced BLX(E) cells were subsequently treated with butein for 48 h. As a results, the EMT-like phenotype was partly reverted to epithelial phenotype (Fig. 4A and B), while removal of TNF-α for 48 h had no obvious effect (data not shown).

3.7. Suppression of NF-κBp65 by transfection with pRNATu6.1/Neo encoding p65 shRNA

We constructed two sublines that stably expressed NF-κBp65 shRNA and one subline that express negative vector

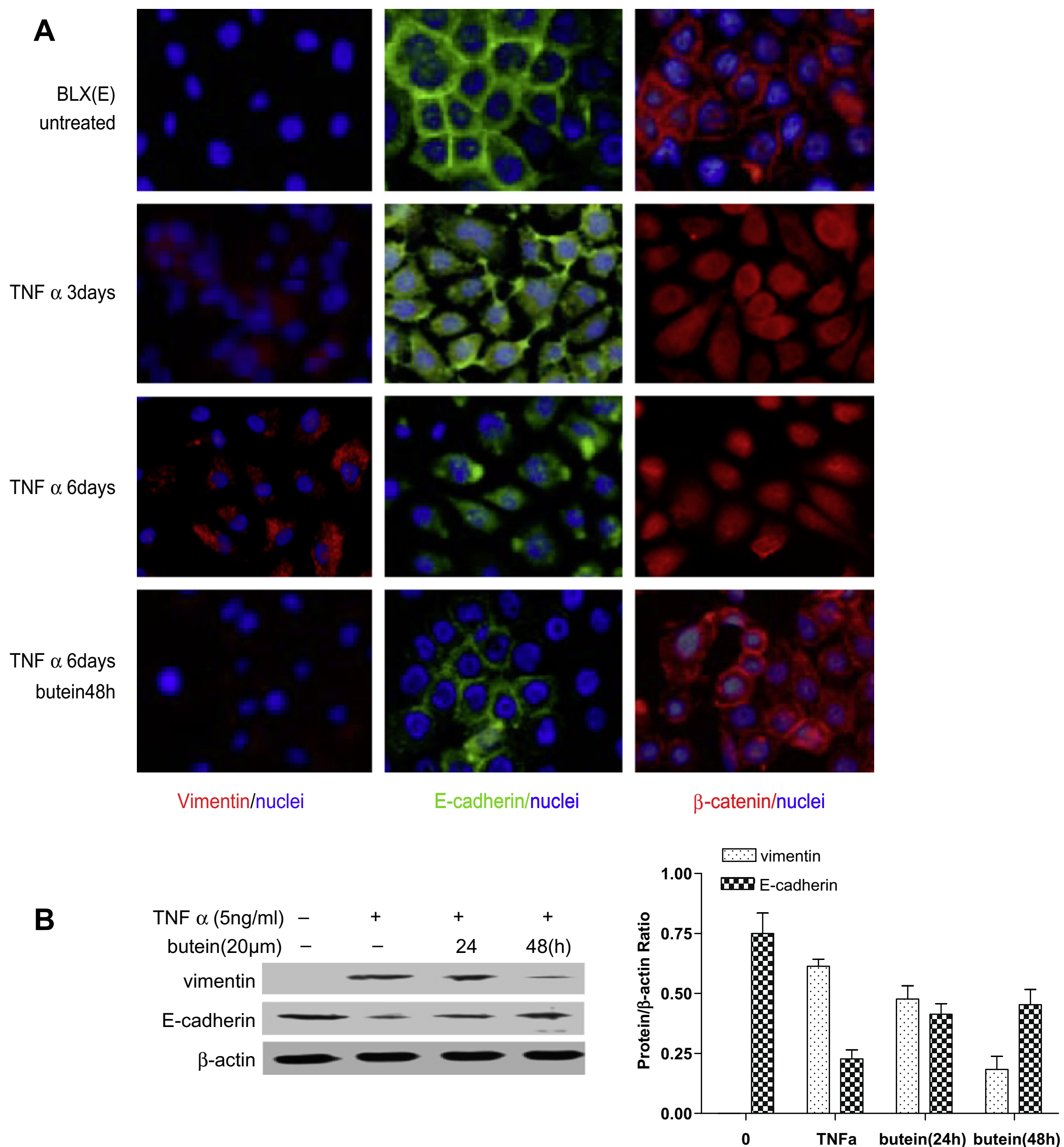

Fig. 4. Butein reverted EMT-like phenotype induced by TNF-α in BLX(E) cells. BLX(E) cells were continuously cultured in medium containing TNF-α (5 ng/ml) for 3 and 6 days, and subsequently treated with or without butein (20 μm) for 48 h. (A) Immunofluorescence analysis showed membrane localization loss of E-cadherin, increased level of vimentin, and nuclear localization of β-catenin at 6 days posttreatment with TNF-α. but butein reverted the morphologic and molecular changes induced by TNF-α. (B) Western blot analysis showed TNF-α downregulate E-cadherin and upregulate vimentin, which can be reverted by butein.

control, designated as shRNAp65-1, shRNAp65-2, and mock control, respectively. Western blot analysis demonstrated that NF-κBp65 protein was significantly suppressed only in one subline pRNATp65-1, but there was no difference between the parental and mock cells (Fig. 5A; $^{*}P < 0.01$).

3.8. Knockdown of NF-κBp65 by RNAi reversed mesenchymal-like phenotype and suppressed motility and invasion capacity of BLS(M) cells

No obvious morphologic changes were observed when the mock cells were compared with the parental cells. In sharp contrast, the shRNAp65-1 cells underwent a significant morphologic transformation; the cells acquired a rounded and less elongated shape (Fig. 5B). Western blot and immunofluorescence results showed shRNAp65-1 cells acquired E-cadherin and significantly down regulated vimentin (Fig. 5C and D).

In vitro motility and invasion assay were performed. Results showed both motility and invasive potential of shRNAp65-1 cells appeared significantly reduced (Fig. 5E; $^{*}P < 0.05$). Scratch-wound assays were also utilized to investigate cell migration. NF-κBp65 knockdown resulted in decreased motility of BLS(M) cells; the recovery of wound was significantly delayed in shRNAp65-1 cells (Fig. 5F; $^{*}P < 0.05$).

3.9. Butein downregulated expression of the genes involved in EMT

To identify the downstream targets of butein, expression of the known transcriptional regulators involved in EMT were

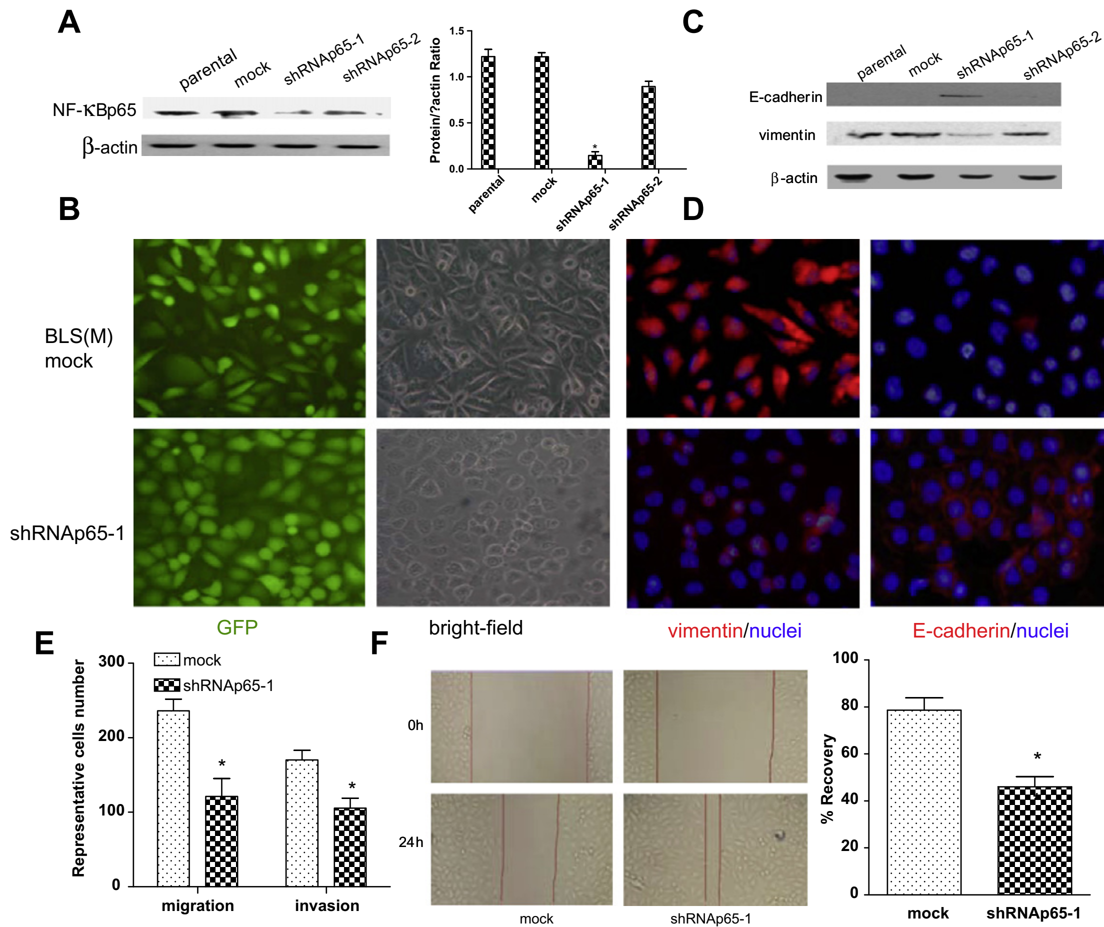

Fig. 5. (A) NF-κBp65 knockdown by RNAi. Western blot analysis indicated NF-κBp65 protein levels are suppressed in shRNAp65-1 cells compared to that of control cells. β-Actin was used as loading control. (B) Green fluorescent and bright field microscopic image of shRNAp65-1 and mock vector cells show a clear change in cell morphology. (C) Western blot and (D) immunofluorescence analysis confirmed reduced expression level of vimentin and gain of E-cadherin in shRNAp65-1 cells. (E) In vitro migration and invasion assay. Representative number of motility or invading cells was counted under the microscope in five random fields at 200×. shRNAp65-1 cells show more motile and invasive than mock cells ($^{*}P < 0.05$). (F) Scratch assay. Images were captured immediately after wounding and 24 h post wounding, representative microscopic image were shown (left). Scratch-wound edges are marked by red lines. Results (percentage of recovery) were represented as means ± S.D. of three independent experiments (right; $^{*}P < 0.05$).

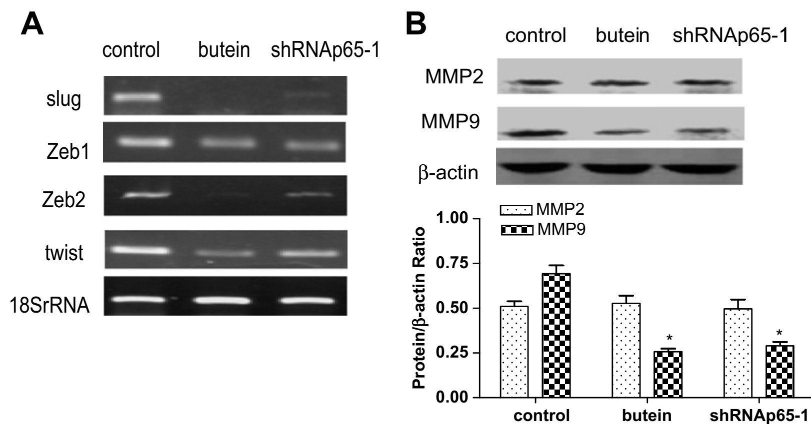

Fig. 6. (A) The inhibition effects of butein on expression of genes involved in EMT. RT-PCR analysis showed butein (20 μm) significantly inhibit the expression of slug, zeb2 and twist. shRNAp65-1 cells showed similar results. (B) The inhibition effect of butein on MMP9 by Western blot analysis ($^{*}P < 0.05$). β-Actin as loading control.

analyzed by RT-PCR. We could not detect the expression of snail in BLS(M) parental cells and butein-treated cells, as well as shRNAp65 cells (data not shown). Expression of slug, zeb2 and twist mRNA were significantly downregulated in cells treated with butein for 48 h compared with the untreated control cells ($P < 0.05$), NF-κBp65 knockdown by RNAi pro-

duced the similar results (Fig. 6A). Next, we also examined the expression of MMP9 and MMP2 by Western blot (Fig. 6B), and found both butein and RNAi targeting NF-κBp65 significantly reduced MMP9 expression ($^{*}P < 0.01$ compared with control group), but had no effect on MMP2.

4. Discussion

In this study, utilizing well-characteristic cell lines of bladder cancer, we have demonstrated a novel anticancer effect of butein and provided possible mechanisms responsible for their anti-invasive effect.

The anti-proliferation effect of butein has been well documented in many types of human cancers [6–10]; our results indicated that butein inhibited cell proliferation of bladder cancer in dose-dependent manner, which were in agreement with previous studies. But the anticancer mechanisms of butein remain elusive. In this study, we found butein greatly reduced the phosphorylation and activation of ERK1/2 in BLS(M) cells. Extracellular signal-regulated protein kinases (ERK)1/2 are important members of the mitogen-activated protein kinase (MAPK) family, which are the key factors of transmitting cell proliferation signals [14]. ERK1/2 is also known to be important for the activation of Nuclear factor κB (NF-κB) [15]. NF-κB is critically involved in the regulation of tumor cell proliferation, apoptosis and oncogenesis [16]. A recent study suggests that anti-tumor and anti-inflammatory activities of butein may be mediated in part through the suppression of the NF-κB activation [17]. Our data further demonstrated that butein could suppress not only constitutive but also inducible NF-κB activation in bladder cancer cells. Therefore, these results suggested that anticancer activity of butein may be involved in both ERK1/2MAPK and NF-κB signaling pathway.

EMT involves diverse signal transduction cascades activated in response to various growth factors and cytokine. These pathways converge to promote cell motility, invasiveness, and metastasis [18]. The Ras pathway has been shown to be of crucial importance in EMT by both in vitro and in vivo studies[19,20]. Moreover, in addition to its well-known antiapoptotic effects, NF-κB has also been implicated in EMT [13,21–23]. NF-κB is essential for both the induction and maintenance of EMT in murine mammary carcinoma model [23]. Since butein significantly suppressed both activation of MAPK pathway and NF-κB activity, we postulated that butein may reverse EMT. As respected, butein could partly reversed mesenchymal-like phenotype of BLS(M) cells as well as the EMT-like phenotype induced by TNF-α in BLX(E) cells, which is judged by morphologic and molecular changes including gain of E-cadherin, reduction of vimentin and membrane localization of β-catenin. β-Catenin is a component of adherent junctions, links transmembrane protein E-cadherin to the actin cytoskeleton, and is also a key effector of the Wnt pathway. The accumulation of free cytoplasmic β-catenin and/or its translocation to the nucleus trigger epithelial–mesenchymal transition [24]. Up-regulation of vimentin can facilitate pseudopod formation and cytoskeletal remodeling [25]. During EMT, loss of intercellular adhesion and the activation of the cytoskeleton ultimately enhance motility and invasion. Reversal of EMT often results in the inhibition of cell metastasis ability [23]. We observed the suppression effect of butein on both migration and matrigel invasion capacity of BLS(M) cells. Thus, it is possible that butein could suppress the bladder cancer invasion and metastasis through reversal of EMT.

We performed RNAi targeting NF-κBp65 in BLS(M) cells, and found NF-κBp65 silencing could obviously inhibit the migration as well as invasion ability, and partly reverse the mesenchymal-like phenotype, which were similar to that observed in butein-treated BLS(M) cells. These data further confirmed that the anti-invasion effect of butein may be associated with the inhibition of NF-κB activity.

Several zinc-finger transcriptional repressors, including slug, zeb1, zeb2, and twist, have been reported to reduce E-cadherin expression and induce EMT [26–29]. Matrix metalloproteinase(MMP)9, as a downstream target of NF-κB, is capable of degrading the ECM and altering cell–cell and cell–ECM interactions, and has been shown to be associated with cancer invasion and EMT [30]. We subsequently examined expression of these EMT-associated genes in butein-treated cells and shRNAp65-1 cells. Downregulation of slug, zeb2, twist and MMP9 was demonstrated in both butein-treated and shRNAp65-1 cells compare to untreated control cells, suggesting the decreased expression of these genes by butein is mediated in part through the inhibition of the NF-κB activity, which may play an important role in mesenchymal to epithelial transition induced by butein.

Taken together, our data for the first time suggested butein could inhibit migration and invasion through ERK1/2 and NF-κB signaling pathway, and this inhibitory effect may be associated with reversal of EMT. Butein should be considered as a possible therapeutic agent for inhibiting the metastasis and invasion of bladder cancer. Further studies in vivo will be required to assess the potential of butein in treatment of cancer.

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