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JNK3 Cooperates With RelA/p65 to Decrease Bel-7402 Cell Adhesion Upon The Inhibition of NF-κB Pathway*

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Abstract The c-Jun amino-terminal kinase (JNK) is an important player in inflammation, proliferation, and apoptosis. Here, by using a yeast two-hybrid technology, p65 subunit of NF-κB transcription factor was identified as a partner of JNK3. We show that JNK3 physically associated with p65 *in vivo* and *in vitro*. Overexpression of JNK3 inhibited NF-κB- dependent transcription induced by TNFα. It was demonstrated that JNK3 decreased NF-κB binding to its cognate DNA sequences and NF-κB target genes expression. Taken together, these data suggest that JNK3 may function *in vivo* as a modulator in suppressing the transcriptional activity of p65.

Key words JNK3, NF-κB, p65, cell adhesion, protein-protein interaction
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The c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases, belong to the mitogenactivated protein kinase (MAPK) group of serine threonine protein kinases [1–2]. JNKs could be activated in response to a variety of stimuli, such as TNFα [3] or UV radiation [4]. And JNK signaling is important in movement of epithelial sheets [5–6], wound healing [7], apoptosis [8–10], cell survival [11], and tumor development [12]. Three JNK isoforms (JNK1, JNK2 and JNK3), which encoded by three distinct genes, have been identified [1–2]: JNK1 and JNK2 are ubiquitously produced, but JNK3 is essentially restricted to neurons [1–2]. JNK1 takes part in the production of neurite-like structures in PC12 cells [13], while JNK2 has been associated with epidermal growth factor (EGF)-mediated growth of A549 cells[14]. JNK3 plays a role in neuronal apoptosis. Targeted disruption of the JNK3 gene would render mice highly resistant to glutamate toxicity [15] and protect mice from brain injury after cerebral ischemia-hypoxia[16]. It has been reported that JNK3 was associated with TNFα-meditated death of oligodendrocytes [17] and increased number and length of neurites during PC12 cells differentiation [18]. Moreover it has been reported that JNK3 was involved in the differentiation and proliferation of C6 glioma cells using pharmacological inhibition and knockdown of JNK3 by RNAi[19]. Since the expression of the JNK3 gene was lost in 10 of 19 human brain cancer cell lines [20], a possible role for JNK3 in the development of tumors could be predicted. However, the mechanism is not clear.

In the present study, we identified the interaction between JNK3 and p65, and found that JNK3 decreased NF-κB-dependent transcription and its DNA-binding activity. Furthermore, JNK3 could decrease the capability of adhesion with ECM in Bel-7402 cells, and the transcription of a few of genes correlated with adhesion were depressed.

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1 Materials and methods

1.1 Cell culture and transfection

Human hepatocarcinoma cell line Bel-7402, and human embryonic kidney cell line HEK293 were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were detached *via* incubation with 0.05% trypsin and 0.04% EDTA in phosphate-buffered saline. Transfections were performed with Vigofect reagent (Vigorous Biotechnology, Beijing, China) according to the manufacturer's protocol.

1.2 Plasmids

The plasmids pCDNA3.1-JNK3, pFlag-JNK3, GST-JNK3, pFlag-p65, pGFP-N1-JNK3 and pRED-N1-p65 were generated in our laboratory. The E-selectin-Luc reporter construct, ICAM-1-Luc reporter construct and IL-6-luc reporter construct was gifted by Prof. LI Hui-Yan.

1.3 Yeast two-hybrid screening

Yeast two-hybrid screening was carried out according to the manufacturer's instructions for the ProQuest™ two-hybrid system (Invitrogen, Carlsbad, CA). To screen proteins that interact with JNK3, yeast MaV203 cells were sequentially transformed with pDBLeu-Keap1 fused in-frame to the DNA binding domain of GAL4, and then with human liver cDNA library (Invitrogen, Carlsbad, CA) constructed in pPC86 vector that codes for the activation domain of GAL4. Controls *A* through *E* correspond to different levels of protein-protein interactions. At least 1×10^6 transformants were screened. Transformants were plated on the SD-Leu-Trp-His+25 mmol/L 3AT plates. Primary positives were selected and tested in three reporter assays, using histidine and uracil prototrophy and β-galactosidase activity. pPC86 plasmids recovered from colonies positive in histidine prototrophy and either or both of uracil prototrophy and β-galactosidase activity were retransformed into MaV203 cells with either pDBLeu-Keap1 or pDBLeu vector to assess specific protein interactions. Nucleotide sequences of the cDNA inserts in pPC86 plasmids were verified by sequencing and analyzed using BLAST search.

1.4 Co-immunoprecipitation

HEK 293 cells were transfected with indicated expression vectors with Vigofect reagent, and cultured for 24 h in DMEM, and lysed for 20 min on ice in lysis buffer(50 mmol/L Tris-HCl(pH7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% TritonX-100) supplemented with protease inhibitor cocktail(Roche, Basel, Switzerland). Soluble cell lysates were incubated with 2 μg indicated antibodies for 2 h at 4℃ followed by incubation at 4℃ with protein A/G agarose beads overnight. Unbound proteins were removed by washing 4 times with lysis buffer. Following SDS-PAGE, immunoprecipitated proteins were transferred onto polyvinylidene difluoride membranes (Amersham life science, Buckinghamshire, England) and probed with various antibodies. The enhanced chemiluminescence (ECL) system (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection.

1.5 Generation of recombinant proteins and GST pull-down assays

GST-JNK3 fusion proteins were expressed in *Escherichia coli* BL21, induced by 0.1 mmol/L isopropyl thio-β-D-galactoside (IPTG) and solubilized from bacteria in lysis buffer (1% Triton X-100 in PBS) by sonication. After centrifugation at 12 000 r/min for 15 min at 4℃ , the supernatant was added to the glutathione-Sepharose 4B beads(Amersham Biosciences, Uppsala, Sweden) and mixed gently at 4℃ overnight. After binding, the beads were washed four times in cold PBS to remove nonspecific binding. GST, GST-JNK3 fusion proteins, as judged by Coomassie bright blue staining, were bound to glutathione-Sepharose beads.

The cell lysis were treated with DNase Ⅰ (TaKaRa, Japan) for 30 min at 37℃ to remove the genomic DNA contamination before mixed with GST fusion protein which adsorbed to Sepharose beads. The binding reaction was carried out overnight at 4℃ in 1 ml binding buffer (20 mmol/L Tris-HCl at pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol and 0.1% NP-40). After thoroughly washing, specifically bound proteins were subjected to 15% SDS-PAGE followed by Western blotting analysis.

1.6 Subcellular localization assays

For JNK3 and p65 location, immunofluorescence was performed. HEK293 cells were seeded in 6-well plates, cultured in DMEM supplemented with 10% fetal bovine serum and transfected with JNK3-GFP and p65-RFP.

Confocal imaging was performed using Zeiss 510 META system. The green fluorescence was excited at 488 nm with 505～530 nm barrier filter and red fluorescence was simultaneously excited at 543 nm

with 560 nm barrier filter.

1.7 Luciferase reporter assay

HEK293 cells were seeded into 24-well plates and transiently transfected with different plasmids as indicated. In each case, vector DNA was added as necessary to achieve a constant amount of transfected DNA (1.25 μg). Luciferase activities were measured as previously described [21]. Luciferase activities were expressed as fold induction relative to values obtained from control cells. The results represent the mean of at least three independent transfection experiments, each carried out in duplicate. Renilla luciferase activity was used as an internal control for transfection efficiency.

1.8 Preparation of nuclear extracts and electrophoreticmobility shift assays (EMSAs)

HEK293 cells were transfected with various amount of pcDNA3.1-JNK3 and nuclear extracts were isolated as described previously [21]. In each case, pcDNA3.1 was added as necessary to keep the total amount of transfected plasmids fixed. The DNA probe was prepared by annealing two oligonucliotides 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ and 3′ TCA ACT CCC CTG AAA GGG TCC G 5′, and labeling them with [γ-^{32}P] ATP by filling in the T4 polynucleotide kinase (Gel Shift Assay System, Promega). Nuclear extracts (10 mg) were incubated with the binding buffer for 10 min, followed by incubation with 0.5 ng of ^{32}P-labeled DNA probe for 30 min at room temperature. The DNA-protein complexes were analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5-Tris-borate/ethylene diaminetetraacetic acid (EDTA) electrophoresis buffer at 300V followed by autoradiography. For competition experiments, extracts were preincubated with a 50-fold excess of unlabeled double-stranded oligonucleotides. In supershift assays, 2 mg of antibodies against p65 or p50 were added to the nuclear extracts 30 min before the addition of radiolabeled probes.

1.9 Total RNA isolation and real-time PCR

Total RNA isolation and reverse-transcription were applied according to the manufacturer's protocol. The cDNA was analyzed using real-time PCR according to the instruction from the kit. In brief, real-time PCR was done using Bio-Rad IQTM5 Multicolor real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Premix Ex TaqTM (2 ×) kit (TaKaRa, Japan). The cycling conditions were as follows: 95℃ for 1 min, 40 cycles of 10 s at 95℃, 30 s at 55℃ and 30 s at 72℃. SYBR Green fluorescence was measured after each elongation step. Specific primers for each gene were as follows: ENG, 5′ GATGCCTGGAGAGTCAGCTC 3′ and 5′ CACTAGCCAGGTCTCGAAGG 3′, TNC, 5′ GTGAAGGAAGACAAGGAGAG 3′ and 5′ TGGGGAGACTGTAATTGAGG 3′, VCAM1, 5′ GGAACGAACACTCTTACCTG 3′ and 5′ GAACACTTGACTGTGATCGG 3′, GAPDH, 5′ TGGCCAAGGTCATCCATGACAACT 3′ and 5′ CACAGTCTTCTGGGTGGCAGTGAT 3′. At the end of PCR, a melting curve analysis was performed by gradually increasing the temperature from 55℃ to 95℃ to determine purity. PCR was set up in triplicates and threshold cycle (C_t) values of the target genes were normalized to the endogenous control. Differential expression was calculated according to the $2^{-\Delta\Delta C_t}$ method.

1.10 Adhesion assays

Quantitative cell adhesion assays were performed essentially as previously described[22]. Wells of 96 well plates were incubated overnight, 4℃, with 1 g/L fibronectin or type Ⅰ collagen (Sigma) in PBS. Wells were blocked for 1 h in 1% BSA in PBS, room temperature. Cells at 80% confluence were harvested using 2 mmol/L EDTA in PBS (20 min, room temperature), washed twice with DMEM serum-free medium containing 1% BSA (Sigma, St. Louis, MO) and resuspended in DMEM. 2.5×10^4 cells were placed into each well. At the times indicated, on-adherent cells were removed by washing with PBS. Adherent cells were quantified by incubation with 10 ml MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide)solution for 4 h at 37℃, after which formazan reaction products in each well were dissolved in 100 ml of dimethyl sulphoxide and A_{490} was measured.

1.11 Data analysis

Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered significant. Data were expressed as $\bar{x} \pm s$.

2 Results

2.1 Identification of p65 as an interacted protein of JNK3

We used a yeast two-hybrid system to analyze the JNK3 interactors. As bait, JNK3 was constructed in the GAL4 DNA-binding domain vector (pDBLeu) to screen human liver cDNA library. The most stringent screening conditions were used to select positive colonies and minimize the false positives in yeast two-hybrid assays. The nucleotide sequences of the positive

clones were analyzed and one of the isolated clones encoded p65 (amino acids 169～301), a subunit of NF-κB transcription factor. In yeast re-transformation assays, yeast cells that cotransformed with JNK3 and p65 were grown in selection medium, but not for the cells transformed with JNK3 p65 alone (Figure 1a), thus indicating the specific interaction of p65 and JNK3.

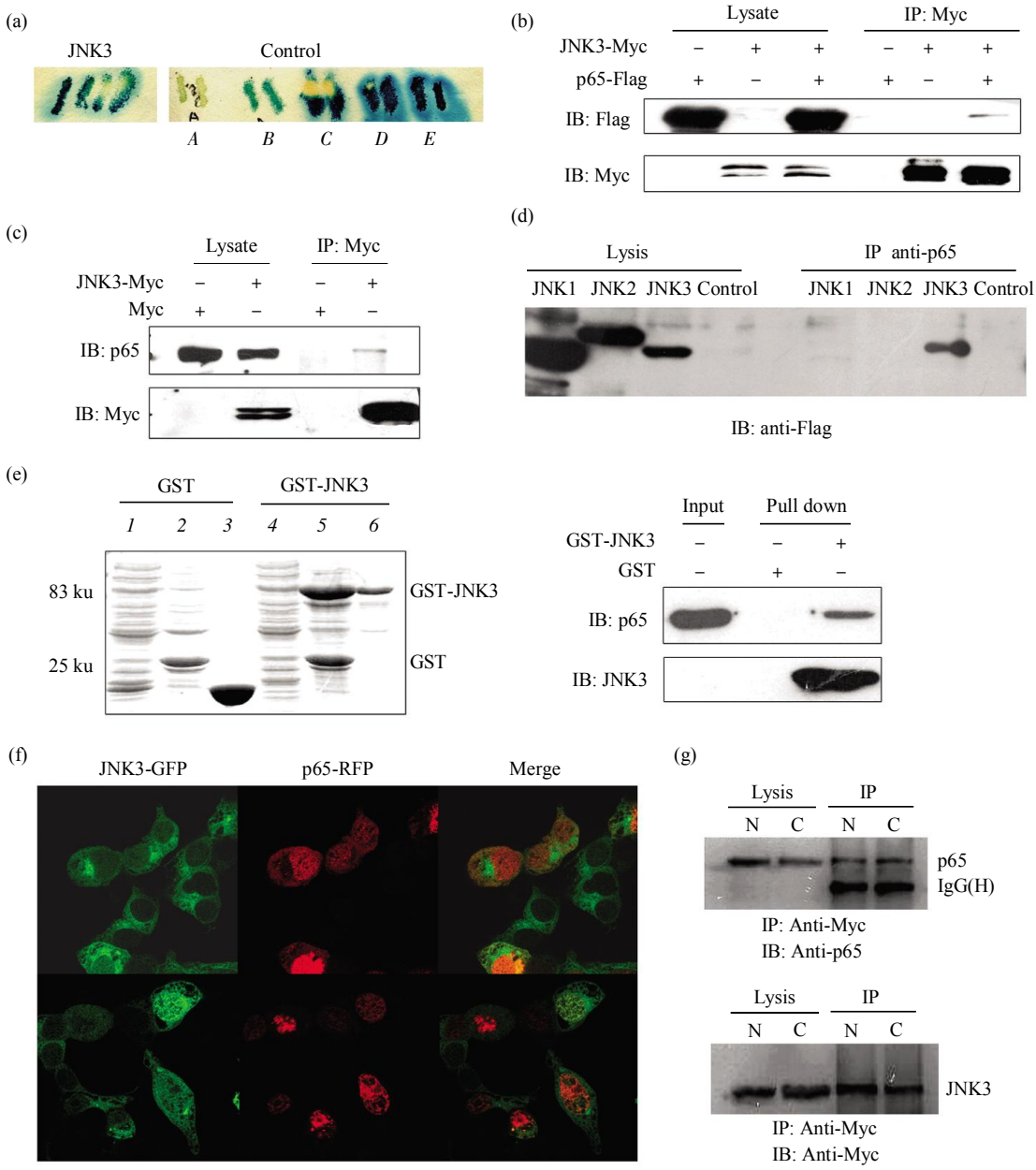

Fig. 1　Identification of p65 as an interacted protein of JNK3

(a) Yeast retransformation assay was performed to confirm the activation of reporter gene. The plasmids were transformated into Yeast strains as indicated, and X-Gal assay was performed. Yeast control strains *A*-*E* were used to compare the phenotypes of the potential positive interaction. Control *A*, no interaction; *B*, weak interaction; *C*, moderately strong interaction; *D*, strong interaction; *E*, very strong interaction. (b) HEK293 cells were transfected with JNK3-Myc and p65-Flag. Then the lysates were immunoprecipitated with anti-Myc antibody. The lysates and immunoprecipitates were detected by Western-blot with anti-Myc or anti-Flag antibodies as indicated. (c) HEK293 cells were transfected with expression plasmids for JNK3-Myc or empty vector (as negative control). Cell lysates were immunoprecipitated with monoclonal anti-Myc antibody. The lysates and immunoprecipitates were detected by Western-blot with anti-Flag and anti-p65 antibodies as indicated. (d) HEK293 cells were transfected with JNK1-Flag or JNK2-Flag or JNK3-Flag or empty vector(as negative control). Then the lysates were immunoprecipitated with anti-p65 antibody. The lysates and immunoprecipitates were detected by Western-blot with anti-p65 and anti-Flag antibodies as indicated. (e) GST, GST-JNK3 fusion proteins were expressed in *E. coli*, purified by glutathione-Sepharose beads. The complexes were washed to remove noninteracting proteins, electrophoresed in SDS-PAGE and visualized by Coomassie bright blue staining. Lysates of HEK293 cells were incubated with GST or GST-JNK3 respectively, whole-cell lysates were extracted and digested with DNase Ⅰ and performed GST pull down. Complexes were detected by Western blotting analysis using anti-p65 antibody and anti-GST antibody. (f) HEK293 cells were co-transfected with GFP-JNK3 and RFP-p65 for 24 h. The locations of JNK3 (green) and p65 (red) were observed through confocal imaging. (g) HEK293 cells were transfected with JNK3-Myc. 24 h later, cell lysates were extracted and immunoprecipitated with monoclonal anti-Myc antibody. The lysates and immunoprecipitates were detected by Western-blot with anti-Myc and anti-p65 antibodies as indicated.

To test for the association of JNK3 and p65 in mammalian cells, a co-immunoprecipitation assay was performed in HEK293 cells. HEK293 cells were transfected with JNK3-Myc and p65-Flag. Then, the cell lysates were subjected to immunoprecipitation with anti-Myc antibody and Western blotting with anti-Flag or anti-Myc antibody (Figure 1b). Immunoblotting analysis showed that JNK3 was coimmunoprecipitated with p65.

Moreover, a half-*in vivo* coimmunoprecipitation was performed. HEK293 cells were transfected with JNK3-Myc or control vector, and then the cell lysates were subjected to immunoprecipitation with anti-Myc antibody and Western blotting with anti-p65 and anti-Myc antibody. Immunoblotting analysis showed that p65 was co-immunoprecipitated with JNK3 (Figure 1c).

In addition, we detected whether p65 interacted with other JNK family members. HEK293 cells were transfected with JNK1-Flag, or JNK2-Flag, or JNK3-Flag, or control vector, and then the cell lysates were subjected to immunoprecipitation with anti-p65 antibody and Western blotting with anti-Flag antibody (Figure 1d). Immunoblotting analysis showed that p65 was specifically co-immunoprecipitated with JNK3, whereas neither JNK1 nor JNK2 was present in p65 immunoprecipitation products.

The interaction of JNK3 and p65 was confirmed *in vitro* by pull down assay. GST-JNK3 fusion protein was expressed in bacteria. HEK293 cell lysates were pulled down with GST-JNK3 or GST alone. We demonstrated that p65 bound with GST-JNK3 and not GST alone (Figure 1e). All of these results indicate that JNK3 physically binds to p65.

To further confirm this association, colocalization of JNK3 and p65 was studied. As shown in Figure 1f, when the images were merged, JNK3 and p65 showed colocalization in nuclear as well as in cytoplasm. We also detected where the interaction between p65 and JNK3 happened. HEK293 cells were transfected with JNK3-Myc, 24 h later, cytoplasm protein and nuclear protein were extracted respectively and immunoprecipitated with anti-Myc antibody. Western-blotting analysis showed that JNK3 could interact with p65 both in nuclear and in cytoplasm (Figure 1g). These results indicate that JNK3 interacts specifically with p65 *in vivo*.

2.2 JNK3 selectively inhibited NF-κB transactivation

It has been reported that JNK3 inhibited p65-mediated 3 × κB-luc reporter activity. We next examined the functional effects of JNK3 on the NF-κB activation by luciferase reporter assays in HEK293 cells using NF-κB target gene promoter reporter. As shown in Figure 2, cotransfection of JNK3 with E-selectin led to a significant inhibition of reporter activity, down to 40% of the basal level. Further increasing the expression of JNK3 led to a

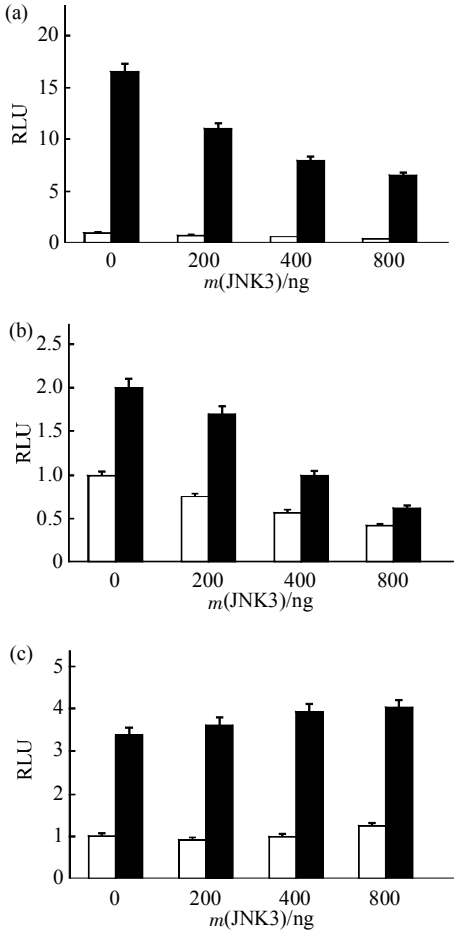

Fig. 2 JNK3 inhibits NF-κB transcription

(a ～c) Various amounts of JNK3 were cotransfected with 400 ng E-selectin-luc (a) or ICAM-1-luc (b) or IL-6-luc (c) in HEK293 cells. Control vector was added as necessary to keep the amount of transfected plasmids constant. 24 h later, the cells were treated with TNFα or left untreated for 12 h before that luciferase activity was measured. For the luciferase assays, the values were normalized to Renilla luciferase activity. Results were presented as fold inductions relative to the activity of cells that were transfected with reporter only and left untreated. Representative data of at least three independent experiments are shown with similar patterns. □: TNFα−; ■: TNFα+.

dose-dependent repressing of basic E-selectin -Luc reporter luciferase activity compared to the control ($P < 0.05$). Similar results were observed when the E-selectin -Luc was activated by TNFα, an efficient NF-κB activator, in contrast to control, the reporter activity of E-selectin was activated to ~16-fold over the basal level, cotransfection of JNK3 decreased the reporter activity down to ~6-fold over the basal level ($P < 0.05$, Figure 2a). Moreover, we tested the ICAM-1 promoter activity in the presence of JNK3, which is another NF-κB target gene and was used to confirm NF-κB repression. The results showed that JNK3 overexpression resulted in 60% decreasing of ICAM-1 promoter activity with or without stimulation of TNFα ($P < 0.05$, Figure 2b).

By using the similar experimental approaches, we showed that transfection of JNK3 did not affect IL-6 promoter activity in the presence or absence of TNFα (Figure 2c). These results indicate that the inhibition of JNK3 on NF-κB is optional, more inclined to adhesion-related genes.

2.3 JNK3 reduced the binding of NF-κB to its target sequences

To understand the mechanism of inhibition of NF-κB activity by JNK3, we examined the effect of JNK3 on the DNA-binding ability of NF-κB. EMSA with the ^{32}P-labeled fragment of DNA containing NF-κB binding site and nuclear extracts from HEK293 cells which were transfected with various amounts of JNK3-Flag was performed. As shown in Figure 3, specific DNA-protein complexes were reproducible detected. When JNK3 was added, the intensities of bands decreased in a dose-dependent manner, indicating that JNK3 inhibited NF-κB binding (lanes *2* ~ *4* in Figure 3). Competition experiments with an excess of nonradioactive-labeled NF-κB recognition sequence completely led to the disappearance of the protein-DNA complexes (lane *5* in Figure 3). For the supershift assay, p65 antibody or p50 antibody was added respectively to identify the protein-DNA complexes (lane *6* and lane *7* in Figure 3).

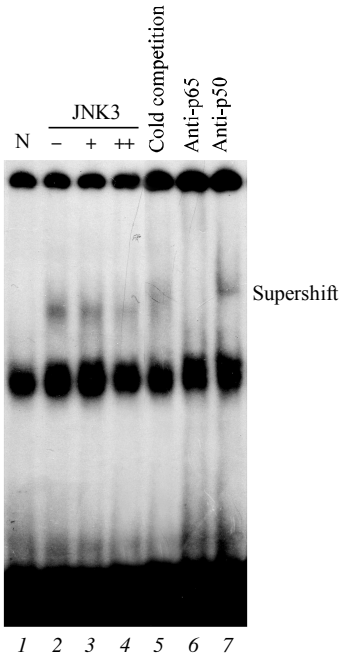

Fig. 3 JNK3 inhibits the binding of NF-κB to its target sequences

HEK293 cells were transfected with various amounts of JNK3. Control vector was added as necessary to maintain the amount of transfected plasmids constant. After 24 h, nuclear extracts were prepared to analyze the binding to ^{32}P-labeled probe by electrophoretic mobility shift assay (EMSA). The DNA probe was prepared by annealing two oligonucliotides 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ and 3′ TCA ACT CCC CTG AAA GGG TCC G 5′. The ^{32}P-labeled oligonucleotides were incubated with the indicated nuclear extracts and/or antibody and resolved on a 4% polyacrylamide gel. For competition assays, a 50-fold molar excess of unlabeled probe was added to the binding reaction mixture, and for the supershift assays, p65 antibody or p50 antibody was added respectively. Lane *1*, binding reaction between water and ^{32}P -labeled NF-κB probe. Lane *2*, binding reaction between ^{32}P -labeled NF-κB probe and nuclear extracts from cells that transfected with 4 μg control vector. Lane *3*, binding reaction between ^{32}P -labeled NF-κB probe and nuclear extracts from cells that co-transfected 2 μg control vector and 2 μg JNK3. Lane *4*, binding reaction between ^{32}P -labeled NF-κB probe and nuclear extracts from cells that transfected with 4 μg JNK3. Lane *5*, competition binding between labeled and unlabeled NF-κB probe with nuclear extracts from cells that transfected with 4 μg JNK3. Lane *6*, supershift reaction between ^{32}P -labeled NF-κB probe, nuclear extracts from cells that transfected with 4 μg JNK3, and the p65 antibody. Lane *7*, supershift reaction between ^{32}P -labeled NF-κB probe, nuclear extracts from cells that transfected with 4 μg JNK3, and the p50 antibody.

2.4 JNK3 decreased NF-κB target genes expression

We then evaluated the effects of JNK3 on the transcription of adhesion-related endogenous NF-κB target genes in Bel-7402 cells, and the mRNA levels of these genes were measured by real-time PCR. As shown in Figure 4, transfection of Bel-7402 cells with JNK3 plasmid down-regulated ENG and VCAM-1 mRNA level, compared with that in cells transfected with the empty vector; the AP-1 target gene TNC mRNA level was up-regulated in the presence of JNK3 ($P < 0.05$). These results suggest that JNK3 is an inhibitor of NF-κB.

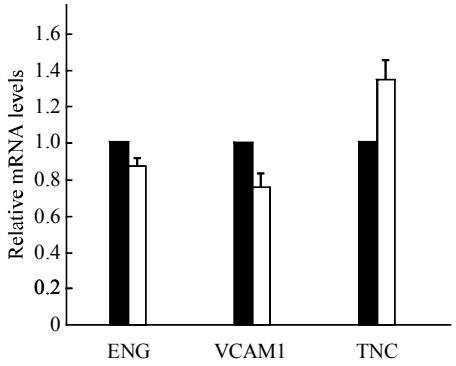

Fig. 4 JNK3 decrease NF-κB target gene expression

HEK293 cells were transfected with JNK3 expression vector or control vector. Real-time PCR was performed using primer sets specific for VCAM-1, ENG, TNC, and GAPDH. GAPDH was used as the internal control. PCR was performed in triplicates, and results were normalized to the endogenous control. Fold induction represented the relative expression of VCAM-1 or ENG mRNA in JNK3 transfected cells over that of controls ($P < 0.05$). The results were the $\bar{x} \pm s$ of triplicate experiments. ■: Control; □: JNK3.

2.5 JNK3 suppressed hepatoma cell adhesion

Finally, we examined the changes of cell adhension ability in Bel-7402 cells upon JNK3 tranfection by using a standard adhesion assay. We found that JNK3 transfection displayed reduced cell abilities to attach to type Ⅰ collagen, compared with that of cells transfected with empty vector (Figure 5). This is consistent with the above results that JNK3 decreased NF-κB target gene VCAM-1 and ENG mRNA level, both of which are involved in cell adhesion.

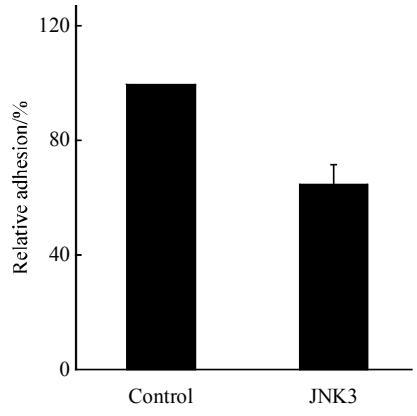

Fig. 5 Effect of JNK3 on Bel-7402 cell adhesion

Bel-7402 cells were transfected with JNK3 expression vector or control vector. Adherent cells were quantified by MTT assay and reaction products in each well were measured at 490 nm.

3 Discussion

JNKs are multifunctional kinases involved in many physiological processes and a large number of JNK substrates have been identified, which are involved in numerous intracellular functions, including: apoptosis, cytoskeletal rearrangements, transcriptional activation, and protein ubiquitination[23–24]. JNK can interact with and phosphorylate the N-terminal transactivation domain of c-Jun and thereby enhance its ability to transactivate related gene expression [24]. Moreover transcription factors such as JunB, JunD, c-Fos, ATF, ElK1, NFAT, and p53 can also be phosphorylated by JNK [11, 25–30]. Interestingly, JNK is able to phosphorylate Bcl-2 and Bcl-xL and diminish their anti-apoptotic activity [31], as well as phosphorylate the pro-apoptotic protein Bim and Bmf to promote their pro-apoptotic effects[32]. In this study, we first identified p65 as a partner of JNK3 by Y2H screening. Then, the interaction was confirmed *in vivo* by co-immunoprecipitation assay and *in vitro* by pull down assay. Moreover, JNK3 repressed the transcriptional activities of NF-κB in cultured cells by luciferase reporter assays and JNK3 decreased NF-κB binding to its cognate DNA sequences. Therefore, we proposed that JNK3 may function *in vivo* as a modulator in suppressing the transcriptional activity of p65.

The proteins of nuclear factor-κB (NF-κB)/Rel family are inducible transcription factors that play a central role in regulating the expression of a wide variety of genes associated with cell proliferation, immune response, inflammation, cell survival, and oncogenesis[33]. RelA/p65 is a subunit of NF-κB family, which shares an N-terminal Rel homology domain (RHD) with other members for DNA binding and homo- and heterodimerization. The activities of p65 are tightly regulated by the interaction with inhibitor of κB (IκB), which masks nuclear localization signal (NLS) and results in the cytoplasmic retention of p65. In most cells, NF-κBs are held captive in the cytoplasm from translocating to the nucleus by the IκB proteins. Dysregulation of NF-κB activation results in a variety of diseases, including various inflammatory diseases and different types of cancers through inducing the expression of target genes involved in cell proliferation, metastasis, angiogenesis, and anti-apoptosis [33]. In this study, we identified that overexpression JNK3 inhibited both E-selectin

reporter and ICAM-1 reporter luciferase activity in the presence or absence of TNFα, whereas IL-6 promoter activity was not repressed by JNK3. E-selectin and ICAM-1 are NF-κB target genes which is involved in carcinoma cell adhesion to the endothelium, an interaction thought to be required for tumor extravasation during metastasis [34]. We further identified another two adhesion related NF-κB target genes VCAM-1 and ENG were down-regulated by JNK3 at mRNA level through real-time PCR. These results suggest that JNK3 plays an important role in adhesion of tumor cell through regulating NF-κB pathway.

It has been reported that JNK is phosphorylated only in cells at the edge of the wound, and inhibition of JNK pathway blocks migration and lamellipodia extension [35]. Phosphorylated JNK is detected in focal adhesions [36], and it promotes cell migration through phosphorylating the focal adhesion protein paxillin [37]. Cell confluence was also accompanied by loss of p-JNK and c-Jun [38], while disruption of cell adhesion was accompanied by JNK activation [39]. Recently, JNK1 was reported to regulate junctions adherents formation through phosphorylating β-catenin [40]. In our study, we also found that JNK3 transfection displayed reduced abilities to attach to type Ⅰ collagen, which raised the possibility that JNK3 might be involved in the development of brain tumors.

In summary, we have identified JNK3 as a novel interactor for p65, which inhibited the binding of NF-κB to cognate DNA sequences and thereby transcriptional repression. The interaction of JNK3 and p65 might provide valuable insights into the regulatory mechanisms of tumor development.

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JNK3 通过与 RelA/p65 相互作用抑制 NF-κB 信号通路减弱 Bel-7402 细胞的黏附能力 *

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摘要　JNK 信号通路在细胞的炎症、增殖与凋亡等生物学过程中发挥了重要的作用．我们采用酵母双杂交技术发现转录因子 p65 是 JNK3 的相互作用蛋白质．体内体外实验均证实 JNK3 与 p65 存在蛋白质相互作用．报告基因实验结果表明过表达 JNK3 抑制 TNFα 诱导 NF-κB 介导的转录激活．EMSA 结果证明 JNK3 减弱 NF-κB 的 DNA 结合能力．实时定量 PCR 结果表明 JNF3 减少 NF-κB 靶基因的表达．综上所述，我们的研究结果表明 JNK3 做为一个调节分子在体内发挥了抑制 p65 转录活性的功能．

关键词　JNK3，NF-κB，p65，细胞黏附，蛋白质相互作用
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