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Pure curcumin increases the expression of SOCS1 and SOCS3 in myeloproliferative neoplasms through suppressing class I histone deacetylases

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Suppressors of cytokine signaling, SOCS1 and SOCS3, are important negative regulators of Janus kinase 2/signal transducers and activators of transcription signaling, which is constitutively activated in myeloproliferative neoplasms (MPNs) and leukemia. Curcumin has been shown to possess anticancer activity through different mechanisms. However, whether curcumin can regulate the expression of SOCS1 and SOCS3 is still unknown. Here, we found that curcumin elevated the expression of SOCS1 and SOCS3 via triggering acetylation of histone in the regions of SOCS1 and SOCS3 promoter in K562 and HEL cells. As a novel histone deacetylases (HDACs) inhibitor, curcumin inhibited HDAC enzyme activities and decreased the levels of HDAC1, 3 and 8 but not HDAC2. Knockdown of HDAC8 by small interfering RNA markedly elevated the expression of SOCS1 and SOCS3. Moreover, ectopic expression of HDAC8 decreased the levels of SOCS1 and SOCS3. Thus, HDAC8 plays an important role in the modulation of SOCS1 and SOCS3 by curcumin. Also, trichostatin A (TSA), an inhibitor of HDACs, increased the levels of SOCS1 and SOCS3. Furthermore, curcumin increased the transcript levels of SOCS1 and SOCS3 and significantly inhibited the clonogenic activity of hematopoietic progenitors from patients with MPNs. Finally, curcumin markedly inhibited HDAC activities and decreased HDAC8 levels in primary MPN cells. Taken together, our data uncover a regulatory mechanism of SOCS1 and SOCS3 through inhibition of HDAC activity (especially HDAC8) by curcumin. Thus, being a relative non-toxic agent, curcumin may offer a therapeutic advantage in the clinical treatment for MPNs.

Introduction

As negative regulators of Janus kinase 2/signal transducers and activators of transcription (JAK2/STAT) signaling, suppressors of cytokine signaling, SOCS1 and SOCS3, play an important role in suppressing cell proliferation caused by constitutive activation of JAK2/STAT signaling in myeloproliferative neoplasms (MPNs) and leukemia (1). The rapid induction of SOCS1 and SOCS3 by a broad spectrum of cytokines results in downregulation of JAK2 and STAT phosphorylation and decreased transcription of target genes (2). SOCS1 directly binds JAK2 (3), whereas SOCS3 indirectly targets JAK2 via binding cytokine receptor such as erythropoietin receptor (4). Thus, SOCS proteins finally degrade JAK2 through E3 ubiquition ligase ECS complex. It is well established that constitutive activation of JAK2/STAT signaling by JAK2V617F (5) and MPL515 (6) is a major causative determinant of MPNs. JAK2V617F mutation enables tyrosine phosphorylation of SOCS3 protein, which finally fails to inhibit constitutive activation of JAK2/STAT signaling pathway in MPNs (7). Therefore, restoring the negative regulation of SOCS1 and SOCS3 proteins on JAK2/STAT signaling may contribute to the treatment of MPNs.

Epigenetic modifications that include acetylation of histone and non-histone proteins play a central role in the development of human cancers. At present, 18 histone deacetylases (HDACs) have been discovered and classified into four groups based on their structural homology. The ubiquitously expressed class I HDACs including HDAC1–3 and 8 are the best characterized proteins (8). It has been reported that HDAC1–3 are implicated in general cellular process such as differentiation, apoptosis and proliferation. Knockdown of HDAC1 led to cell growth inhibition, cell cycle arrest and an increase in the percentage of apoptotic cells in human tumors (9). Furthermore, aberrant expression of HDACs had been detected in various tumors. For example, HDAC1 was found to be overexpressed in prostate cancer (10). HDAC8 was overexpressed in neuroblastoma and glioma (11). Emerging studies have indicated targeting HDACs provides an ideal strategy for tumors especially hematological malignancies. In fact, HDAC inhibitors such as vorinostat had been approved by the Food and Drug Administration for treating advanced and refractory cutaneous T-cell lymphoma (12). Although HDAC inhibitors exhibit selective toxicity against tumor cells at nanomolar concentration, their prolonged use in patients leads to fatigue, severe immune suppression and gastrointestinal side effects (13). It would be advantageous to identify HDAC inhibitors that are effective but minimally toxic. Plant-derived compounds such as sulforaphane and green tea polyphenols have the ability to inhibit HDAC activity because they have structural features compatible with HDAC inhibitors (14,15).

Curcumin, a major component of the spice turmeric derived from the plant *Curcuma longa*, has been demonstrated to possess cancer preventive and therapeutic activity (16). Several studies have indicated that curcumin has strong antiproliferative, antioxidant and proapoptotic activities via targeting multiple downstream cancer-related signaling molecules including growth factors, transcription factors and genes regulating cell proliferation and apoptosis (17–19). Curcumin has been shown to modulate JAK2/STAT signaling in variety of cancer cells. Curcumin inhibited constitutively active NF-kappaB and STAT3 pathways in Hodgkin's lymphoma cells (20). Moreover, curcumin induced a decrease of nuclear STAT3, STAT5a and STAT5b without affecting the phosphorylation levels of STAT1, STAT3 or STAT5 in K562 cells (21). However, the underlying mechanism that curcumin modulates JAK2/STAT signaling through upregulation of SOCS1 and SOCS3 remains to be defined.

Recently, curcumin has been recognized as an epigenetic agent (22). Curcumin has the ability to restore the expression of some tumor suppressor genes through epigenetic modulations including DNA methylation and chromatin remodeling. Curcumin reduced the promoter hypermethylation and finally resulted in the increased expression of FANCF in SiHa cells (23). Furthermore, curcumin effectively decreased the level of M.SssI, which is an analog of DNA methyltransferase 1 (23). Curcumin inhibited cell growth and induced apoptosis via inhibiting class I HDACs and increasing the level of acetylated histone H4 (ac-H4) in Raji cells (24). Therefore, epigenetic modulation by curcumin plays an important role in cancer treatment.

The results of our present study demonstrate that curcumin elevates the expression of SOCS1 and SOCS3 in K562, HEL, 32D and primary MPN cells. Curcumin inhibits activity of HDAC enzyme

Abbreviations: ac-H4, acetylated histone H4; ChIP, chromatin immunoprecipitation; ET, essential thrombocytosis; HDAC, histone deacetylase; IL-3, interleukin 3; IMF, idiopathic myelofibrosis; JAK2/STAT, Janus kinase 2/signal transducers and activators of transcription; MPN, myeloproliferative neoplasm; mRNA, messenger RNA; PV, polycythemia vera; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; TSA, trichostatin A.

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and reduces the levels of HDAC1, 3 and 8. Knockdown of HDAC8 markedly elevates the expression of SOCS1 and SOCS3. Thus, curcumin upregulates the levels of SOCS1 and SOCS3 probably through inhibiting HDAC activity (especially HDAC8) in K562 and HEL cells. Without severe side effects, curcumin may become a useful agent for the treatment of MPNs.

Materials and methods

Cell lines and primary MPN cells

K562, HEL and murine interleukin 3 (IL-3)-dependent 32D cell lines were employed for the present study. K562 and HEL cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) in humidified 37°C incubator with 5% CO_2. 32D cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum plus 10 ng/ml IL-3 (PeproTech, Rocky Hill, NJ). Primary MPN cells were obtained from 34 MPN patients (Supplementary Table 1, available at *Carcinogenesis* Online) including 12 patients with polycythemia vera (PV), 7 with idiopathic myelofibrosis (IMF) and 15 with essential thrombocytosis (ET). All patients gave informed consent in accordance with the Declaration of Helsinki. All manipulations were approved by the Medical Science Ethic Committee of Wenzhou Medical College. Bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation (GE Healthcare, Uppsala, Sweden) and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen) in humidified 37°C incubator with 5% CO_2. Pure curcumin (Sigma–Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide as 20 mM stock solution and kept at −20°C. Trichostatin A (TSA, Sigma–Aldrich) was dissolved in ethanol and kept at −20°C until use. Cell viability was determined by the trypan-blue exclusion assay, and growth inhibition rate was calculated according to viable cell number of treated cells against number of untreated cells.

Plasmids transfection

Full-length SOCS1 (NM_003745), SOCS3 (NM_003955) and HDAC8 (NM_018486) cDNA were synthesized and cloned into pEGFP-N1 vector (Clontech, Palo Alto, CA). The primers were designed to clone the full-length cDNA (Supplementary Table 2, available at *Carcinogenesis* Online). The sequence and orientation of the SOCS1, SOCS3 and HDAC8 inserts were confirmed by DNA sequencing. pEGFP-SOCS1, pEGFP-SOCS3, pEGFP-HDAC8 and pEGFP-N1 were transfected into K562 and HEL cells by Lipofectamine™ LTX and PLUS™ Reagents (Invitrogen).

Small interfering RNA transfection

Small interfering RNA (siRNA) sequences targeting class I HDACs (Supplementary Table 2, available at *Carcinogenesis* Online) and unspecific control siRNA (scramble) were synthesized from Invitrogen. siRNA-HDACs and scramble were transfected into K562 and HEL cells by Hiperfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. We labelled FAM to all siRNAs to easily measure the transfection efficiencies by flow cytometry.

Chromatin immunoprecipitation assays

The acetylation levels of gene promoter associated histones H3 and H4 were examined by chromatin immunoprecipitation (ChIP) and quantitative real-time PCR assay. ChIP analysis was performed using the acetyl-histone H3/H4 ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, treated and untreated K562 cells were cross-linked with 1% formaldehyde for 10 min. Nuclear extracts were prepared and chromatin was sonicated to generate 200–1000 bp DNA fragments. Protein–DNA complexes were immunoprecipitated with 5 μg of specific antibodies (antiacetylated histone H3, antiacetylated histone H4 and non-relevant rabbit immunoglobulin G). The DNA–protein cross-link was reversed by heating at 65°C for 4 h, and then DNA was purified. Standard PCR reactions were performed with primer sequences (Supplementary Table 2, available at *Carcinogenesis* Online) specific for the SOCS1 and SOCS3 promoter.

HDAC activity detection

HDAC activity was measured by the colorimetric HDAC Activity Assay Kit (Upstate Biotechnology) according to manufacturer's protocol. Briefly, cells were incubated with curcumin for 24 h and then lysed with RIPA lysis buffer supplemented with protease inhibitors. The cell lysates and positive HeLa nuclear extract were incubated with assay buffer containing HDAC assay substrate for 60 min at 37°C. The reaction was then terminated with HDAC activator solution and absorbance was read at 405 nm.

Messenger RNA extract and quantitative real-time PCR

Total RNA was extracted by TRIzol (Invitrogen) following the manufacture's protocol. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primers of SOCS1, SOCS3 and other gene transcripts were indicated in Supplementary Table 2, available at *Carcinogenesis* Online. GAPDH housekeeping gene was used for normalization.

Western blotting

Western blotting analysis was performed using standard techniques. The following antibodies were used: SOCS1, SOCS3, JAK2, p-JAK2, STAT5 and p-STAT5 (Cell Signaling Technology, Beverly, MA) and HDAC1, HDAC2, HDAC3 and HDAC8 (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected by chemiluminescence phototope-HRP kit (Cell Signaling Technology). Blots were stripped and reprobed with β-actin antibody (Santa Cruz Biotechnology) as an internal control. All experiments were repeated three times with the similar results. Signal intensity of proteins was normalized against β-actin using Quantity One (Bio-Rad, Richmond, CA).

Colony assay

Bone marrow was obtained from six patients (two PV and four ET) with MPNs. Mononuclear cells were separated by Ficoll-Paque liquid and suspended in IMDM (Gibco). Viable cells were counted and diluted to a concentration of 2×10^4 cells/ml. Cells were diluted 1:10 in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum (Invitrogen) with or without 20 μM curcumin. Cells were plated in 35 mm dishes with Methocult H4434 Classic (Stem Cell Technologies, Vancouver, British Columbia, Canada) for 14 days of incubation at 37°C with 5% CO_2. Colony (>40 cells) was counted as one colony.

Statistical analysis

The significance of the difference between groups was determined by Student's *t*-test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS software (version 13; SPSS, Chicago, IL).

Results

Curcumin upregulates the expression of SOCS1 and SOCS3 in K562 and HEL cells and inhibits JAK2/STAT5 signaling in HEL cells but not in K562 cells

To determine whether curcumin modulated the expression of SOCS1 and SOCS3, the messenger RNA (mRNA) and protein levels of SOCS1 and SOCS3 were measured by quantitative real-time PCR and western blotting in K562 and HEL cells treated with non-cytotoxic dose of curcumin (20 μM) for 12 and 24 h (21). Because 20 μM curcumin effectively inhibited the growth of K562 and HEL cells but not obviously induced cell death at 12 and 24 h by trypan-blue exclusion assay (Figure 1A and B), this concentration of curcumin was selected for the following test. The protein levels of SOCS1 and SOCS3 in curcumin-treated K562 and HEL cells were significantly elevated compared with untreated cells (Figure 1C and E). Also, the mRNA levels of SOCS1 and SOCS3 were increased by 3–8-fold in curcumin-treated K562 and HEL cells than untreated cells (Figure 1D and F). Therefore, these data suggest that curcumin elevates the transcript and protein levels of SOCS1 and SOCS3 in K562 and HEL cells.

Previous study indicated that constitutive activation of JAK2/STAT5 phosphorylation was necessary and sufficient for some aspects of the MPN phenotype *in vitro* and *in vivo* (25). Next, we determined whether curcumin inhibited JAK2/STAT5 phosphorylation via increasing the levels of SOCS1 and SOCS3, which negatively regulated JAK2/STAT5 signaling (26). We found curcumin markedly reduced the production of JAK2, p-JAK2^{Tyr1007}, STAT5 and p-STAT5^{Tyr694} in HEL cells (Figure 1H). However, curcumin only decreased the levels of total JAK2 and STAT5 but not p-JAK2^{Tyr1007} and p-STAT5^{Tyr694} in K562 cells (Figure 1G). Therefore, these data suggest curcumin inhibits JAK2/STAT5 phosphorylation in HEL cells but not in K562 cells.

Curcumin blocks HDAC enzyme activity, inhibits class I HDAC expression and knockdown of HDAC8 upregulates the expression of SOCS1 and SOCS3

Being an epigenetic agent, curcumin has been shown to inhibit HDAC enzyme activity in medulloblastoma (27). We then investigated

whether curcumin might have HDAC inhibitor activity in K562 and HEL cells. HDAC activity was measured in K562 and HEL cells treated with 5, 10, 20 and 40 μM curcumin and 0.5 μM TSA for 24 h. As indicated in Figure 2A, 20 and 40 μM curcumin and 0.5 μM TSA significantly decreased the enzyme activity of HDACs ($P < 0.01$). TSA treatment was used as positive control. Thus, curcumin treatment resulted in dose-dependent inhibition of HDAC enzyme activity. We further investigated whether curcumin modulated the levels of class I HDACs including HDAC1–3 and 8. We found curcumin significantly decreased the transcript and protein levels of HDAC1, 3 and 8 but not the level of HDAC2 (Figure 2B and C). Furthermore, knockdown of an individual HDAC1, 3 and 8 by siRNAs was performed to decide which individual HDACs were responsible for modulation of SOCS1 and SOCS3. FAM-labelled siRNAs were transfected into K562 and HEL cells and the transfection efficiencies were about 64.5–78.6% as determined by FAM measurement by flow cytometry (data not shown). Our data demonstrated that knockdown of HDAC8 resulted in the increased protein levels of SOCS1 and SOCS3 (Figure 2D) as well as mRNA levels of SOCS1 and SOCS3 (Figure 2E and F). In contrast, knockdown of HDAC1 and 3 by siRNAs failed to alter the protein (Supplementary Figure 1A and B, available at *Carcinogenesis* Online) and mRNA (Supplementary Figure 1C and D, available at *Carcinogenesis* Online) levels of SOCS1 and SOCS3.

Ectopic expression of HDAC8 decreases SOCS1 and SOCS3 expression in K562 and HEL cells

To further explore the effect of HDAC8 on SOCS1 and SOCS3 expression, K562 and HEL cells were transfected with pEGFP-HDAC8 for 48 h. The transfection efficiencies were about 68.2% for K562 cells and 62.3% for HEL cells, respectively, as determined by green fluorescent protein measurement by flow cytometry (data not shown). We found the mRNA and protein levels of SOS1 and SOCS3 were markedly decreased in K562 (Figure 3A and C) and HEL (Figure 3B and D) cells by overexpression of HDAC8 compared with negative control. These results indicate that HDAC8 plays an important role in the modulation of SOCS1 and SOCS3 by curcumin.

Curcumin triggers acetylation of histone in the regions of SOCS1 and SOCS3 promoter

Low levels of acetylation on histones H3 and H4 (ac-H3 and ac-H4) are linked to HDACs-mediated gene silence. Curcumin has been shown to increase the level of ac-H4 in Raji cells (24). We then explored whether curcumin could increase the levels of ac-H3 and ac-H4 in K562 and HEL cells. The levels of ac-H3 and ac-H4 were markedly increased in curcumin-treated K562 and HEL cells than untreated cells (Figure 4B). Although curcumin effectively blocked HDAC activity (Figure 2A) and increased the levels of ac-H3 and ac-H4, whether curcumin modulated the levels of histone acetylation in SOCS1 and SOCS3 promoter was not determined. To test it, we performed ChIP to detect the levels of histone acetylation in SOCS1 and SOCS3 promoter regions. As shown in Figure 4C, the band densities of SOCS1-associated acetylated histone proteins were obviously higher in chromatin that was extracted from curcumin-treated K562 cells than in chromatin that was extracted from untreated cells. Furthermore, immunoprecipitated DNA from K562 and HEL cells was analyzed by quantitative real-time PCR. The levels of ac-H3 and ac-H4 in SOCS1 promoter regions (−503 to −54 nucleotides, Figure 4A), as well as SOCS3 promoter regions (−765 to −160 nucleotides, Figure 4A), were increased by 3–6-fold in curcumin-treated cells compared with untreated cells (Figure 4D and E). These results demonstrate that curcumin leads to the increased hyperacetylation of histones in SOCS1 and SOCS3 promoter regions.

Pure curcumin increases the expression of SOCS1 and SOCS3 and inhibits the colony formation in primary MPN cells

To further investigate whether curcumin could regulate the expression of SOCS1 and SOCS3 in primary MPN cells, we determined the expression of SOCS1 and SOCS3 in mononuclear cells from

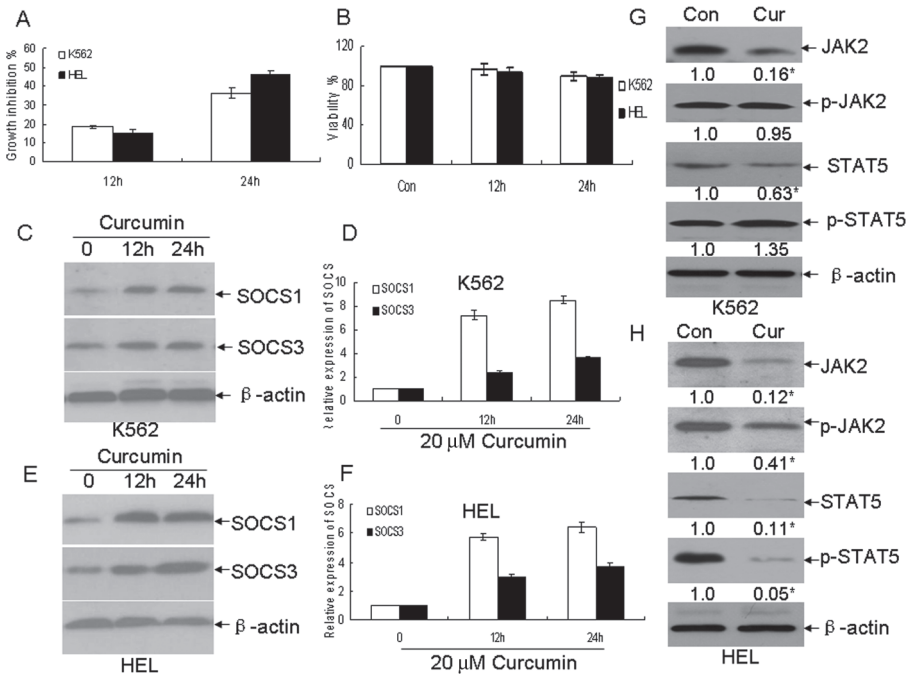

Fig. 1. Curcumin upregulates the expression of SOCS1 and SOCS3 and inhibits JAK2/STAT5 signaling in HEL cells but not in K562 cells. K562 and HEL cells were treated with 20 μM curcumin for 12 and 24 h. (**A** and **B**) Cell growth inhibition and viability were counted by trypan-blue exclusion assay. (**C** and **E**) The protein levels of SOCS1 and SOCS3 were detected by western blotting. β-Actin antibody served as an internal control. (**D** and **F**) The mRNA levels of SOCS1 and SOCS3 were measured by quantitative real-time PCR in K562 and HEL cells treated with 20 μM curcumin for 12 and 24 h. Results are expressed as relative expression compared with untreated cells. Each value is the mean ± SD of three experiments. (**G** and **H**) K562 and HEL cells were treated with 20 μM curcumin for 24 h. The indicated protein of JAK2/STAT5 signaling was detected by western blotting. Representative western blots are shown ($n = 3$). The relative intensity of each band after normalization for β-actin is shown under each blot as the fold change compared with untreated control, which was assigned an unit 1.0 in each case ($n = 3$). *$P < 0.01$ versus untreated control.

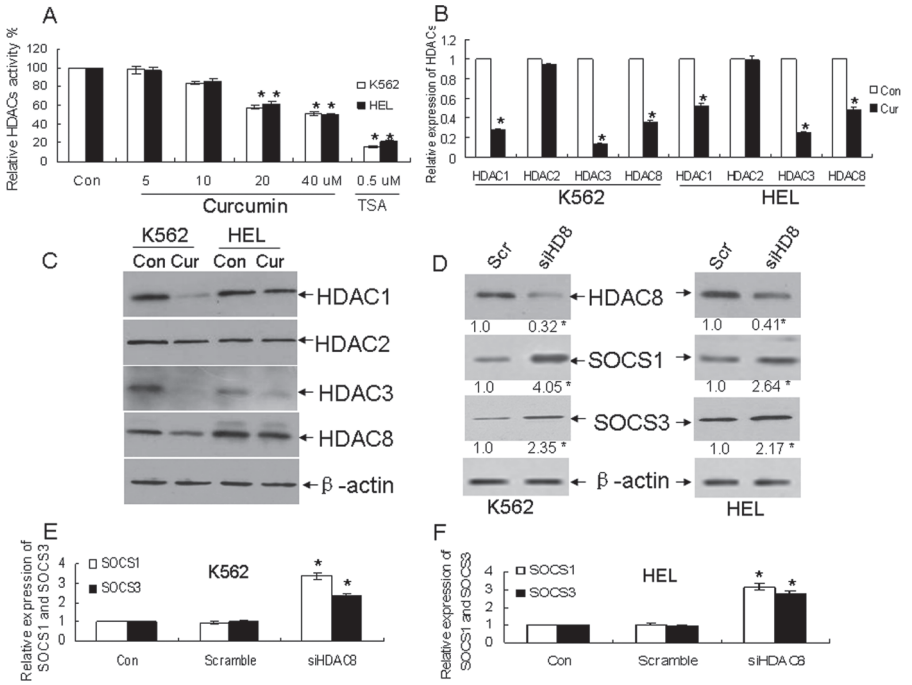

Fig. 2. Curcumin inhibits HDAC activity, reduces the levels of HDAC8 and knockdown of HDAC8 increases the expression of SOCS1 and SOCS3. (**A**) HDAC activity was measured by colorimetric HDAC Activity Assay Kit after K562 and HEL cells were treated with indicated concentration of curcumin and 0.5 μM TSA for 24 h. *$P < 0.01$ versus untreated cells. The mRNA and protein levels of HDAC8 were measured by quantitative real-time PCR (**B**) and western blotting (**C**) in K562 and HEL cells treated with 20 μM curcumin for 24 h. *$P < 0.01$ versus untreated cells. (**D**) Western blotting was performed with extracts from K562 and HEL cells that were transfected with scramble or siRNA against HDAC8 for 72 h. The relative intensity of each band after normalization is shown under each blot. *$P < 0.01$ versus scramble. (**E** and **F**) Quantitative real-time PCR was performed with total mRNA isolated from K562 and HEL cells that were treated as in (D). *$P < 0.01$ versus scramble.

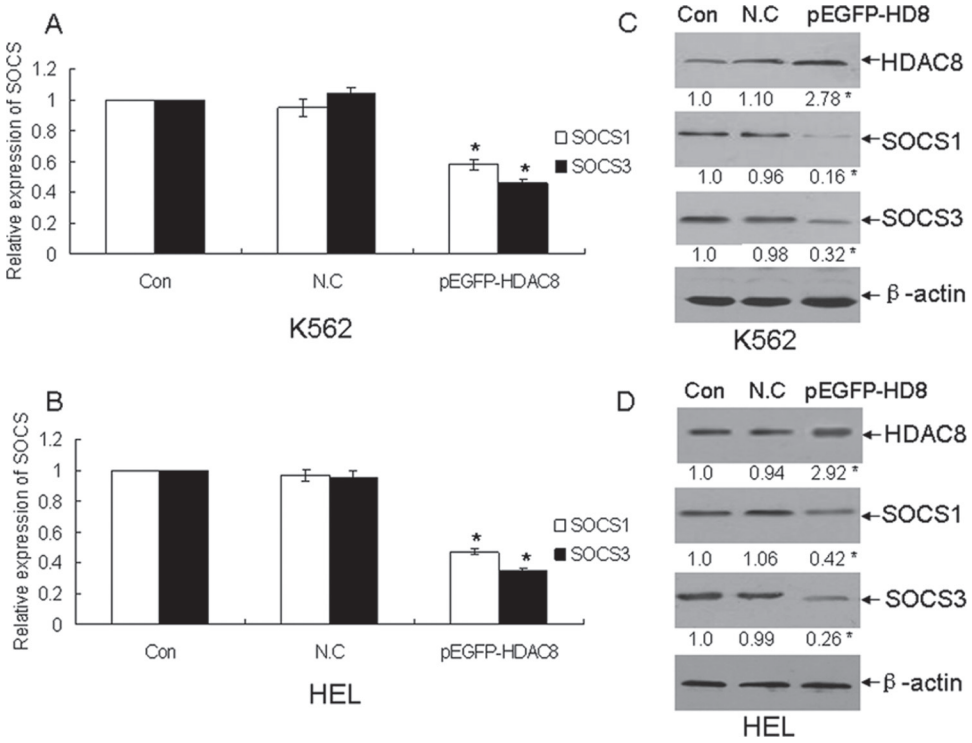

Fig. 3. Ectopic expression of HDAC8 decreases the expression of SOCS1 and SOCS3. (**A** and **B**) Quantitative real-time PCR was performed with total mRNA isolated from K562 and HEL cells that were transfected with negative control (pEGFP-N1) or pEGFP-HDAC8 for 48 h. *$P < 0.01$ versus N.C. (**C** and **D**) Western blotting was performed with extracts from K562 and HEL cells, which were treated as in (A) and (B). The blots were then probed with antibody against SOCS1, SOCS3, HDAC8 and β-actin. The relative intensity of each band after normalization is shown under each blot. *$P < 0.01$ versus N.C.

bone marrow of 34 MPN patients before and after 20 μM curcumin treatment. The detailed data of these MPN patients are shown in Supplementary Table 1, available at *Carcinogenesis* Online. Curcumin increased the SOCS1 expression in 9 of 12 (75%) patients with PV, 5 of 7 (71.4%) with IMF and 10 of 15 (66.7%) with ET (Supplementary Figure 2A–C, available at *Carcinogenesis* Online). The levels of SOCS1 were increased by 2.25-, 1.86- and 2.85-fold in PV, IMF and ET patients, respectively (Figure 5A). In all MPNs, the levels of SOCS1 were increased by curcumin in 24 of 34 MPN patients (70.5%) and the levels of SOCS1 were increased 2.43-fold in curcumin-treated cells compared with untreated cells (Figure 5A). Also, curcumin increased the SOCS3 expression in 8 of 12 (66.7%) patients with PV, 4 of 7 (57.1%) with IMF and 10 of 14 (71.4%) with ET (Supplementary Figure 2D–F, available at *Carcinogenesis* Online). The levels of SOCS3 were increased by 2.76-, 1.97- and 2.1-fold in PV, IMF and ET patients, respectively (Figure 5B). In all MPNs, the level of SOCS3 was increased by curcumin in 22 of 34 (64.7%) MPN patients and the level of SOCS3 increased 2.22-fold in curcumin-treated cells compared with untreated cells (Figure 5B). These results indicate that curcumin elevates the levels of SOCS1 and SOCS3 in primary MPN cells.

Furthermore, we determined the effect of curcumin on the clonogenic activity of fresh bone marrow mononuclear cells from six MPN patients (two with PV and four with ET). As shown in Figure 5C and D, treatment with 20 μM curcumin resulted in significantly reduced colony formation in six primary MPN cells. In case 6, curcumin completely inhibited the colony formation.

Pure curcumin reduces the activities of HDACs and inhibits the expression of HDAC8, which is inversely correlated with SOCS1 and SOCS3 in primary MPN cells

Although curcumin reduced HDAC activities and HDAC8 levels in K562 and HEL cells, whether curcumin decreased HDAC activities and HDAC8 levels in primary MPN cells was not determined. We then measured HDAC activities and HDAC8 levels in curcumin-treated bone marrow mononuclear cells from 34 MPN patients. Curcumin individually decreased HDAC activities in 9 of 12 (75%) patients with PV, 5 of 7 (71.4%) with IMF, 13 of 15 (86.7%) with ET and 27 of 34 (79.4%) with MPN (Supplementary Figure 3A–C, available at *Carcinogenesis* Online). Totally, HDAC activities were decreased about 43.5% in patients with PV, 36.3% in patients with IMF, 59.8% in patients with ET and 45.6% in all MPN patients (Figure 6A). Meanwhile, curcumin reduced HDAC8 levels in 10 of 12 (83.3%) patients with PV, 5 of 7 (71.4%) with IMF, 11 of 15 (73.3%) with ET and 26 of 34 (76.5%) with MPN (Supplementary Figure 3D2–F, available at *Carcinogenesis* Online). Finally, HDAC8 levels were decreased about 58.7% in patients with PV, 41.8% in patients with IMF, 49.7% in patients with ET and 52.1% in all MPN patients (Figure 6B). These data demonstrate that curcumin can effectively reduce HDAC activities and HDAC8 levels in mononuclear cells from MPN patients.

Our results indicate knockdown of HDAC8 increases the expression of SOCS1 and SOCS3 and ectopic expression of HDAC8 decreases the level of SOCS1 and SOCS3. To further consolidate these findings, we explore whether there is an inverse correlation with HDAC8 and SOCS1 or SOCS3 in these 34 MPN patients. The levels of SOCS1, SOCS3 and HDAC8 were measured and normalized to normal cells. As shown in Figure 6C and D, when the relative expression levels of HDAC8 were plotted against that of SOCS1 or SOCS3 in each patient, a significant inverse correlation was found (HDAC8 versus SOCS1: $R = -0.809$, $P < 0.05$; HDAC8 versus SOCS3: $R = -0.70$, $P < 0.05$). In conclusion, the levels of HDAC8 are inversely correlated with SOCS1 or SOCS3 in primary MPN cells.

Discussion

Both dietary and environmental factors have been shown to induce epigenetic changes, which finally result in stable transmission of cellular traits without an alteration in DNA sequence or amount. Epigenetic deregulation frequently participates in tumorigenesis through inactivation of tumor suppressor genes. Curcumin and other dietary compounds can reverse these epigenetic changes. Thus, curcumin has potential to be an effective agent in the prevention and treatment of diverse tumor including MPNs and leukemia.

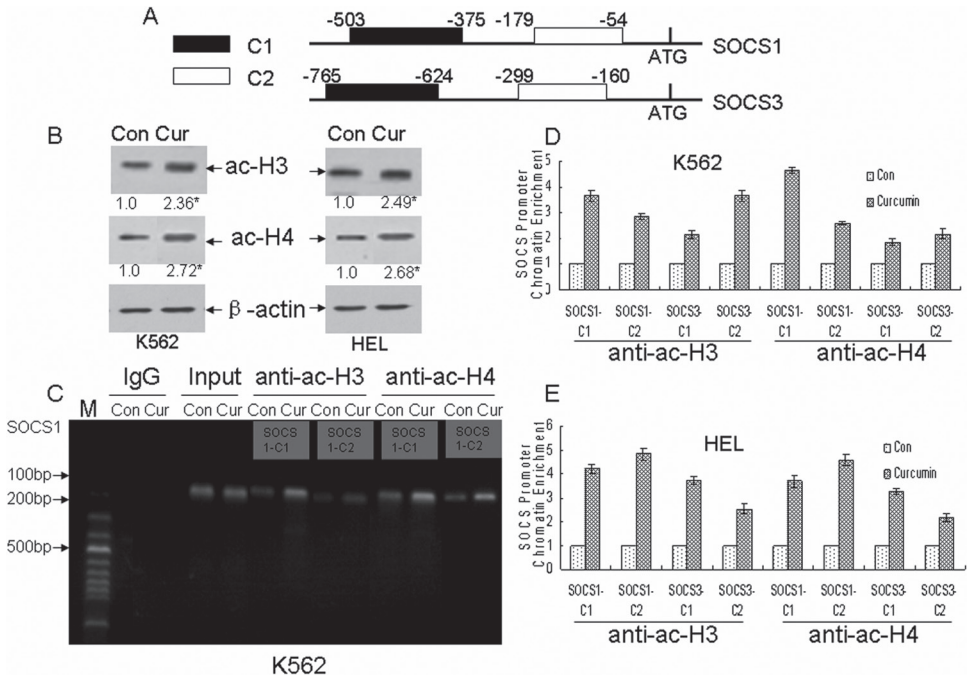

Fig. 4. Curcumin induces the accumulation of ac-H3 and ac-H4 in SOCS1 and SOCS3 promoter regions. (**A**) A schematic representation of the promoter regions amplified by ChIP-PCR assay. (**B**) Total levels of ac-H3 and ac-H4 were measured by western blotting in K562 and HEL cells treated with 20 μM curcumin for 24 h. The relative intensity of each band after normalization is shown under each blot. (**C**) Soluble chromatin from K562 cells treated with or without curcumin was immunoprecipitated with anti-ac-H3 and anti-ac-H4 antibodies and then analyzed by agarose gel electrophoresis. (**D** and **E**) Immunoprecipitated DNA from K562 and HEL was analyzed by quantitative real-time PCR.

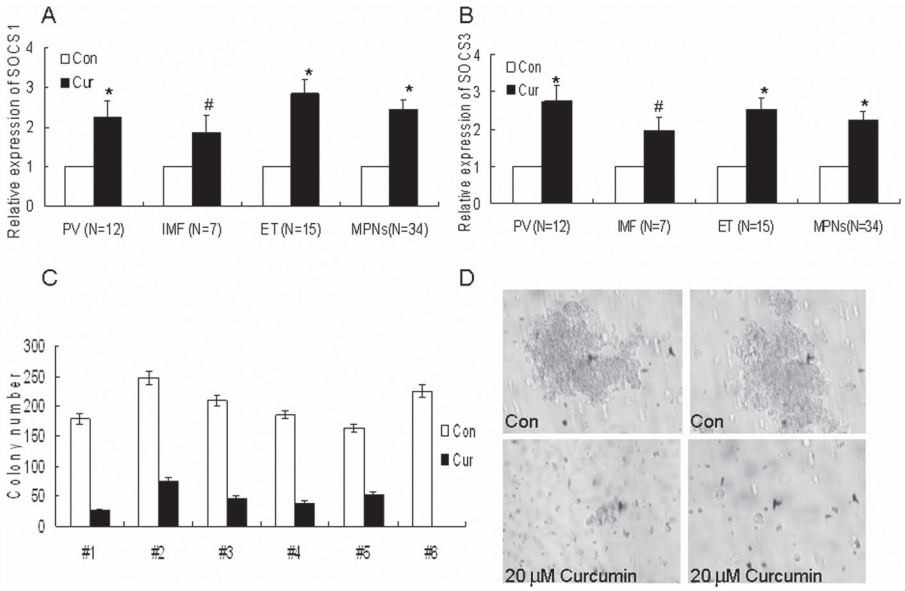

Fig. 5. Curcumin increases the levels of SOCS1 and SOCS3 and inhibits the colony formation in primary MPN cells. (**A** and **B**) About (2–5) × 10^5/ml bone marrow mononuclear cells isolated from 34 MPN patients including 12 PV, 7 IMF and 15 ET were treated with 20 μM curcumin for 24 h, and then SOCS1 and SOCS3 levels were measured by quantitative real-time PCR. *$P < 0.01$ and $^{\#}P < 0.05$ versus normal control. (**C**) Bone marrow mononuclear cells from six MPN patients (two PV and four ET) were cultured with 20 μM curcumin for colony assay as described in Material and methods. The number of colonies containing >40 cells in each dish was counted. All experiments were done twice using triplicate plates per experimental point. (**D**) Representative images of colony for the case 1 patient. Images were visualized with a LEICA DMIRB (LEICA, Wetzlar, Germany) microscope equipped with a ×40/0.5 numerical aperture objective lens (LEICA) and were captured through Canon digital camera.

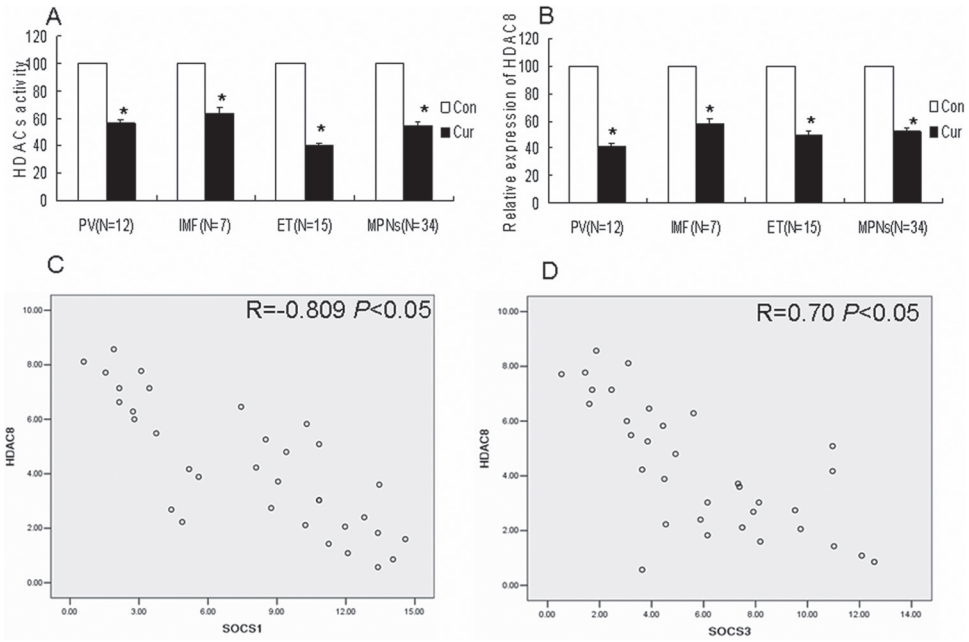

Fig. 6. Curcumin reduces the activity of HDACs and inhibits the expression of HDAC8 in primary MPN cells. HDAC activity (**A**) and the mRNA levels of HADC8 (**B**) were detected in bone marrow mononuclear cells that were isolated from 34 MPN patients including 12 PV, 7 IMF and 15 ET and were treated with 20 μM curcumin for 24 h. *$P < 0.01$ versus normal control. (**C** and **D**) The *y*-axis or *x*-axis represented the relative mRNA levels of HDAC8 or SOCS1/3, respectively, which were normalized against housekeeping gene (GAPDH) and calculated to normal cells in 34 primary MPN cells. A statistically significant inverse correlation between HDAC8 and SOCS1/3 expression was observed by Pearson's method.

Curcumin can inhibit proliferation and induce apoptosis via suppressing constitutive activation of JAK/STAT signaling in a number of cancer cell lines. Curcumin inhibited cell cycle, migration, cell proliferation, invasion and angiogenesis through reducing the levels of p-JAK2 and p-STAT3 in small cell lung cancer (28). Curcumin inhibited lysophosphatidic acid-induced STAT3 phosphorylation and IL-6 and IL-8 secretion, resulting in blocked ovarian cancer cell motility (29). Curcumin also induced a dose-dependent decrease in JAK3, STAT3 and STAT5 phosphorylation, finally resulting in the induction of growth arrest and apoptosis in T-cell leukemia (30). However, the mechanism that curcumin inhibits JAK2/STAT signaling is still not well delineated. As demonstrated in Supplementary Figure 4,

available at *Carcinogenesis* Online, curcumin decreased HDAC activity and reduced HDAC8 level. Curcumin treatment upregulated the expression of SOCS1 and SOCS3 via increasing hyperacetylation of histones in SOCS1 and SOCS3 promoter regions. Furthermore, TSA treatment (Supplementary Figure 5, available at *Carcinogenesis* Online) and HDAC8 knockdown elevated the expression of SOCS1 and SOCS3. Thus, we conclude that curcumin increases the expression of SOCS1 and SOCS3 via inhibiting HDAC activity (especially HDAC8) in both K562 and HEL cells. As negative regulators of JAK2/STAT signaling, overexpression of SOCS1 and SOCS3 reduced the levels of p-JAK2 and p-STAT5 in K562 and HEL cells (Supplementary Figure 6A and B, available at *Carcinogenesis* Online). Therefore, we hypothesized that curcumin inhibited JAK2 and STAT5 phosphorylation through increasing the expression of SOCS1 and SOCS3 in both K562 and HEL cells. However, our results indicated curcumin only inhibited JAK2 and STAT5 phosphorylation in HEL cells but not in K562 cells. Consistent with a previous report (21), curcumin failed to decrease the JAK2 and STAT5 phosphorylation in K562 cells. Guerini *et al.* (31) also reported that HDAC inhibitor ITF2357 (Givinostat) only inhibited JAK2/STAT5 signaling in HEL cells but not in K562 cells. Because K562 cells are positive with constitutively active tyrosine kinase BCR-ABL, which plays a key role in the pathogenesis of chronic myeloid leukemia via eliciting a variety of leukemogenic signals including JAK2/STAT5, Ras/MAPK, Myc and others (32), we speculate that the inhibition of JAK2/STAT5 signaling by curcumin is prevented by tyrosine kinase BCR-ABL. In order to further prove curcumin regulated JAK2/STAT5 signaling by upregulation of SOCS1 and SOCS3, the IL-3-dependent murine myeloid cell line 32D cells were treated with 10 μM curcumin for 24 h because 10 μM curcumin effectively inhibited cell growth by 46.3% but slightly decreased viability (data not shown) (33). JAK2/STAT5 signaling is activated by IL-3 in 32D cells. We found curcumin increased the expression of SOCS1 and SOCS3 and reduced the levels of p-JAK2 and p-STAT5 (Supplementary Figure 7A and B, available at *Carcinogenesis* Online). Moreover, HDAC activity and HDAC8 level were reduced by curcumin treatment in 32D cells (Supplementary Figure 7A and C, available at *Carcinogenesis* Online). As SOCS1 and SOCS3 are negative regulators of JAK2/STAT signaling, curcumin may inhibit JAK2/STAT5 signaling via upregulation of SOCS1 and SOCS3 in 32D cells. In summary, our findings might reflect a general mechanism of curcumin's regulation of JAK2/STAT5 signaling.

Epigenetic inactivation of SOCS1 and SOCS3 in MPNs has been largely investigated. Capello *et al.* (34) reported SOCS3 methylation frequently occurred in MPNs (41.1%) and acute myeloid leukemia post-MPNs (58.8%) resulting in transcriptional silence. In contrast, SOCS1 methylation was observed in only fraction of MPNs (13.4%) and acute myeloid leukemia post-MPNs (15%) (34). However, Fourouclas *et al.* (35) indicated that SOCS3 methylation only occurred in 23% patients with IMF but not in patients with PV and ET. SOCS3 transcription levels were not silenced in JAK2 V617F positive patients. The inconsistencies of SOCS3 methylation levels in MPNs are probably due to detecting CpG island methylation in different promoter regions of SOCS3 or due to different patient selection criteria. However, whether HDACs affected the expression of SOCS1 and SOCS3 was not fully elucidated. Xiong *et al.* (36) reported that TSA inhibited JAK2/STAT signaling in colorectal cancer cells through upregulation of SOCS1 and SOCS3. Another HDAC inhibitor, vorinostat, also increased the levels of SOCS1 and SOCS3 in a murine model of PV (37). Our results also indicate that TSA increases the expression of SOCS1 and SOCS3 in K562 and HEL cells (Supplementary Figure 5, available at *Carcinogenesis* Online). In conclusion, the levels of SOCS1 and SOCS3 are modulated by epigenetic regulation including DNA methylation and HDACs.

Recently emerging studies have indicated that curcumin modulates multiple biological processes through its activity as an epigenetic agent. Curcumin has been shown to block HDAC activity in Raji cells and medulloblastoma cells (24,27). Curcumin induced apoptosis and cell cycle arrest at G_2/M in medulloblastoma cells through blocking HDAC activity and decreasing HDAC4 expression (27). Furthermore, curcumin enhanced the anticancer effects of HDAC inhibitors. Curcumin in combination with TSA produced greater antiproliferative and apoptotic effects than either agent alone in SkBr3 and 435eB breast cancer cells (38). Curcumin, at subtoxic concentration, markedly sensitized tumor cells to vorinostat- and panobinostat-induced growth inhibition and apoptosis (39). Moreover, curcumin/HDAC inhibitors combination greatly reduced pharmacologically achievable concentrations, which were ineffective when each drug was used alone (39). Curcumin was found to be highly potent to interact HDAC8 and inhibit its activity through molecular docking test compared with sodium butyrate (40). Our results also indicated that curcumin reduced HDAC activity and decreased the class I HDAC expression (HDAC1, 3 and 8), which is consistent with a previous report (24). TSA (Supplementary Figure 5, available at *Carcinogenesis* Online) and HDAC8 knockdown by siRNA increased the expression of SOCS1 and SOCS3 in K562 and HEL cells. Furthermore, curcumin increased the levels of ac-H3 and ac-H4 and finally led to the increased hyperacetylation of histones in SOCS1 and SOCS3 promoter regions. Taken together, curcumin upregulates the levels of SOCS1 and SOCS3 through inhibiting HDAC activity (especially HDAC8).

HDAC8 is a member of class I HDACs, but it has a distinct biological function from HDAC1–3 (41). Only HDAC8, but not other HDACs, was significantly correlated with advanced disease stage in neuroblastoma cells (42). Moreover, knockdown of HDAC8 led to growth inhibition and cell cycle arrest in cultured neuroblastoma cells (42). Recently, HDAC8 has been reported to modulate p53 level. Upon knockdown of each class I HDAC, only HDAC8 knockdown resulted in decreased expression of wild-type and mutant p53 proteins and transcripts (43). However, the role of HDAC8 in MPNs and leukemia remains largely unknown. Our results indicate that knockdown of HDAC8 increases the expression of SOCS1 and SOCS3 and ectopic expression of HDAC8 decreases the expression of SOCS1 and SOCS3. Furthermore, HDAC8 was downregulated by curcumin. Therefore, HDAC8 plays an important role in the upregulation of SOCS1 and SOCS3 by curcumin. However, it is not clear whether HDAC8 affects the accumulation of ac-H3 and ac-H4 at the SOCS1 and SOCS3 promoter regions. Our data indicated that HDAC8 knockdown increased the histone acetylation (Supplementary Figure 8A and B, available at *Carcinogenesis* Online) and overexpression of HDAC8 decreased the histone acetylation at the SOCS1 and SOCS3 promoter regions in K562 and HEL cells (Supplementary Figure 8C and D). Thus, HDAC8 modulates the expression of SOCS1 and SOCS3 via regulating the acetylation levels of histone at the site of SOCS1 and SOCS3 promoter.

The use of pan-HDAC inhibitors to treat cancer in clinic is promising. However, considering the effects of pan-HDAC inhibitors on cancer are not specific and unselective inhibition of HDACs causes a variety of side effects, the application of HDAC inhibitors in clinic is limited. Knockdown experiments of selective HDAC isoforms had revealed that HDAC8 was essential for cell survival and HDAC8 knockdown reduced the proliferation of colon, lung and cervical cancer cells (44). HDAC8-specific inhibitor PCI-34051, which had >200-fold selectivity over the other HDAC isoforms, induced caspase-dependent apoptosis in T-cell lymphomas and leukemia (45). HDAC8 inhibition by siRNA or selective compound also inhibited proliferation, reduced clonogenic growth and promoted differentiation in cultured neuroblastoma cells (11). Therefore, inhibition of HDAC8 by curcumin or specific HDAC8-targeting agent might be beneficial for the treatment of patients with MPNs.

Several studies have indicated that dietary compounds possess anticancer activity through inhibiting HDACs. For example, MCP30, which was isolated from dietary bitter melon seeds, induced apoptosis via inhibiting HDAC1 activity in malignant prostate cancer cells (46). We report for the first time that curcumin elevates the expression of SOCS1 and SOCS3 via reducing HDAC activities and HDAC8 levels in K562, HEL, 32D and primary MPN cells. HDAC8 knockdown increases the expression of SOCS1 and SOCS3 through triggering acetylation of histone in the regions of SOCS1 and SOCS3 promoter.

Curcumin inhibits JAK2/STAT5 signaling in HEL and 32D cells. Therefore, in combination with other modes of therapy, curcumin has the potential to develop into a therapeutic agent for MPNs and leukemia without severe side effects.

Supplementary material

Supplementary Figures 1–8 and Tables 1 and 2 can be found online at http://carcin.oxfordjournals.org/

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