

## Honokiol Inhibits Constitutive and Inducible STAT3 Signaling via PU.1-Induced SHP1 Expression in Acute Myeloid Leukemia Cells

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Constitutive and inducible activation of signal transducer and activator of transcription 3 (STAT3) signaling facilitates the carcinogenesis in most human cancers including acute myeloid leukemia (AML). Negative regulators, such as protein tyrosine phosphatases SHP1, inhibit the activated STAT3 signaling. In this study, we investigated the effect of honokiol (HNK), a constituent of *Magnolia officinalis*, on the STAT3 signaling. STAT3 signaling and SHP1 expression were measured by quantitative real-time PCR and western blotting in leukemic cell lines and primary AML blasts treated with HNK. HNK decreased the phosphorylated STAT3 but not the total STAT3 through increasing the expression of SHP1. In addition, HNK inhibited transcription activity of STAT3, reduced nuclear translocation of STAT3, and decreased the expression of STAT3 target genes. Knockdown of SHP1 by small hairpin RNA (shRNA) or treatment with vanadate, a protein tyrosine phosphatases inhibitor, abolished HNK-induced STAT3 inhibition, suggesting that SHP1 plays an important role in the inhibition of STAT3 signaling by HNK. Further, HNK increased the expression of transcript factor PU.1, which had been reported to activate the expression of SHP1 via binding SHP1 promoter region. Knockdown of PU.1 reversed HNK-induced upregulation of SHP1 and inactivation of STAT3 signaling. Finally, HNK increased the expression of PU.1 and SHP1 in hematopoietic progenitors isolated from patients with AML. In conclusion, our data have shown a regulatory mechanism underlying the inhibition of STAT3 signaling by HNK. Therefore, as a relative non-toxic compound, HNK may offer a therapeutic advantage in the clinical treatment for AML.

**Keywords:** acute myeloid leukemia; honokiol; PU.1; SHP1; STAT3

Tohoku J. Exp. Med., 2015 November, 237 (3), 163-172. © 2015 Tohoku University Medical Press

### Introduction

The signal transducer and activator of transcription (STAT) signaling regulates cell proliferation, survival, differentiation, and immune response by the transmission of cytokine signals from cell surface to the nucleus. Constitutive activation of STAT signaling has often been discovered in hematopoietic malignancies including acute myeloid leukemia (AML), T-cell large granular lymphocytic leukemia, Hodgkin lymphoma and myeloproliferative neoplasm (Lin et al. 2000; Van Roosbroeck et al. 2011; Teramo et al. 2013). Mutations of upstream receptors such as FMS-like tyrosine kinase 3- internal tandem duplication (FLT3-ITD), c-KIT, and granulocyte colony stimulating factor receptor (G-CSFR), and NRAS caused constitutive

activation of STAT signaling, which finally resulted in uncontrolled proliferation and prevention of apoptosis in AML (Weiler et al. 1996; Dong et al. 1997; Stirewalt and Radich 2003; Bacher et al. 2006). Moreover, BCR-ABL oncoprotein and other translocations also activated this pathway (Frank and Varticovski 1996). Constitutively activated JAK2/STAT signaling, which was mainly caused by the JAK2 V617F mutation and the W515L mutation of the myeloproliferative leukemia (MPL) gene, was also found in myeloproliferative neoplasm and was considered as the main pathogenesis for patients with myeloproliferative neoplasm (James et al. 2005). Therefore, emerging evidence has supported this pathway has an important role in oncogenesis and has been extensively validated as a molecular therapeutic target pathway for human cancer treatment

Received July 6, 2015; revised and accepted September 15, 2015. Published online October 14, 2015; doi: 10.1620/tjem.237.163.

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(Bromberg et al. 1999).

SHP1 is encoded by the protein tyrosine phosphatase, non-receptor type 6 (PTPN6) gene and primarily expressed in hematopoietic and epithelial cells. SHP1 decreases cell activation by binding and de-phosphorylating the receptor associated tyrosine kinases, such as JAK2/STAT signaling, subsequently leading to the inhibition of STAT signaling. However, loss of SHP1 has been associated with aberrant activation of JAK2/STAT signaling in several hematological malignancies, such as multiple myeloma (Chim et al. 2004a), anaplastic large-cell lymphoma (Han et al. 2006), cutaneous T-cell lymphoma (Witkiewicz et al. 2007), and acute leukemia (Chim et al. 2004b). The mechanism of SHP1 loss was due to the epigenetic silencing by aberrant hypermethylation in promoter region rather than inactivation mutation (Chim et al. 2004a, b). Therefore, restoring expression of SHP1 might facilitate the inactivation of JAK2/STAT signaling in hematological malignancies.

Honokiol (HNK), a small molecular weight natural product derived from the stem and bark of the plant *Magnolia officinalis* (Fried and Arbiser 2009), has been reported to possess potent anti-neoplastic and anti-angiogenic properties through targeting multiple signaling pathways including PI3K/Akt, STAT3, nuclear factor-kappa B (NF-kappaB), epidermal growth factor receptor (EGFR), and mammalian target of rapamycin (m-TOR) (Arora et al. 2012). For example, HNK induced G1 cell cycle arrest and apoptosis by reducing the expression of cyclins D, cellular inhibitor of apoptosis 2 (cIAP2), X-linked inhibitor of apoptosis protein (XIAP), and survivin in adult T-cell leukemia (Ishikawa et al. 2012). HNK suppressed transcription factor NF-kappaB activation and NF-kappaB-regulated gene expression through the inhibition of IkappaB kinases (Tse et al. 2005). Furthermore, HNK inhibited cell growth and induced apoptosis via decreasing expression of phosphorylated STAT3 and phosphorylated ERK1/2 in human Barrett's epithelial and esophageal adenocarcinoma cells (Yu et al. 2012). HNK inhibits STAT3 signaling via increasing SHP1 expression in hepatocellular carcinoma cells (Rajendran et al. 2012). However, the mechanism by which HNK induces SHP1 expression in AML cells remains unclear.

In the present study, we investigated the action of HNK on STAT3 signaling in leukemic cells. HNK inhibited p-JAK2 and p-STAT3 via PU.1-induced upregulation of SHP1. Our findings provide new insight into the mechanism of HNK action in leukemic cells and suggest a novel approach to prevention and/or therapy of AML.

## Materials and Methods

### Cell lines and primary AML blasts

Human leukemic cell lines (HEL and THP1), multiple myeloma U266 cell line, and IL-3 dependent murine myeloid cell line 32D from Shanghai cell bank of Chinese Academy of Sciences were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in humidified 37°C

Table 1. AML Patients' characteristics.

Characteristic	All patients N (%)
Overall	30
Gender	
Male	17 (56.7)
Female	13 (43.3)
Age (range)	52 (41-68)
FAB	
M0-M1	1 (3.3)
M2	7 (23.3)
M3	4 (13.3)
M4	10 (33.3)
M5	8 (26.7)
Cytogenetics	
Normal karyotype	16 (53.3)
t (15; 17)	4 (13.3)
t (8; 21)	3 (10)
Inv (16)	2 (6.7)
+8	1 (3.3)
+11	1 (3.3)
Not available	3 (10)

incubator with 5% CO<sub>2</sub>. Primary AML cells (blasts% > 70%, Table 1) were obtained from 30 AML patients which all gave informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Medical Science Ethic Committee of Wenzhou Medical University. Bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation (GE Healthcare, Uppsala, Sweden) and were cultured in the same condition as described above. Purified HNK (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethyl alcohol and kept at -20°C until use. Sodium orthovanadate (Selleck, Shanghai, China) was dissolved in distilled water and kept at -20°C until use.

### mRNA extract and quantitative real-time PCR

Total RNA from HNK-treated or untreated cells was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentration and quality were quantified by measuring the absorbance at 260 nm with Beckman DU6400 spectrophotometer (Beckman Counter, Miami, FL, USA) and gel analysis. Relative expression was calculated using the 2<sup>-ΔΔCt</sup> method. The primers of SHP1 and other gene transcripts are indicated in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used for normalization.

### STAT3 luciferase reporter assay

293T cells were seeded in 24-well plates for 24 h before transfection. The STAT3-responsive elements linked to a luciferase reporter gene and inter control vector were transfected into 293T cells using Hiperfect transfection reagent (Qiagen, Valencia, CA, USA). After 24 h transfection, cells were treated for HNK. Cell lysates were harvested and firefly and renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Table 2. The sequences of primers for quantitative real-time PCR and construction of plasmids.

Primer	Sequence (5'-3')	Position in mRNA sequence
SHP1-L	TTT CAA GAA GAC GGG GAT TG	821
SHP1-R	CGG ACT CCT GCT TCT TGT TC	951
PU.1-L	GCA AGA AGA AGA TCC GCC TG	723
PU.1-R	CCT TGT GCT TGG ACG AGA AC	845
Bcl-2-L	ATC CAG GAT AAC GGA GGC TG	1,058
Bcl-2-R	GAA ATC AAA CAG AGG CCG CA	1,129
Bcl-xl-L	TAT TGG TGA GTC GGA TCG CA	848
Bcl-xl-R	GTT CTC CTG GAT CCAAGG CT	921
Cyclin D1-L	CCC CGCACG ATT TCA TTG AA	676
Cyclin D1-R	CGT GTT TGC GGA TGA TCT GT	753
Survivin-L	CAG ACTTGG CCCAGT GTT TC	276
Survivin-R	TTG GTT TCC TIT GCA TGG GG	356
GAPDH-L	GGT CGG AGT CAA CGG ATT TG	203
GAPDH-R	ATG AGC CCCAGC CTT CTC CAT	521
sh-SHP1	GCA AGA ACC GCT ACA AGA A	
sh-PU.1	GCC CTA TGA CAC GGA TCT ATA	
MSCV-PU.1-L	GAA GAT CTG ACC GAC TCG GAG CCC GGC TGG	
MSCV-PU.1-R	CGG AAT TCC GGC GGG GGC TGC GGG CTC AGT	

#### Immunocytochemistry for STAT3 localization

HNK-treated leukemic cells were plated on a glass slide and fixed with 4% formaldehyde for 30 minutes, followed with methyl alcohol for 20 minutes. Slides were blocked with 5% albumin for 2 h, and then incubated with a rabbit polyclonal anti-human STAT3 antibody (dilution 1:100) overnight. Slides were washed and then incubated with anti-rabbit immunoglobulin-FITC (dilution 1:100) for 1 h and counterstained for nuclear by DAPI (0.2 µg/mL) for 15 min. The staining was analyzed under a fluorescence microscope (Olympus BX51, Tokyo, Japan) and Imstar FISH Progress software to capture the pictures.

#### Apoptosis detection

AML blasts were plated in triplicate at  $2 \times 10^5$  cells/ml. After being treated with HNK, apoptosis was detected by Annexin V/PI assay (Invitrogen, Carlsbad, CA, USA) and samples were analyzed by flow cytometry (Becton Dickinson, San Diego, CA, USA) within 30 min after staining.

#### Plasmid construction

To produce PU.1 overexpression plasmid, human PU.1 coding sequence (NM\_003120) was amplified by PCR and then cloned into retrovirus vector pMSCV-puro (Clontech, Palo Alto, CA, USA). Gene-specific short hairpin RNAs (shRNAs) for SHP1 and PU.1 were designed and cloned into retroviral vector pSIREN-RetroQ (Clontech). Control shRNA is a nonfunctional construct provided from Clontech. All the primer sequences for plasmid construction were indicated in Table 2.

#### Western blot analysis

Western blot analysis was performed using standard techniques. The following antibodies were used: SHP1, JAK2, p-JAK2<sup>Tyr1007</sup>, STAT3, p-STAT3<sup>Tyr705</sup>, and PU.1 (Cell Signaling Technology, Beverly, MA, USA); and Bcl-xl, Bcl-2, cyclin D1, regulatory factor X-1

(RFX-1), and survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As necessary, blots were stripped and reprobed with β-actin antibody (Santa Cruz Biotechnology) as an internal control. Signals were detected with chemiluminescence reagents (Cell Signaling Technology). All experiments were repeated three times with the similar results.

#### Statistical analysis

The significance of the difference between groups was determined by Student's t-test. A *P* value of less than .05 was considered statistically significant. All statistical analyses were performed with SPSS software (Chicago, IL, USA).

## Results

### HNK inhibits constitutive and inducible STAT3 phosphorylation

In order to determine whether HNK inhibited STAT3 signaling in leukemic cells, HEL (Wang et al. 2009) and THP1 cells (Vuletic et al. 2011) presenting constitutive activation of STAT3 were treated with 40 µM HNK for 24 h, because 40 µM HNK significantly induced cell growth arrest (Li et al. 2015). HNK obviously decreased the production of p-JAK2<sup>Tyr1007</sup> and p-STAT3<sup>Tyr705</sup> but did not affect the total protein levels of JAK2 and STAT3 in HEL and THP1 cells (Fig. 1A, B). Next, we explored whether HNK inhibited the inducible activation of STAT3. IL-3-treated 32D cells, in which STAT3 was activated, were incubated with HNK. Similarly, HNK reduced JAK2 and STAT3 phosphorylation but not the total levels of JAK2 and STAT3 (Fig. 1C). We further used multiple myeloma U266 cell line, which is characterized by high levels of phosphorylated STAT3 triggered by the autocrine secreted IL-6. Also,

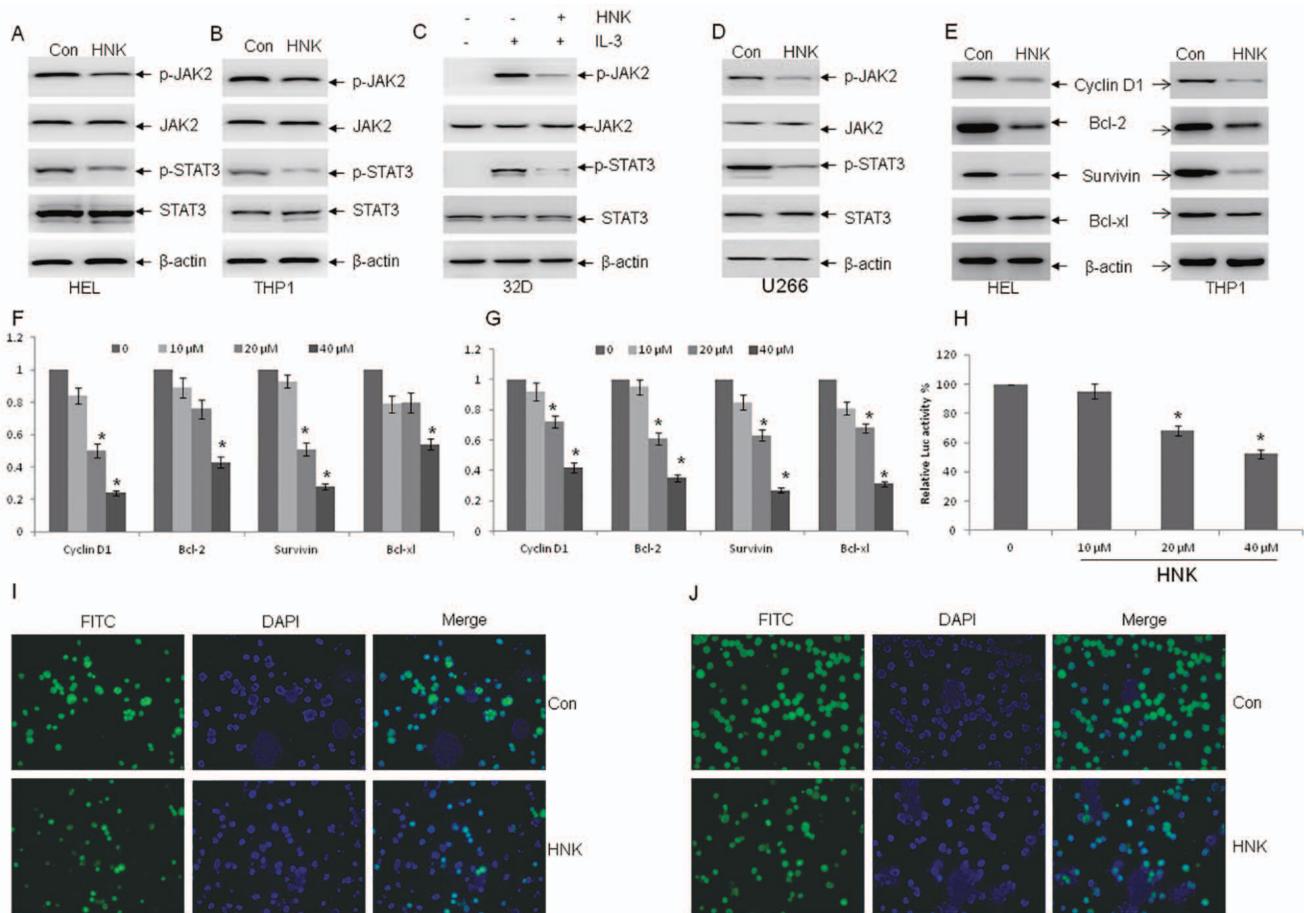


Fig. 1. HNK inhibits constitutive and inducible activation of STAT3 signaling in leukemic cell lines.

(A and B) Protein levels of JAK2/STAT3 signaling were detected by Western blot analysis in HEL and THP1 cells treated with 40  $\mu$ M HNK for 24 h. (C) IL-3-dependent murine myeloid cell line 32D cells were treated with 40  $\mu$ M HNK for 24 h and protein levels of JAK2/STAT3 signaling were measured by Western blot analysis. (D) U266 cells were treated with 40  $\mu$ M HNK for 24 h and protein levels of JAK2/STAT3 signaling were measured by Western blot analysis. (E) HEL and THP1 cells were treated with 40  $\mu$ M HNK for 24 h. STAT3 target genes including cyclin D1, Bcl-2, Bcl-x1, and Survivin were measured by Western blot analysis. (F and G) The mRNA levels of STAT3 target genes were detected by qRT-PCR in HEL (F) and THP1 cells (G).  $P < 0.05$  verse untreated cells. (H) 293T cells were cotransfected with plasmid STAT3-luciferase (STAT3-Luc) and normal control for 24 h, and treated with indicated concentrations of HNK for another 24 h. Whole-cell extracts were then prepared and analyzed for Firefly luciferase activities and Renilla luciferase activities. Histograms show the Firefly luciferase activities normalized to Renilla luciferase activities.  $P < 0.05$  verse untreated cells. (I and J) HEL (I) and THP1 cells (J) were treated with 40  $\mu$ M HNK for 24 h followed by fixation and permeabilization. STAT3 (green) was stained with anti-STAT3 followed by FITC-conjugated secondary antibody (first panels). The nucleus (blue) was stained with DAPI (second panels). The merged images of the first and second panels were indicated in the third panels.

the phosphorylated JAK2 and STAT3 but not the total levels of JAK2 and STAT3 were significantly decreased by HNK (Fig. 1D).

STAT3 phosphorylation modulates cell proliferation and apoptosis through affecting the expression of a variety of genes such as Bcl-x1, Bcl-2, cyclin D1, and survivin. We then investigate whether HNK affects the expression of STAT3 target genes. As indicated in Fig. 1E, HNK inhibited the protein levels of STAT3 target genes. Similarly, HNK decreased the mRNA levels of STAT3 target genes in a concentration-dependent manner (Fig. 1F, G). Furthermore, HNK inhibited STAT3 luciferase activity in a concentration-dependent manner in 293T cells transfected with

STAT3-Luc (Fig. 1H). Because STAT3 dimerization caused by tyrosine phosphorylation finally results in STAT3 translocation to the nucleus, immunocytochemical analysis was used to determine whether HNK reduced nuclear translocation of STAT3. As indicated in Fig. 1I and J, HNK decreased the translocation of STAT3 to the nucleus in HEL and THP1 cells.

#### *HNK increases the expression of SHP1 in AML cell lines*

Several negative regulators such as suppressors of cytokine signaling (SOCS1 and SOCS3) (Yoshikawa et al. 2001) and SHP1 inhibit the activated STAT3 signaling. We then asked whether HNK inhibited STAT3 signaling

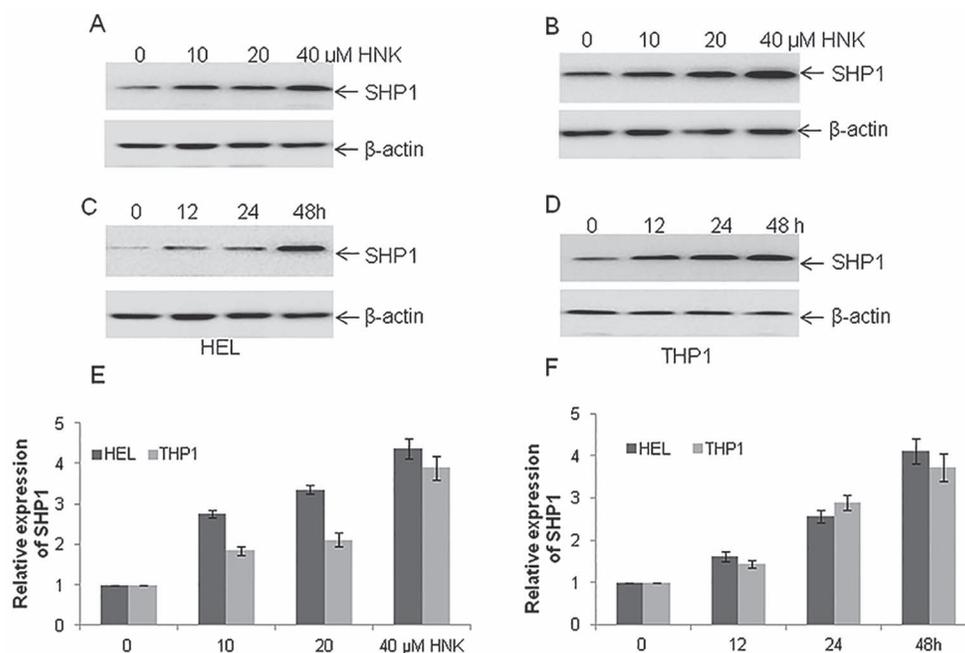


Fig. 2. The effect of HNK on the expression of SHP1.

(A and B) HEL and THP1 cells were treated with indicated concentrations of HNK for 24 h and SHP1 protein levels were detected by Western blot analysis. (C and D) The protein levels of SHP1 were detected in HEL and THP1 cells treated with 40  $\mu$ M HNK for the indicated times. (E) HEL and THP1 cells were treated with indicated concentrations of HNK for 24 h and SHP1 mRNA levels were detected by qRT-PCR. (F) The mRNA levels of SHP1 were detected in HEL and THP1 cells treated with 40  $\mu$ M HNK for the indicated times.

through increasing the expression of these negative regulators. HNK increased the protein levels of SHP1 in concentration- and time-dependent manner in leukemic cells (Fig. 2A-D). Similarly, HNK increased the mRNA levels of SHP1 in a concentration- and time-dependent manner (Fig. 2E, F). While, HNK failed to modulate the protein level of SOCS1/3 in HEL and THP1 cells (Data not shown).

#### *PTP inhibitor vanadate and SHP1 knockdown reverse the inhibition of STAT3 signaling by HNK*

To determine whether SHP1 plays an important role in HNK-induced inhibition of STAT3 signaling, HEL and THP1 cells were co-treated with PTP inhibitor vanadate and HNK for 24 h. As indicated in Fig. 3A and B, vanadate reversed HNK-induced inhibition of p-STAT3 but did not modulate the total level of STAT3. Further, we explore whether SHP1 knockdown by shRNA could abrogate the inhibitory effect of HNK. The protein and mRNA levels of SHP1 were effectively reduced in sh-SHP1-transfected cells than those in sh-NC-transfected cells (Fig. 3C, D). Also, HNK-induced upregulation of SHP1 and inhibition of STAT3 phosphorylation but not the total level of STAT3 were obviously reversed in sh-SHP1-transfected cells compared with negative control (Fig. 3E, F).

#### *HNK increases PU.1 expression and knockdown of PU.1 reverses HNK-induced SHP1 upregulation and STAT3 inhibition*

Previous studies showed several transcript factors such

as PU.1 (Wlodarski et al. 2007) and RFX-1 (Su et al. 2014) activated the expression of SHP1. We then determined whether HNK induced the expression of these transcript factors. As indicated in Fig. 4A and B, HNK significantly increased the protein and mRNA levels of PU.1 but not RFX-1 expression. In agreement with previous report (Wlodarski et al. 2007), ectopic overexpression of PU.1 upregulated SHP1 level in HEL cells (Fig. 4C) and knockdown of PU.1 by shRNA downregulated the expression of SHP1 in THP1 cells (Fig. 4D), suggesting that SHP1 is regulated by PU.1. Next, we determined whether PU.1 was mediated in HNK-induced upregulation of SHP1 and inhibition of STAT3 signaling. HNK failed to increase the expression of SHP1 in sh-PU.1-transfected cells compared with negative control (Fig. 4E, F). Similarly, HNK-induced inhibition of STAT3 signaling was reversed in leukemic cells transfected with sh-PU.1 compared with negative control (Fig. 4G, H).

#### *HNK increases the levels of SHP1 and PU.1 in primary AML blasts*

To determine whether HNK increased SHP1 and PU.1 expression in primary AML blasts, mRNA levels of SHP1 and PU.1 were detected in bone marrow mononuclear cells treated with HNK for 24 h. HNK significantly increased the mRNA levels of SHP1 and PU.1 by 2.18-fold and 1.83-fold, respectively (Fig. 5A, B). Similarly, HNK increased the protein levels of SHP1 and PU.1 in primary AML blasts and correspondingly decreased p-STAT3 protein level (Fig.

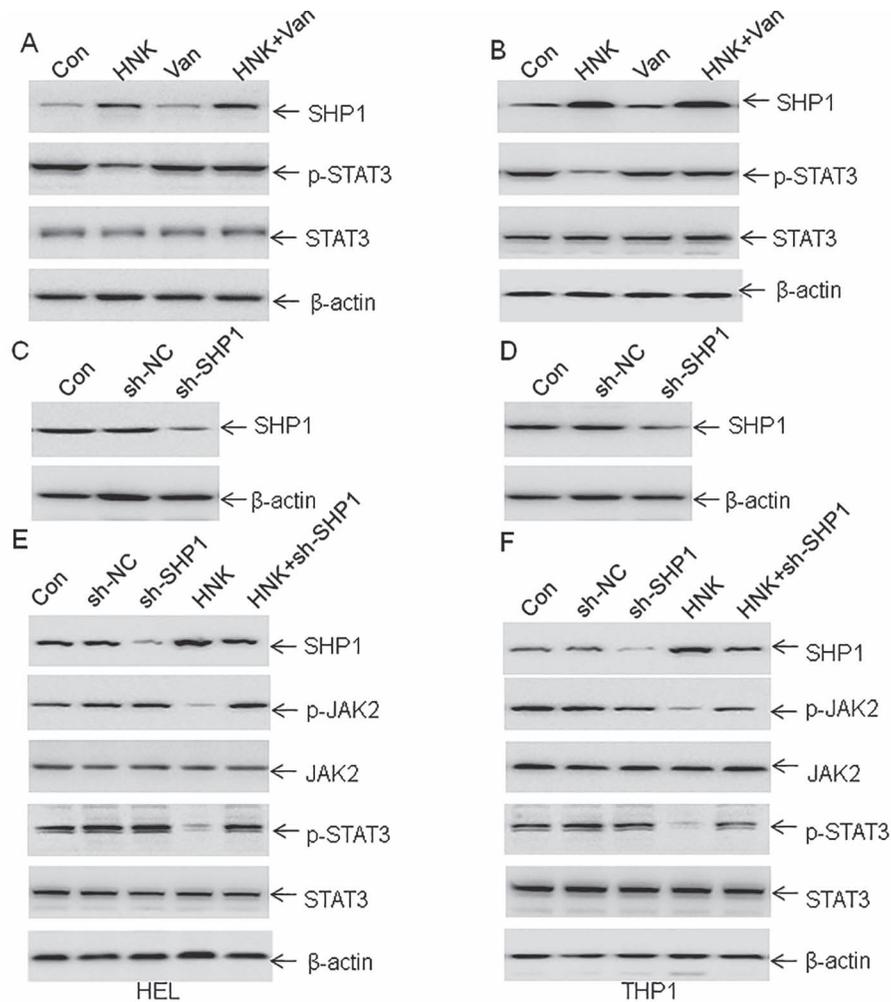


Fig. 3. Vanadate treatment and SHP1 knockdown inhibit HNK-induced inactivation of STAT3 signaling pathway. (A and B) HEL and THP1 cells were treated with 50 μM vanadate and 40 μM HNK for 24 h, the protein levels of SHP1, STAT3, and p-STAT3 were detected. (C and D) HEL and THP1 cells were transfected with retrovirus vector pSIREN-NC (sh-NC) or pSIREN-SHP1 (sh-SHP1). Positive clones were selected by puromycin. Western blot analysis was performed to measure SHP1 protein levels. (E and F) The positive clones were incubated with 40 μM HNK and the indicated proteins were detected.

5C). Further, HNK induced apoptosis in 5 of 6 AML blasts (Fig. 5D).

### Discussion

Agents suppressing STAT3 activation pathway have potential for cancer prevention and treatment. This study was aimed to investigate the inhibition of STAT3 signaling by HNK in AML cells. We found HNK inhibited constitutive and inducible STAT3 signaling through increasing the expression of SHP1. SHP1 knockdown and SHP1 inhibitor vanadate reversed HNK-induced STAT3 inhibition, thereby indicating the important role of SHP1 in the inactivation of STAT3 signaling. Furthermore, HNK increased the expression of transcription factor PU.1, which activates SHP1 level through binding SHP1 promoter region (Wlodarski et al. 2007). Collectively, HNK inhibited constitutive and inducible STAT3 signaling through PU.1-induced SHP1 expression.

STAT3 phosphorylation plays critical roles in the proliferation and survival of various tumor including hematological malignancies. Constitutive and inducible activation of STAT3 were frequently occurred in hematological malignancies. JAK2 V617F, K539L, T875N, and other mutations often led to constitutive activation of STAT3 signaling in myeloproliferative neoplasm and AML (Zou et al. 2011). HNK inhibited constitutively activated STAT3 signaling not only in HEL cells harboring JAK2V617F mutation but also in THP1, 32D, and U266 cells without JAK2V617F mutation, suggesting that HNK suppressed constitutively activated STAT3 signaling independent of JAK2V617F mutation. Furthermore, HNK inhibited inducible activation of STAT3 in 32D cells and U266 cell lines, in which STAT3 was activated by IL-3 and IL-6, respectively. In addition, HNK inhibited STAT3-regulated genes, confirming the effects of inactivation of STAT3 signaling. Overall, HNK suppresses both constitutive and inducible STAT3 activa-

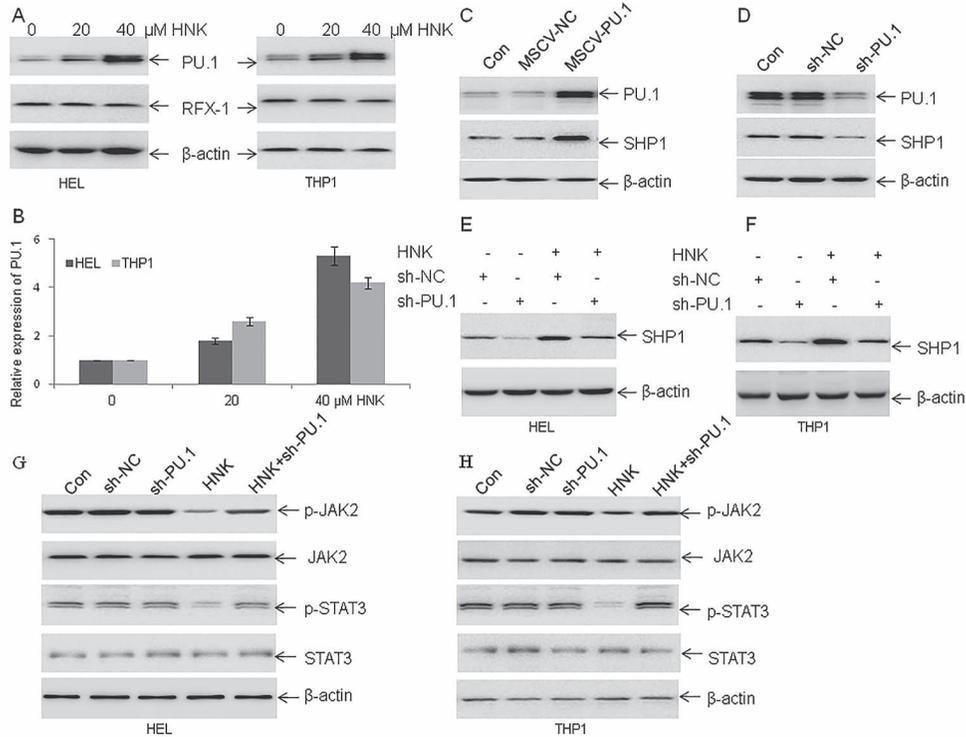


Fig. 4. The role of PU.1 in HNK-induced STAT3 inhibition.

(A and B) The mRNA and protein levels of PU.1 were detected in HEL and THP1 cells treated with indicated concentrations of HNK for 24 h. (C) HEL cells were transfected with retroviral vector MSCV-NC and MSCV-PU.1. After positive clones were selected with puromycin, PU.1 and SHP1 were detected. (D) THP1 cells were transfected with retroviral vector pSIREN-NC (sh-NC) or pSIREN-PU.1 (sh-PU.1). PU.1 and SHP1 were detected in positive clones selected by puromycin. (E and F) SHP1 was detected in HEL and THP1 cells which were transfected with sh-NC or sh-PU.1, followed with treatment with 40 μM HNK for 24 h. (G and H) JAK2/STAT3 signaling was detected in HEL and THP1 cells which were transfected with sh-NC or sh-PU.1, followed with treatment with 40 μM HNK for 24 h.

tion leading to the other downstream effects.

SHP1, an important negative regulator of STAT signaling, is an attractive target for the dysregulation of STAT signaling. However, hypermethylation of SHP1 which led to the low expression of SHP1 was frequently occurred in lymphoma (Oka et al. 2002), leukemia (Oka et al. 2002), and high-risk myelodysplastic syndrome (Zhang et al. 2012). Thus, restoration of SHP1 expression by demethylation agent, such as 5-aza-2'-deoxycytidine, contributed to the increase of SHP1 and restored the negative regulation activity of STAT signaling. However, SHP1 hypermethylation was not found in THP1 and HEL cells using methylation-specific polymerase chain reaction (Data not shown). Thus, we speculate that HNK increases the expression of SHP1 not through demethylation of SHP1. In addition to SHP1, SOCS1 and SOCS3 also negatively regulate STAT signaling. Our data showed HNK increased the expression of SHP1 but not SOCS1/3 protein levels and SHP1 knockdown reversed HNK-induced inhibition of STAT3 signaling, indicating that SHP1 plays critical role in the action of HNK.

Considering that SHP1 act as the key negative regulator of numerous signaling transduction pathways, SHP1 was modulated by several transcript factors, such as PU.1

(Wlodarski et al. 2007) and RFX-1 (Su et al. 2014). PU.1, which plays a crucial role in differentiation and function of the lymphoid and myeloid cells, activated SHP1 expression via binding SHP1 p2 promote region in malignancy hematopoietic cells (Wlodarski et al. 2007). HNK increased PU.1 expression and knockdown of PU.1 reversed the upregulation of SHP1 and the inhibitory effect of STAT3 signaling induced by HNK, suggesting that PU.1 plays an important role in the action of HNK. However, the underlying mechanism by which PU.1 is regulated by HNK remains to be elucidated.

HEL and THP1 cells present easily detectable levels of endogenous p-STAT3, suggesting that p-STAT3 is significantly overexpressed in these two cell lines. Although the knockdown of PU.1 leading to the reduction of SHP1 will increase the expression of p-STAT3 in theory, we failed to find the increase of p-JAK2 and p-STAT3 (Fig. 4G, H). We speculate that it is not easy to observe the increase of p-STAT3, which is highly expressed in background concentration. In contrast, it is easy to observe the reduction of p-STAT3 in HEL and THP1 cells treated with HNK.

HNK has been reported to regulate a series of signal pathway including PI3K/mTOR pathway (Crane et al. 2009), p38 MAP kinase pathway (Deng et al. 2008), and

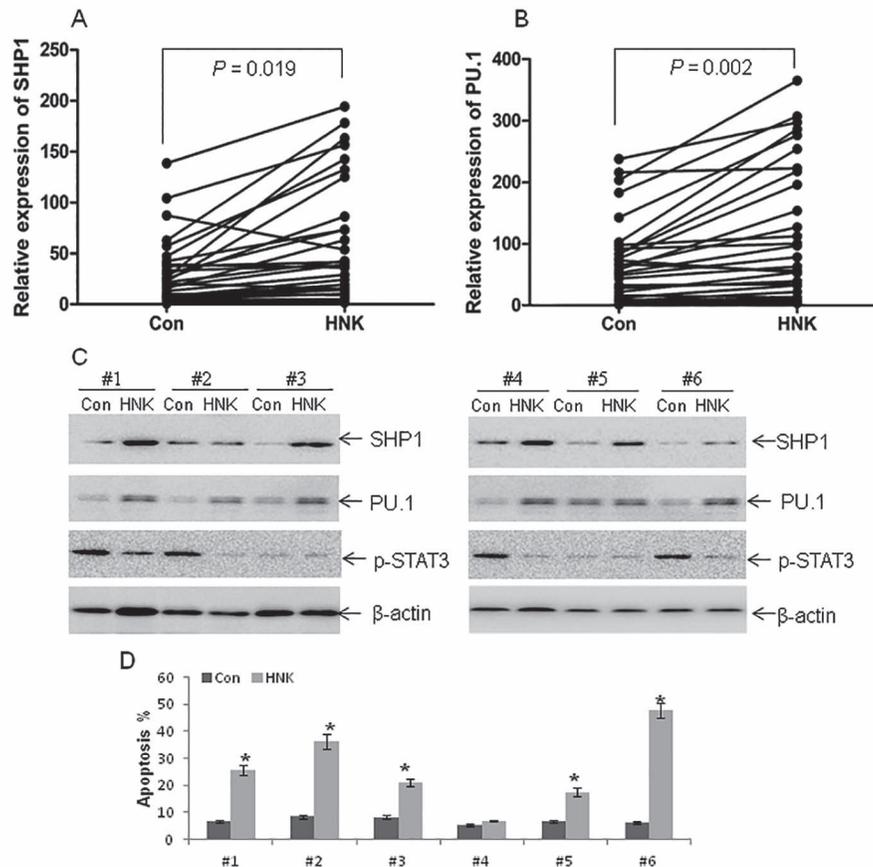


Fig. 5. The effect of HNK on the expression of SHP1 and PU.1 in primary AML blasts. (A and B) SHP1 and PU.1 mRNA levels were detected in bone marrow mononuclear cells, which were isolated from 30 patients with AML, followed with treatment with 40  $\mu$ M HNK for 24 h. (C and D) Bone marrow mononuclear cells were isolated from 6 AML patients and treated with 40  $\mu$ M HNK for 24 h. The protein levels of SHP1, PU.1, and p-STAT3 were measured by western blot analysis (C) and apoptosis was detected by annexin V/PI assay in these blast cells (D). \* $P < 0.01$  verse untreated cells.

STAT signaling (Rajendran et al. 2012) in various cancer cells. Our previous data also indicated that HNK induced apoptosis and cell growth arrest in leukemic cells by inhibiting histone deacetylases (HDAC) activity (Li et al. 2015). However, inhibition of HDAC activity by HDAC inhibitor trichostatin A (TSA) failed to modulate the expression of SHP1 (Data not shown), suggesting that HNK induced the expression of SHP1 not through inhibiting HDAC activity. STAT3 signaling modulated cell growth and apoptosis through STAT3 target genes, which were also decreased by HNK in leukemic cells, indicating that HNK induced cell growth arrest and apoptosis partly through these STAT3 target genes.

Our previous study indicated that 40  $\mu$ M HNK induced apoptosis in bone marrow mononuclear cells from patients with AML, but did not induce apoptosis in normal hematopoietic progenitors from umbilical cord blood (Li et al. 2015). Similarly, other study showed that 40  $\mu$ M HNK failed to produce a significant reduction of cell viability in normal peripheral blood mononuclear cells (Ishikawa et al. 2012). Thus, HNK presents low toxic effect on normal

human cells and in addition, HNK prevents normal cells against drug-induced cytotoxicity (Choi 2011).

The identification of novel anti-cancer compounds derived from existing natural sources gives an enormous opportunity to improve the existing standard of care for AML. Our data indicate HNK inhibited STAT3 signaling via PU.1-induced expression of SHP1. Collectively, the anti-cancer effects of HNK might be due, in part, to the inhibition of STAT3 signaling. Overall, in combination with other modes of therapy or not, HNK has the potential to develop into a therapeutic agent for leukemia.

#### Author Contributions

Bi, L.X. and Yu, Z.J. carried out western blotting. Wu, J.B. carried out PCR and data analysis. Yu, K. participated in sample collection and analysis of data. Hong, G.L. constructed the plasmids. Lu, Z.Q. and Goa, S.M. designed experiments and drafted the manuscript.

#### Acknowledgments

This work was supported by National Natural Science

Foundation of China (81200350); The key construction academic subject (traditional Chinese medicine) of Zhejiang Province (2012-XK-A28).

### Conflict of Interest

The authors declare no conflict of interest.

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