# Interleukin-17 augments tumor necrosis factor α-mediated increase of hypoxia-inducible factor-1α and inhibits vasodilator-stimulated phosphoprotein expression to reduce the adhesion of breast cancer cells

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**Abstract.** Interleukin-17 (IL-17) and tumor necrosis factor (TNF)-α are able to cooperatively alter the expression levels of a number of genes. In the present study, the mRNA expression levels of hypoxia-inducible factor (HIF)-1α were analyzed in MDA-MB-231 breast cancer cells following treatment with IL-17, TNF-α or the combination of IL-17 and TNF-α. The protein expression levels of HIF-1α and vasodilator-stimulated phosphoprotein (VASP) were evaluated using western blot analysis. The adhesive ability of the cells was determined using an MTT assay following treatment with HIF-1α-small interfering RNA and short hairpin RNA-VASP that were used to suppress the expression levels of HIF-1α and VASP protein, respectively. These results demonstrated that IL-17 augmented TNF-α-induced gene expression of HIF-1α. The combination of IL-17 and TNF-α promoted an increase in HIF-1α expression and a decrease in VASP expression and a reduction in the adhesive ability of cells. These results demonstrated that IL-17 effectively enhanced the TNF-α-induced increase in HIF-1α and inhibited VASP expression, thus reducing the adhesion of MDA-MB-231 cells.

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## Introduction

Tumor necrosis factor (TNF)-α is a cytokine with numerous functions that serves an important role in cell survival, apoptosis, inflammation and immunity (1). TNF-α has been demonstrated to possess anti-tumor activity as it has cytostatic and cytotoxic effects on a number of cancer cell lines (2). However, previous studies have demonstrated that TNF-α may also affect matrix degradation and mediate the development of tumor metastases (3,4). Additionally, TNF-α regulates the expression levels of cell adhesion proteins and therefore may serve a role in determining the metastatic phenotype of tumor cells (5,6).

Interleukin (IL)-17 is a proinflammatory cytokine that is secreted by T helper 17 cells and it has been identified to have an important role in the host defense through its involvement in inflammatory and autoimmune diseases, including inflammatory bowel disease (7), multiple sclerosis (8) and rheumatoid arthritis (9). Notably, IL-17 has a complex role in tumor initiation, development and metastasis (10-12).

A number of previous studies have demonstrated that the combination of IL-17 and TNF-α promote the upregulation of gene expression (13,14), whereas treatment with only one of the two cytokines does not affect gene expression. Other previous studies have indicated that IL-17 augments the expression of TNF-α-induced genes, including granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) (15), keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, prostaglandin E2 (PGE2) and vascular endothelial growth factor (VEGF) (16). A previous study has suggested the potential hypothesis that IL-17 may promote the stability of TNF-α-induced mRNA (17). However, previous studies investigating the combination of IL-17 and TNF-α have focused on inflammatory angiogenesis (16), investigating the role of these cytokines in inflammation (14,15) and autoimmune diseases, including rheumatoid arthritis (13,18) and psoriasis (19). Therefore, the current study focused on the combination of IL-17 and TNF-α and its effect on tumor cells.

Vasodilator-stimulated phosphoprotein (VASP) is an Ena/VASP protein family member that has been associated with the microfilament system via promoting actin polymerization in a number of cell types (20,21). VASP has also been associated with the regulation of adherens junctions in epithelial cells (22). Clinical studies have revealed that VASP may be involved in the invasive biological behavior of lung adenocarcinomas, potentially through the regulation of focal adhesions, intracellular actin filament formation and cell migration (23). In previous studies, TNF-α was established to inhibit VASP expression via the TNF-α/hypoxia-inducible factor (HIF)-1α/VASP signaling pathway and modulate the adhesive and proliferative ability of breast cancer cells (24,25).

In the present study, the MDA-MB-231 breast cancer cell line was used to investigate the effects of co-stimulation with IL-17 and TNF-α on the levels of HIF-1α and VASP expression, as well as the cell adhesive ability.

## Materials and methods

*Cell culture, transfection and treatment.* The MDA-MB-231 human breast cancer cell line was obtained from the Department of Pathology and Pathophysiology, School of Medicine, Wuhan University (Wuhan, China). MDA-MB-231 cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% $CO_2$. The human HIF-1α CDS fragment (NM_001530.3) was amplified using polymerase chain reaction (PCR) from the pCGN-HAM-HIF-1α plasmid (24) using the following primers: Forward, 5'-CCG GAATTCCATGGAGGGCGCCGGCGGCGCGAACG-3' and reverse, 5'-CGCGGATCCGTTAACTTGATCCAAAGC TCTGAGT-3'. The PCR reaction system consisted of 100 ng of pCGN-HAM-HIF-1α plasmid DNA, 2 μl of forward primer (10 μmol/l), 2 μl of reverse primer (10 μmol/l), 25 μl of 2 GC buffer I (Takara Biotechnology Co., Ltd., Dalian, China) and 2 μl of Ex Taq DNA polymerase (cat. no. RRX001A; Takara Biotechnology Co., Ltd.). Thermocycling conditions were as follows: An initial 5 min incubation at 95°C; followed by 30 cycles of 15 sec at 95°C, 20 sec at 57°C and 150 sec at 72°C; and lastly 10 min at 72°. The PCR product was identified via agarose gel electrophoresis on a 1% gel. The fragment was inserted in the pEGFP-C1 (Clontech Laboratories, Inc., Mountainview, CA, USA) vector between the *Eco*RI and *Bam*HI restriction sites. All primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The DNA sequence of all constructs was verified by sequencing (Sangon Biotech Co., Ltd.). Transfection of the empty pEGFP-C1 vector was used as the control. Transient transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were cultured in serum-free RPMI-1640 for >8 h prior to treatment with IL-17 (Cell Signaling Technology, Inc., Danvers, MA, USA) and TNF-α (Invitrogen; Thermo Fisher Scientific, Inc.), or control medium (equal volumes of normal saline solution to IL-17 and/or TNF-α).

*Reverse transcription-quantitative PCR (RT-qPCR).* MDA-MB-231 cells were incubated for 6 h with 100 ng/ml IL-17, and various concentrations of TNF-α (0.1, 1 and 10 ng/ml) or combination of doses of IL-17 (1, 10 and 100 ng/ml) and TNF-α (0.1, 1 and 10 ng/ml). Subsequently, the cells were harvested for RT-qPCR analysis of HIF-1α mRNA. To further investigate the time course of HIF-1α induction following IL-17 and TNF-α stimulation, the transcript levels of HIF-α were measured during a 12 h period. The quantity of HIF-1α mRNA in MDA-MB-231 cells was determined at several time points (3, 6, 9 and 12 h) following treatment with 100 ng/ml IL-17 or 1 ng/ml TNF-α alone or in combination. Total RNA was extracted according to the manufacturer's protocol. In order to lyse the cells, 1 ml/well TRIzol® (Ambion; Thermo Fisher Scientific, Inc.) was added to the 6-well plate, which was then incubated and agitated for 5 min at room temperature. A total of 4 μg RNA was then reverse-transcribed to synthesize first-strand cDNA (final volume 20 μl) using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed in the presence of SYBR green using a Bio-Rad IQ5 Real-Time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers for human HIF-1α were as follows: Sense, 5'-GAAAGCGCAAGTCCT CAAAG-3'; antisense, 5'-TGGGTAGGAGATGGAGATGC-3'. The primers for β-actin were as follows: Sense, 5'-CATTAA GGAGAAGCTGTGCT-3'; antisense, 5'-GTTGAAGGTAGT TTCGTGGA-3'. The total volume for the RT-qPCR reaction was 20 μl. This consisted of 1 μl of a 1/5 water dilution of cDNA, 1 μl of forward primer (10 μmol/l), 1 μl of reverse primer (10 μmol/l), 10 μl of master mix (Bio-Rad Laboratories Inc.) and 7 μl nuclease-free water. RT-qPCR was performed using the following thermal cycling program: An initial 2 min incubation at 95°C followed by 40 cycles of 10 sec at 95°C, 15 sec at 52°C and 20 sec at 72°C. Fluorescence readings were taken during the extension step (72°C incubation). Following the cycling, melting was performed from 72 to 95°C at 0.5°C/sec, to obtain a melting curve. PCR reactions were run in triplicate within each experiment and the experiments were repeated >3 times. Results were calculated according to the $2^{-\Delta\Delta Cq}$ relative quantization method (26) using the β-actin gene for calibration.

*RNA interference.* The MDA-MB-231 cells were cultured in 6-well culture plates for 24 h at 37°C and the cells were cultured in antibiotic-free and serum-free RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) for >8 h at 37°C prior to TNF-α and IL-17 treatment. The cells were transiently transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. RNA extraction and RT-qPCR were performed as previously described (27). Briefly, the shRNA duplexes targeting VASP (GenBank accession no. BC038224) had the following sequence: 5'-TGCTGTAAAGCATCACAGTGGCCCGGGTTTTGGCCA CTGACTGACCCGGGCCAGTGATGCTTTA-3'. This sequence was inserted into the pcDNA6.2-GW/EmGFP vector (Invitrogen; Thermo Fisher Scientific, Inc.) to produce pcDNA6.2-GW/EmGFP-shR-VASP insert-containing vectors as has been previously described (27). A scrambled shRNA (5'-GAAATGTACTGCGCGTGGAGACGTTTT GGCCACTGACTGACGTCTCCACGCAGTACATTT-3')

was obtained from Invitrogen (Thermo Fisher Scientific, Inc.) and used as the negative control in all experiments. The small interfering (si) RNA duplex oligonucleotides targeting HIF-1α (GenBank accession no. NM_001530) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence for HIF-1α-siRNA was: 5'-CUGAUGACCAGC AACUUGAdTdT-3'. A scrambled-siRNA, sequence 5'-AGU UCAACGACCAGUAGUCdTdT-3', was utilized as a control. Following incubation for 8-9 h at 37°C and 5% $CO_2$, the transfection reagent was removed. Medium with antibiotic and serum was added to the plate and the cells were cultured for 24 h at 37°C.

*Western blotting*. Following treatment with 100 ng/ml IL-17, 1 ng/ml TNF-α or the combination for 6 h, the total protein of the cells was extracted. The MDA-MB-231 cells were washed three times with ice-cold PBS and lysed in a modified radioimmunoprecipitation assay buffer (Biyuntian, Shanghai, China) containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM $Na_3VO_4$, 1 mM EDTA, 1 mM EGTA and 1x protease inhibitor cocktail. The protein concentration was measured with the Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal quantities of total protein (10 $\mu$g) were separated by 10% (v/v) SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche Applied Science, Pleasanton, CA, USA). Membranes were blocked for 1 h at room temperature with 5% powdered skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBST) and probed with VASP antibody (dilution, 1:1,000; cat. no. 3112; Cell Signaling Technology, Inc.), HIF-1α antibody (dilution, 1:1,000; cat. no. ab210073; Abcam, Cambridge, UK) or GAPDH antibody (dilution, 1:5,000; cat. no. A0080, ABclonal Biotech Co., Ltd., Cambridge, MA, USA) at 4°C overnight and washed with TBST. This was followed by incubation with horseradish peroxidase (HRP)-linked secondary antibodies (dilutions 1:40,000; cat. nos. 7071 and 7072, Cell Signaling Technology, Inc.) for 1 h at room temperature. Washing was performed with TBST and detection occurred using enhanced chemiluminescence (ECL) western blotting detection reagents (Advansta, Inc., California, USA). The experiments were repeated 3 times with similar results. The bands were visualized using WesternBright ECL HRP substrate (Advansta, Inc.) and developed using Kodak film (Kodak, Rochester, NY, USA) in a darkroom. Quantification of band densities was performed using Image J (version no., 1.6.0_20, National Institutes of Health, Bethesda, MD, USA).

*Cell adhesion assay*. Following transfection, the MDA-MB-231 cells were seeded at a density of 1x10⁵ per ml (100 $\mu$l per well) in 96-well plates coated with fibronectin (100 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA). Following a 2 h incubation at 37°C in an incubator containing 5% $CO_2$, the cells were washed with PBS to remove non-adherent cells. A total of 10 $\mu$l MTT (5 mg/ml, Amresco, LCC, Solon, OH, USA) was added to each well. Following 4 h of additional incubation, the supernatant was discarded and 100 $\mu$l dimethyl sulfoxide was added to each well to dissolve the formazan crystals and culture plates were agitated on a horizontal shaker for 10 min. Absorbance values were determined by using an ELISA reader (Infinite®

200 PRO; Tecan Group Ltd., Männedorf, Switzerland) at a wavelength of 490 nm. The percentage of adhesive cells was calculated according to the following formula: Percentage of adhesion=[optical density (OD) 490 of cells treated/OD490 of cells untreated]x100%. Three independent experiments were performed in triplicate.

*Statistical analysis*. All data were expressed as the mean + standard deviation. The statistical significance of the differences observed between experimental groups was determined using the Student's *t*-test. Data were analyzed using GraphPad Prism software version 6.0.2 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

*IL-17 augments TNF-α-induced HIF-1 α gene expression in MDA-MB-231 breast cancer cells*. As presented in Fig. 1, compared with the control group, stimulation with IL-17 alone did not affect the expression of HIF-1α mRNA and the use of TNF-α alone at various concentrations had a minimal effect on HIF-1α mRNA levels. Similarly, the effects of treatment with low dose (1 ng/ml) IL-17 and TNF-α on expression levels of the HIF-1α gene were also mild. However, the moderate (10 ng/ml) and the high (100 ng/ml) concentrations of IL-17 significantly increased (P<0.01) the ability of TNF-α to induce HIF-1α mRNA expression levels compared with TNF-α alone in a dose-dependent manner.

To further investigate the time course of HIF-1α induction following IL-17 and TNF-α stimulation, the transcript level of HIF-α was measured during a 12 h period. The results presented in Fig. 1B demonstrate that the expression levels of HIF-1α were not affected by IL-17 or TNF-α alone. However, a rapid and sustained increase in HIF-1α mRNA levels was identified following stimulation with the combination of drugs compared with the control treatments. The highest level of HIF-1α mRNA was observed at 6 h following treatment and this was ~10X higher compared with the control cells.

*The combination of IL-17 and TNF-α reduces VASP expression levels and suppresses cell adhesion*. The adhesive ability of tumor cells to the extracellular matrix (ECM) may be an important event in the progression to metastasis (28). VASP possesses an important role in the regulation of cell adhesion (29). Previous studies have established that HIF-1α binds to the VASP promoter and this inhibits VASP transcription, which suppresses cell adhesion (24). To investigate the potential role of IL-17 and TNF-α in cell adhesion, the protein expression levels of HIF-1α and VASP were examined in MDA-MB-231 cells treated with IL-17, TNF-α or a combination of the two. The HIF-1α and VASP expression levels were detected using western blotting. As presented in Fig. 2A, stimulation with IL-17 or TNF-α alone did not affect HIF-1α or VASP expression levels. However, the combination of IL-17 and TNF-α significantly increased HIF-1α expression levels by 38.5% (P<0.01) and decreased VASP expression levels by 51% (P<0.01). These results were significantly different compared with the TNF-α treatment group. Additionally, the adhesive ability of cells was evaluated. It was observed that the
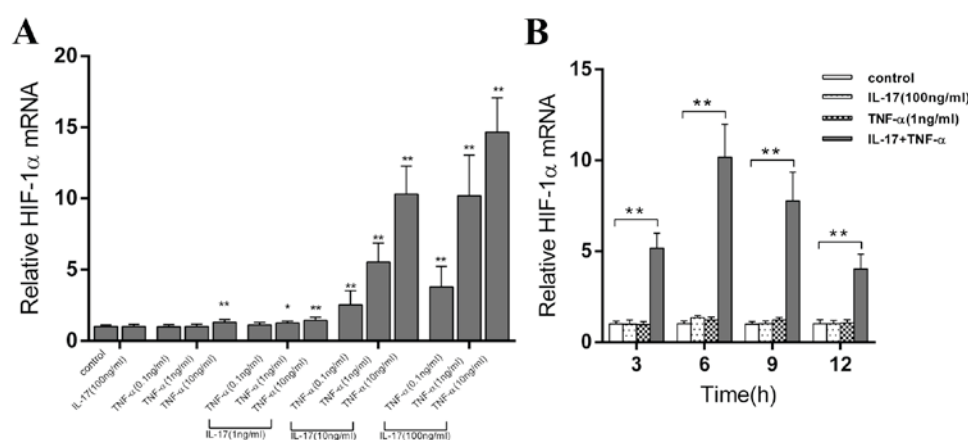
Figure 1. IL-17 augments TNF-α-induced HIF-1α gene expression in MDA-MB-231 breast cancer cells. (A) A range of concentrations of IL-17 and TNF-α alone or in combination were used to induce the expression levels of HIF-1α in MDA-MB-231 cells. The cells were stimulated for 6 h and the HIF-1α mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. The data values represent the mean + standard deviation of 4 separate experiments. IL-17 or TNF-α groups were compared with the control group. IL-17 and the specified doses of TNF-α were compared with the specified doses of TNF-α alone. (B) MDA-MB-231 cells were treated for 3, 6, 9 and 12 h with 100 ng/ml IL-17, 1 ng/ml TNF-α or the two in combination, and the HIF-1α mRNA levels were evaluated. $^*P<0.05$, $^{**}P<0.01$. IL-17, interleukin-17; TNF, tumor necrosis factor; HIF, hypoxia-inducible factor.
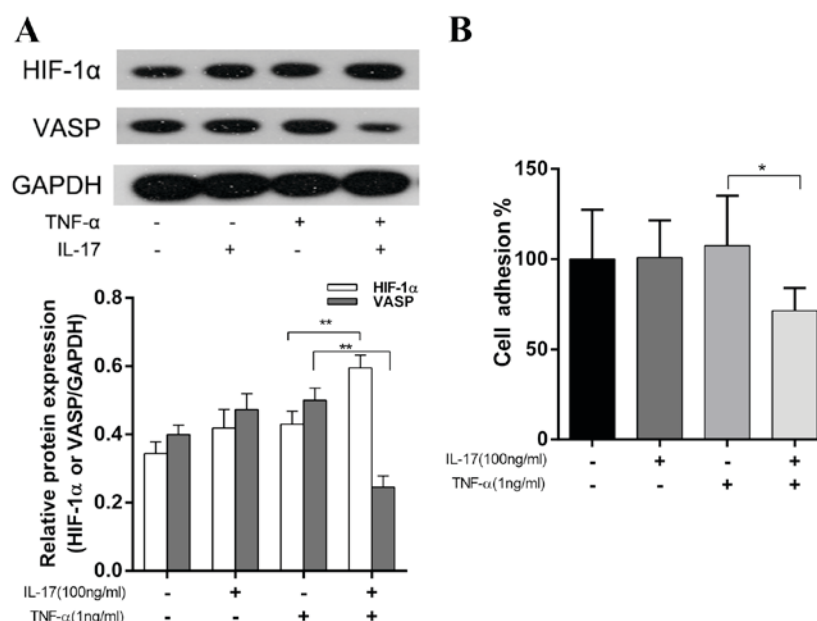


Figure 2. Combined treatment with IL-17 and TNF-α decreases VASP expression levels and reduces cell adhesion. MDA-MB-231 cells were treated with 100 ng/ml IL-17, 1 ng/ml TNF-α or the two in combination for 6 h. (A) The HIF-1α and VASP protein expression levels of total cell protein were detected by western blotting. (B) The cell adhesive ability was examined using an MTT assay. The results were presented as the mean + standard deviation of 3 independent experiments. $^*P<0.05$, $^{**}P<0.01$. IL-17, interleukin-17; TNF, tumor necrosis factor; HIF, hypoxia-inducible factor; VASP, vasodilator-stimulated phosphoprotein.

cell adhesion ability in the IL-17 and TNF-α treatment group was reduced by 33.5% (P<0.05) compared with the TNF-α treatment group (Fig. 2B). These results were in accordance with the effects on VASP expression levels observed following this combination drug treatment (Fig. 2A).

*HIF-1α-siRNA knockdown in MDA-MB-231 cells reduces cell adhesive abilities and the inhibition of VASP expression following IL-17 and TNF-α treatment.* To evaluate the association between IL-17 and TNF-α combination drug treatment and HIF-1α and VASP expression levels, MDA-MB-231 cells were transfected with HIF-1α-siRNA. MDA-MB-231 cells

were also transfected with scrambled-siRNA as a control. It was observed that, following knockdown of HIF-1α expression levels, VASP protein expression levels were significantly increased (P<0.05) compared with the control group (Fig. 3A). By contrast, the cells transfected with pEGFP-Cl-HIF-1α in order to overexpress HIF-1α exhibited a significant decrease (P<0.01) in VASP protein compared with the empty vector pEGFP-C1 control group (Fig. 3A). These results indicate that HIF-1α may be involved in the regulation of VASP expression levels. The role of HIF-1α in the effect of IL-17 and TNF-α combination treatment on VASP expression levels was investigated. MDA-MB-231 cells were transfected
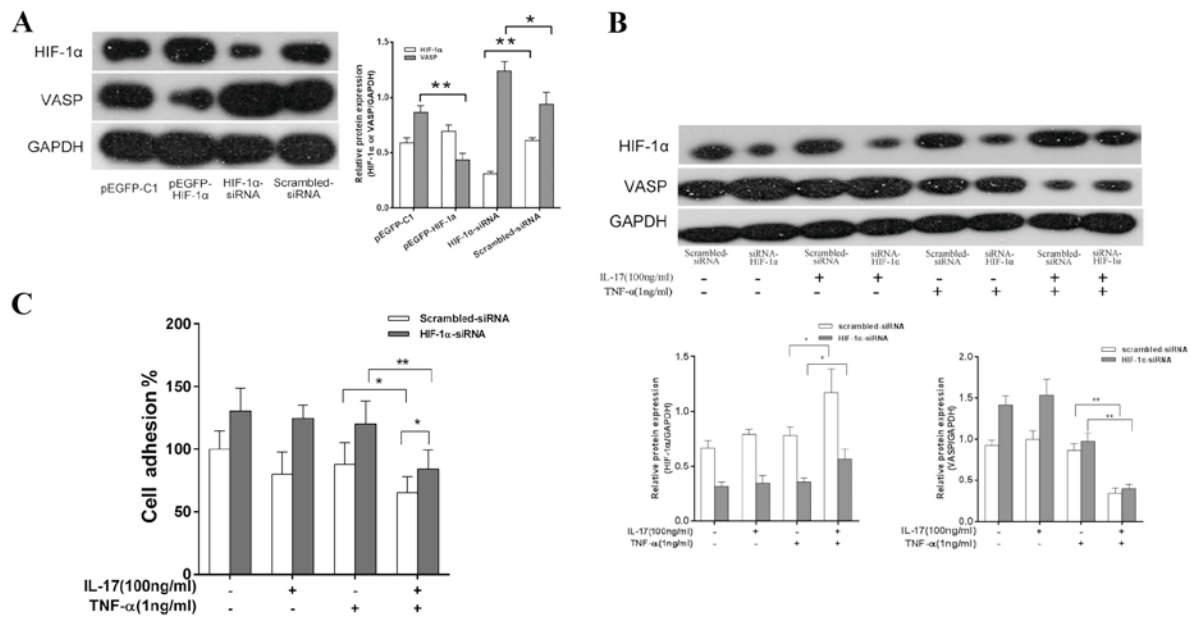
Figure 3. Transfection with HIF-1α-siRNA mitigated the reduction of VASP expression and promoted adhesive ability in the MDA-MB-231 cells treated with IL-17 and TNF-α. (A) MDA-MB-231 cells were transfected with siRNA-HIF-1α or pEGFP-C1-HIF-1α for 24 h. Scrambled siRNA and pEGFP-C1 vector transfection was used as the controls. The HIF-1α and VASP protein expression levels were determined using western blotting. GAPDH was used as a loading control. (B) Cells were treated 100 ng/ml IL-17 or 1 ng/ml TNF-α or in combination following transfection with siRNA-HIF-1α for 24 h. Scrambled siRNA was used as a control. The HIF-1α and VASP protein expression levels were determined using western blotting. (C) Cell adhesion was examined using an MTT assay. The results were presented as the mean + standard deviation of 3 independent experiments. *P<0.05, **P<0.01. IL-17, interleukin-17; TNF, tumor necrosis factor; HIF, hypoxia-inducible factor; VASP, vasodilator-stimulated phosphoprotein; siRNA, small interfering RNA.

with HIF-1α-siRNA or scrambled-siRNA and treated with 100 ng/ml IL-17, 1 ng/ml TNF-α or IL-17 and TNF-α. Western blotting was used to detect HIF-1α and VASP protein expression levels and the adhesive ability of cells was also evaluated. As presented in Fig. 3B, there were no significant differences in the protein levels of VASP or HIF-1α in the IL-17 or TNF-α treatment groups compared with the control group. However, compared with the TNF-α alone treatment group, in the IL-17 and TNF-α combination treatment group, the HIF-1α expression levels in cells transfected with scrambled-siRNA were significantly increased (P<0.05) and the VASP protein levels were significantly decreased (P<0.01). Additionally, the increase of HIF-1α levels (P<0.05) and decrease in VASP expression levels (P<0.01) following the combination treatment were reduced in the cells transfected with HIF-1α-siRNA compared with those treated with TNF-α alone. Similarly, to cells treated with a combination of IL-17 and TNF-α, the adhesive ability of MDA-MB-231 cells transfected with HIF-1α-siRNA was significantly increased (P<0.05) compared with cells transfected scrambled-siRNA (Fig. 3C).

*IL-17 and TNF-α enhance the reduction of VASP expression levels and decrease the adhesive ability of the MDA-MB-231 cells transfected with shRNA-VASP.* To investigate the association between VASP and HIF-1α, MDA-MB-231 cells were transfected with shRNA-VASP (pcDNA6.2-GW/EmGFP-shR-VASP vector) to inhibit VASP expression levels. The cells were transfected with scrambled shRNA (pcDNA6.2-GW/EmGFP-MIR) as a control. Following incubation for 6 h with either the vehicle control, 100 ng/ml IL-17, 1 ng/ml TNF-α or IL-17 and TNF-α, the

HIF-1α and VASP expression levels in MDA-MB-231 cells were evaluated using western blotting and the adhesive ability of the cells was also analyzed. As presented in Fig. 4A, VASP expression levels in cells transfected with shRNA-VASP were decreased by 67.3% compared with those transfected with scrambled-shRNA in the vehicle control groups (P<0.01), which suggests that shRNA-VASP was effectively transfected and exhibited a clear inhibitory effect on VASP expression levels. HIF-1α protein expression levels in cells transfected with scrambled-shRNA or shRNA-VASP in the vehicle control groups were not significantly different. In the IL-17 and TNF-α combination treatment group, VASP expression levels in the shRNA-VASP and scrambled-shRNA transfected cells were reduced by 33.2 and 77.9%, respectively (P<0.05 and P<0.01), compared with cells in the TNF-α treatment group. The variation in the degree of protein reduction between the two methods of knockdown indicates that treatment with shRNA-VASP and the combination of IL-17 and TNF-α may provide more effective inhibition of VASP expression compared with shRNA-VASP treatment alone. This indicates that the combination of IL-17 and TNF-α may provide a robust method for inhibiting VASP expression levels. The adhesive ability of cells was decreased in the groups that also exhibited a reduction in VASP expression levels and the strength of the effect was concordant with the level of VASP reduction (Fig. 4B).

## Discussion

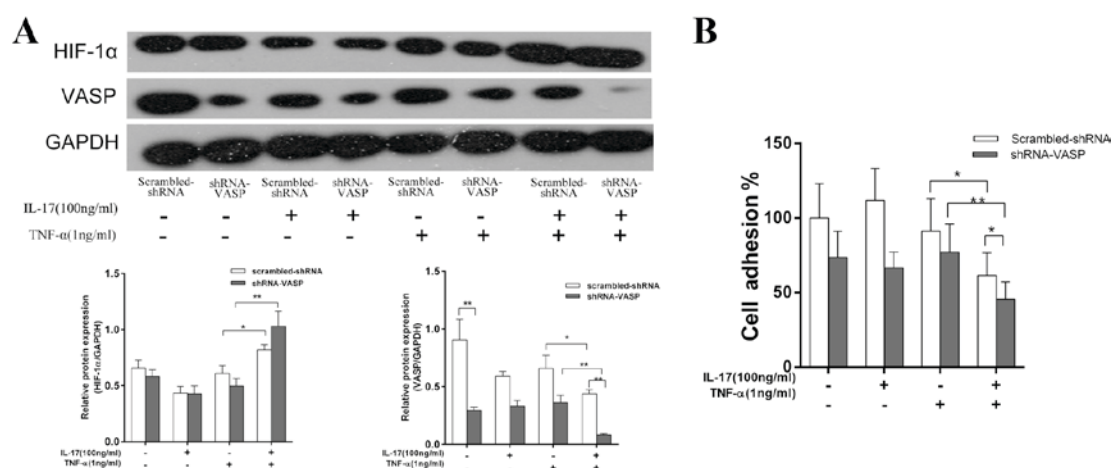IL-17 and TNF-α are associated and typically observed in acute and chronic inflammation and the effects of combined

Figure 4. IL-17 and TNF-α enhance the suppression of VASP expression levels and decrease the adhesive ability of MDA-MB-231 cells following transfection with shRNA-VASP. (A) The cells were treated 100 ng/ml IL-17, 1 ng/ml TNF-α or the two in combination following transfection with shRNA-VASP for 24 h. Scrambled-shRNA was used as a control. The HIF-1α and VASP protein expression levels were determined using western blotting. (B) Cell adhesion was examined using an MTT assay. The results were presented as the mean + standard deviation of three independent experiments. $^*P<0.05$, $^{**}P<0.01$. IL-17, interleukin-17; TNF, tumor necrosis factor; HIF, hypoxia-inducible factor; VASP, vasodilator-stimulated phosphoprotein; shRNA, short hairpin RNA.

IL-17 and TNF-α treatment is, therefore, biologically relevant to the interaction between inflammation and cancer (30). A previous study has established that the combination of IL-17 and TNF-α induces the activation of certain genes, including HIF-1α (13), neutrophil gelatinase-associated lipocalin (14), whereas IL-17 or TNF-α alone did not produce a significant effect. Other previous studies (15,16,18) have also demonstrated that IL-17 augments TNF-α-induced gene expression, including G-CSF, GM-CSF, KC, MIP-2, PGE2 and VEGF. A potential underlying mechanism that has been hypothesized is that IL-17 may promote the stability of TNF-α-induced mRNA (7).

In the current study, MDA-MB-231 cells were incubated with a number of doses of TNF-α (0.1, 1, 10 ng/ml) and the results demonstrate that HIF-1α mRNA expression levels were increased in the high dose group (10 ng/ml). The low, moderate and high doses of IL-17 (1, 10, 100 ng/ml, respectively) were also investigated in combination with each of the TNF-α doses. These results indicated that HIF-1α mRNA levels were increased in the combined IL-17 and TNF-α groups in a dose-dependent manner. The increase of HIF-1α mRNA levels was markedly increased in cells treated with the moderate (1 ng/ml) and high (10 ng/ml) doses of TNF-α in combination with IL-17. Therefore, these results suggest that IL-17 may increase the effect of TNF-α on its mediation of HIF-1α mRNA expression levels (Fig. 1A).

A number of distinct physiological and pathological processes are dependent on the adhesive and migratory abilities of cells, including the immune response, tissue morphogenesis and cancer metastasis (31). Cell-to-cell and cell-to-ECM interactions are important in the ability to metastases and a number of families of adhesion molecules, including cadherins, selectins, integrins and the immunoglobulin superfamily (28,32,33) mediate these interactions. Additionally, adhesion dynamics require coordination with new protrusions containing F-actin being assembled, which are controlled by various elongating and actin nucleating molecules (34). It has been demonstrated that a reduction in the adhesive ability of tumor cells to attach

to ECM proteins may provide novel targets for therapeutic intervention (28). VASP is a member of the Ena/VASP protein family, which is associated with a number of diseases, including cancer, thrombosis, cardiomyopathy, arteriosclerosis and nephritis (35). Ena/VASP proteins have been established as regulators of actin-associated processes, including axon outgrowth and guidance, epithelial cell adhesion, cell motility, cell polarity and pathogen F-actin tail formation (36). In epithelial cells, VASP has also been identified as contributing to cell-cell adhesion via the regulation of actin polymerization and bundling (37). VASP affects F-actin filament elongation and bundling by localizing to regions of dynamic actin reorganization, including focal adhesions and filopodia at the leading edge in motile cells (38,39).

In previous studies, high-dose TNF-α has been indicated to mediate VASP expression via the TNF-α/HIF-1α/VASP signaling pathway, promoting the alteration of cell adhesiveness, whereas low-doses of TNF-α did not exhibit a significant effect (24,25). In the present study, TNF-α (1 ng/ml) was not able to significantly affect HIF-1α mRNA expression levels compared with the control group; however, when used in combination with IL-17 (100 ng/ml), breast cancer cells exhibited alterations to the protein expression levels of HIF-1α and VASP and the ability of cells to adhere. These results indicated that the combination TNFα and IL-17 treatment significantly increased the protein expression levels of HIF-1α and decreased the protein expression levels of VASP and subsequently led to a decline in the adhesive ability of cells.

To further investigate the distinct association between HIF-1α and VASP following treatment with a combination of IL-17 and TNF-α, MDA-MB-231 cells were transfected with HIF-1α-siRNA and shRNA-VASP. The results indicated that HIF-1α-siRNA decreased the level of HIF-1α and increased VASP protein expression, which promoted MDA-MB-231 cell adhesion. Following stimulation with IL-17 and TNF-α, HIF-1α expression was increased and the VASP expression levels were decreased. However, this inhibitory effect was altered by the knockdown of HIF-1α. Additionally, it was

also observed that the knockdown of VASP by VASP shRNA, markedly reduced the adhesion of the MDA-MB-231 cells and reduced the levels of VASP protein expression compared with the scrambled shRNA control. The levels of HIF-1α were not altered following transfection. These results indicate that HIF-1α may be upstream of VASP in signaling pathways that control the adhesion of MDA-MB-231 cells. The treatment of IL-17 and TNF-α was able to inhibit VASP expression levels to a higher extent compared with shRNA-VASP alone, which suggests that IL-17 and TNF-α may be used to further inhibit VASP expression levels. These results demonstrate that HIF-1α may mediate the repression of VASP expression levels via IL-17 and TNF-α in MDA-MB-231 cells.

In conclusion, the current study has demonstrated that IL-17 may enhance the TNF-α-induced increase HIF-1α inhibition VASP expression, which reduces the adhesive ability of MDA-MB-231 breast cancer cells. Therefore, targeting IL-17 and TNF-α may provide a novel insight into potential anti-tumor signaling pathways.

## Acknowledgements

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