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ORIGINAL PAPER

DNA methylation regulates constitutive expression of Stat6 regulatory genes *SOCS-1* and *SHP-1* in colon cancer cells

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Abstract
Purpose Stat6 signaling is active in cancer cells and IL-4-induced Stat6 activities or Stat6 activational phenotypes vary among cancer cells. This study aimed at investigating possible mechanism(s) involved in the formation of varying Stat6 activities/phenotypes.
Methods Stat6 regulatory genes, SOCS-1 and SHP-1, were examined for mRNA expression using RT-PCR, and their promoter DNA methylation was assayed by methylation-specific PCR in Stat6-phenotyped colon cancer cell lines. DNA methylation was then verified by sequencing. RT-PCR assay and Western blotting were used to detect the expression of SOCS-1 and SHP-1 after demethylation using 5-aza-2′-deoxycytidine.
Results Compared with Stat6^{null} Caco-2 cells, Stat6^{high} HT-29 cells showed decreased constitutive expression of SOCS-1 and SHP-1, which correlated with DNA hypermethylation in these genes' promoters. Interestingly, demethylation in HT-29 cells recovered the constitutive expression of SOCS-1 and SHP-1.
Conclusions These findings suggest that DNA methylation controls the constitutive expression of negative Stat6 regulatory genes, which may affect Stat6 activities.

Keywords Colon cancer · DNA methylation · IL-4 · SOCS-1 · SHP-1 · Stat6

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Introduction

Functionally active Stat6 signaling pathway plays an important role in biology in a variety of cell types, including cancer cells and immune cells (Bruns and Kaplan 2006; Ansel et al. 2006). The IL-4/Stat6 signaling pathway is composed of at least six associated molecules including IL-4, IL-4Rα, common γ chain (γc), Jak1, Jak3, and Stat6, which forms a cascade of interactions upon activation by IL-4 (Nelms et al. 1999). Activated Stat6 binds to the promoter of IL-4-responsive genes by which it may up- or down-regulate gene expression. Thus far, at least 35 genes have been shown to be Stat6-regulated (Hebenstreit et al. 2006) and this number is likely to increase (Zhang et al. 2008) suggesting the pathway's broad importance in physiology and pathophysiology.

The Stat6 pathway has been extensively studied in gene knockout animals. On the front of cancer studies, it is important to note that mice lacking Stat6 manifest enhanced tumor immunity to both primary and metastatic mammary carcinomas, and induce spontaneous rejection of

implanted tumors (Kacha et al. 2000; Ostrand-Rosenberg et al. 2000). We have shown that human breast cancer and colon cancer cell lines lacking Stat6 signaling (Stat6^{null}) exhibit increased susceptibility to spontaneous apoptosis and less invasiveness/metastasis (Zhang et al. 2008; Li et al. 2008), in keeping with the observations in Stat6 knockout mice. Furthermore, constitutive activation of Stat6 is frequently observed in a number of human malignancies (Bruns and Kaplan 2006). Collectively, these observations strongly suggest a role of Stat6 in the front line of carcinogenesis. We therefore favor the hypothesis that a functionally active Stat6 signaling is beneficial to cancer cells at several stages of carcinogenesis including cancer transformation, growth and metastasis, possibly by promoting an exaggerated Th2 environment, gaining resistance to apoptosis and escaping the host immunosurveillance (Bruns and Kaplan 2006; Nelms et al. 1999; Li et al. 2008; Ostrand-Rosenberg et al. 2004). Accordingly, we have proposed that Stat6 activities may serve as a biomarker capable of forecasting cancer cell's fates for their susceptibility to apoptosis and invasive/metastatic ability (Li et al. 2008).

The controlling mechanisms of Stat6 signaling have been well investigated and there are at least two molecules that are known to negatively regulate the Stat6 pathway. Suppressor of cytokine signaling-1 (SOCS-1) is demonstrated to be a potent inhibitor of the IL-4/Stat6 pathway by suppressing the activation of Jak1, Jak3, and Stat6 in response to IL-4 (Losman et al. 1999; Hebenstreit et al. 2005; Dickensheets et al. 2007). Activated Stat6, on the other hand, induces the expression of SOCS-1, which in turn inhibits further activation of Stat6, forming a negative feedback control to modulate proper activation of the Stat6 (Hebenstreit et al. 2005; Dickensheets et al. 2007). SHP-1 (SH2-containing phosphatase-1) is a protein tyrosine phosphatase that has been indicated in negative regulation of Stat6 activation by dephosphorylating the tyrosine of activated Jak3 (Haque et al. 1998; Rane and Reddy 2002; Hanson et al. 2003).

It is an interesting phenomenon that human EBV-B cell lines show dramatic differences in Stat6 activity by EMSA assay and we have defined three naturally occurring IL-4-induced Stat6 activational phenotypes, termed as Stat6^{high}, Stat6^{low}, and Stat6^{null} (Zhang et al. 2003). The phenomenon also appears to be true in breast and colon cancer cells (Zhang et al. 2008; Li et al. 2008). Attempts to understand molecular mechanisms that may have generated these Stat6 phenotypes have failed to correlate them with constitutive Stat6 protein levels and polymorphisms of the *IL4RA* gene (Zhang et al. 2003). Recently, we have phenotyped two human colon cancer cell lines for their Stat6 activities, of which HT-29 carries active Stat6^{high} phenotype and Caco-2, inactive Stat6^{null} phenotype (Li et al. 2008). Interestingly, negative regulator genes *SOCS-1* and *SHP-1* show underexpression in the Stat6^{high} HT-29 cells but overexpression in the Stat6^{null} Caco-2 cells (Yuan et al. 2008).

Although it is not certain if differentially expressed *SOCS-1* and *SHP-1* may be involved in the formation of Stat6 phenotypes, the observation is sufficiently interesting for further investigation. In carcinogenesis, aberration of DNA methylation, referred to as epigenetic alterations, is a common event that causes altered gene expression which is often utilized to benefit transformed cancer cells (Ushijima 2005). Relevant to our studies are the observations that *SOCS-1* gene has been found to be hypermethylated in a number of cancers including hepatocellular carcinoma (Yoshikawa et al. 2001), hepatoblastomas (Nagai et al. 2003), gastric cancer (Oshimo et al. 2004), and colon cancer (Fujitake et al. 2004). *SHP-1* gene silencing due to promoter methylation has also been reported in lymphomas and leukemia as well as in many haematopoietic cell lines (Valentino and Pierre 2006). Taken the above findings together, we favor the hypothesis that DNA methylation may be a mechanism resulting in differential expression of Stat6 regulatory genes *SOCS-1* and *SHP-1*, which in turn influences Stat6 activities.

In the current study, we focus on the differential expression of Stat6 regulatory genes and their DNA methylation status in relation to Stat6 phenotypes and show that (1) HT-29 defined as active Stat6^{high} phenotype shows much decreased constitutive mRNA expression of Stat6 negative regulator genes *SOCS-1* and *SHP-1*, (2) Stat6^{high} HT-29 cells exhibit DNA hypermethylation in the promoters of *SOCS-1* and *SHP-1* genes compared with those in Stat6^{null} Caco-2 cells, (3) DNA demethylation using 5-Aza-CdR upregulates the mRNA expression of otherwise hypermethylated *SOCS-1* and *SHP-1* in HT-29 cells. These findings may have important implications in Stat6 regulatory mechanisms in relation to constitutively activated Stat6 in cancers.

Materials and methods

Cell lines

Epstein-Barr virus (EBV)-transformed lymphoblastoid B (EBV-B) cell lines were established and phenotyped for Stat6 activity previously (Zhang et al. 2003). Human colon cancer cell lines HT-29 and Caco-2 were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium supplemented with 1% calf serum, 2.05 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2.

Determination of Stat6 activational phenotypes

Phenotyping of IL-4-activated Stat6 was performed using a semi-quantitative EMSA method, we developed earlier (Zhang et al. 2003), and colon cancer cell lines HT-29 and Caco-2 were phenotyped for Stat6 activity in a previous report (Li et al. 2008).

RNA isolation and semiquantitative RT-PCR

HT-29 and Caco-2 cells were cultured in standard culture flask at a concentration of 2×10^6 cells/flask and allowed for spontaneous growth for 4 days. Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacture's instructions. The first strand of cDNA was carried out using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, USA). PCR was performed in a final volume of 25 µl containing 1.5 µl cDNA, 10 pmol of each primer, 2× *Taq* PCR Mix 12.5 µl and DEPC-treated water (Tiangen Co., Beijing, China). For quantitative analysis, RT-PCR products were scanned using a gel imaging scanning system (GeneGenius from Syngene, England). The obtained area readings of target genes were compared with those of human *GAPDH* gene and the results were expressed as a ratio. Individual RT-PCR tests were repeated on at least three independent occasions. Primer sequences and PCR parameters used in RT-PCR were shown in Table 1.

DNA isolation and sodium bisulphite conversion

Genomic DNA was isolated from at most 5×10^6 cells with DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer's instruction. Genomic DNA (1.1 µg) was modified with sodium bisulphate using the EZ DNA Modification-Gold™ Kit (Zymo research Biotech Co., CA, USA). For accuracy, equal amount of DNA from different samples was adjusted. Untreated DNA from normal human placenta tissues was used as control for unmethylated alleles, while DNA treated in vitro with Sss1 methyltransferase (New England Biolabs, Inc., MA, USA) was used as positive control for methylated alleles.

Methylation-specific polymerase chain reaction (MSP)

The methylation-specific polymerase chain reaction (MSP) for promoter methylation was performed as described (Herman et al. 1996) and modified DNA was subjected to two separate PCRs. MSP primers were designed to amplify the methylated (M-MSP) and unmethylated (U-MSP) alleles (Table 1). Methylation-specific primers for *SOCS-1* and *SHP-1* were previously described by others (Weber et al. 2005; Oka et al. 2002). MSP was performed in a thermal cycler (PTC-200, USA) with the following cycling conditions: 95°C for 5 min, 40 cycles of 95°C for 30 s, specific annealing temperature (SOCS-1 at 60°C, SHP-1 at 58°C, SOCS-3 at 60°C) for 1 min, 72°C for 1 min, and a final extension of 10 min at 72°C. Individual MSP tests were repeated on three independent occasions.

DNA sequencing

To confirm CpG methylation, MSP products were purified, sequenced bidirectionally, and analyzed on an automated DNA sequence analyzer (Applied Biosystems, CA, USA).

Table 1 PCR primer sequences and reaction conditions

Genes	Sequences (5′–3′)	PCR conditions[a]	Size (bp)
SOCS-1	F: GGAGCGGATGGGTGTAGGGG	64 (30)	178
	R: GAGGTAGGAGGTGCGAGTTCAG		
SHP-1	F: GTCGGAGTACGGGAACATCACC	61 (30)	387
	R: CCCAGGGCTTTATTTACAAGAGGAG		
SHP-2	F: ATGAGGAGACACGGGTAGGACT	62 (35)	303
	R: GCTATGTGTGAAAGTTGATCCC		
GAPDH	F: CATGAGAAGTATGACAACAGCCT	56 (30)	113
	R: AGTCCTTCCACGATACCAAAGT		
SOCS-1 (M-MSP)	F:TGAAGATGGTTTCGGGATTTACGA	60 (40)	183
	R: ACAACTCCTACAACGACCGCACG		
SOCS-1 (U-MSP)	F: TGAAGATGGTTTTGGGATTTATGA	60 (40)	184
	R: CACAACTCCTACAACAACCACACAC		
SHP-1 (M-MSP)	F: GAACGTTATTATAGTATAGCGTTC	58 (40)	159
	R: TCACGCATACGAACCCAAACG		
SHP-1 (U-MSP)	F: GTGAATGTTATTATAGTATAGTGTTTGG	56 (40)	162
	R: TTCACACATACAAACCCAAACAAT		

[a] Annealing Temperature in °C (number of cycles)

F forward primer, *R* reverse primer

Sequences were compared with wild-type sequence of SOCS-1 and SHP-1.

DNA demethylation using 5-Aza-CdR

Demethylation agent 5-Aza-CdR (10 μM, Sigma Co., St. Louis, IL, USA) was used to treat HT-29 cells at 37°C for 4 days (Oka et al. 2002). Cells were then harvested on day 4 and tested for mRNA expression by RT-PCR and protein expression analysis by Western blotting.

Western Blot analysis

Total proteins with and without 5-Aza-CdR treatment were prepared using Total Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China) and quantified. Equal amounts of proteins were subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and transferred to PVDF membrane (Ameresco, USA). The membrane was incubated with blocking buffer for 2 h first at room temperature, and then incubated overnight at 4°C with the primary polyclonal antibody against SHP-1 (Upstate, NY, USA) or β-actin (Santa Cruz Biotechnology, CA, USA) as a normalizing reference. Quantitative densitometry was performed using a computer-based image analysis system (GeneGenius from Syngene, England).

Statistical analysis

Statistical analysis was performed using independent-samples t test (SPSS statistical software Version 15.0) and significance was defined as a $P < 0.05$ for all analyses and data were presented as mean ± standard deviation (M ± SD).

Results

Phenotyping of IL-4-induced Stat6 activity in HT-29 and Caco-2 human colon cancer cell lines

By reference to an EBV-B cell line with confirmed IL-4-induced Stat6^{high} activational phenotype (DNA binding activity) as a standard control (Zhang et al. 2003), we were able to assign Stat6 activational phenotypes for colon cancer cell lines using EMSA assay (Fig. 1). As seen in Fig. 1, HT-29 was assigned as Stat6^{high} phenotype and Caco-2, Stat6^{null} phenotype. Cells carrying Stat6^{high} phenotype have been shown to exhibit resistance to apoptosis and increased metastatic capability in breast and colon cancer cells (Zhang et al. 2008; Li et al. 2008).

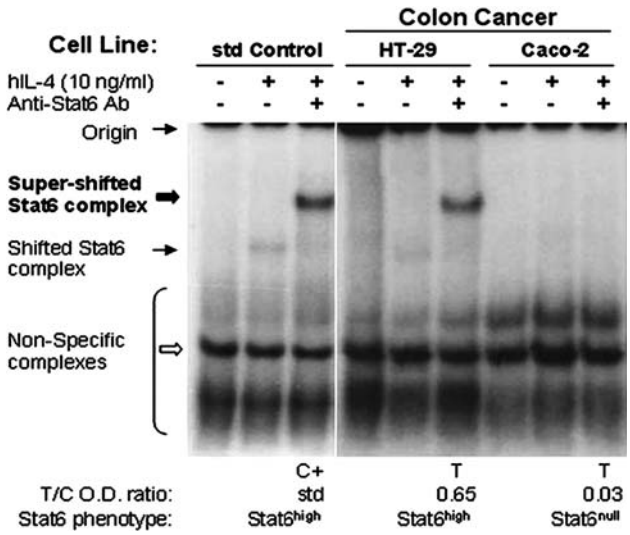

Fig. 1 Colon cancer cell lines HT-29 and Caco-2 show distinct IL-4/Stat6 EMSA profiles corresponding to differential Stat6 phenotypes. The criteria of assigning Stat6 phenotypes were established previously (Zhang et al. 2003). The phenotyping of these two cell lines was reported by us in a previous report (reproduced from Li et al. 2008 with permission)

HT-29 (Stat6^{high}) cells constitutively express less mRNA of SOCS-1 and SHP-1 than Caco-2 (Stat6^{null}) cells

SOCS-1 and SHP-1 are known negative regulators of the Stat6 pathway. It was interesting to note that, as shown in Fig. 2, HT-29 cells carrying active Stat6^{high} phenotype correlated with decreased constitutive mRNA levels of its negative or inhibiting regulatory genes *SOCS-1* and *SHP-1*. Similarly, a reciprocal pattern of increased mRNA expression of *SOCS-1* and *SHP-1* in Caco-2 cells carrying inactive Stat6^{null} phenotype was interesting (Fig. 2). No difference was observed in mRNA expression of *SHP-2* between HT-29 and Caco-2 cells (Fig. 2).

Decreased expression of SOCS-1 and SHP-1 correlates with promoter DNA hypermethylation in HT-29 cells

The above observations were interesting in that two regulatory genes, *SOCS-1* and *SHP-1*, simultaneously showed decreased mRNA expression in HT-29 cells as compared with that in Caco-2 cells (Fig. 2). As DNA methylation was frequently observed to be correlated with gene transcription silencing, especially in cancer cells, we then asked whether DNA methylation would be one of the mechanisms responsible for the differential mRNA expression between cell lines as shown in Fig. 2. Using MSP method, we demonstrated that all two genes SOCS-1 (Fig. 3a, b) and SHP-1 (Fig. 4a, b) exhibited higher DNA methylation in their promoter regions. The DNA methylation in these genes was later confirmed by DNA sequencing (Figs. 3c, 4c).

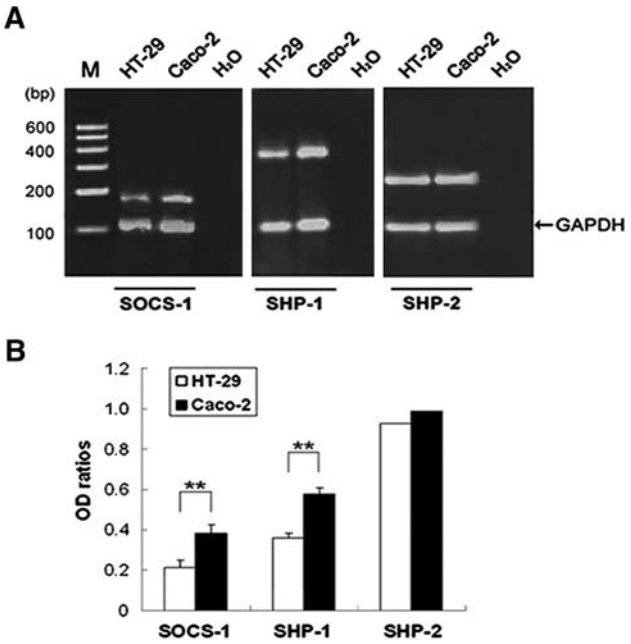

Fig. 2 Active Stat6^{high} HT-29 cells express less constitutive mRNA of Stat6 negative regulators *SOCS-1* and *SHP-1* than inactive Stat6^{null} Caco-2 cells. **a** Gel images of RT-PCR products of *SOCS-1, SHP-1*, and *SHP-2*. The OD readings of individual target gene products were compared with those of GAPDH products within the same lane and the results were expressed as a ratio of target gene/GAPDH. **b** Summary presentation of OD ratios from at least three independent culture/RT-PCR tests for each gene. $^{**}P<0.01$ by statistical analyses

DNA demethylation using 5-Aza-CdR recovers mRNA expression of SOCS-1 and SHP-1 in HT-29 cells

In order to verify if hypermethylation was indeed responsible for decreased expression of two regulatory genes, we tested reversibility of gene expression by DNA demethylation using 5-Aza-CdR treatment. As seen in Fig. 5, when HT-29 cells, which showed gene hypermethylation (Figs. 3, 4), were treated with 10 μM of 5-Aza-CdR for 4 days in culture media, the mRNA expression was upregulated in two genes *SOCS-1* and *SHP-1*. This recovered expression after DNA demethylation was also demonstrable at protein level at least for one gene tested (*SHP-1*; Fig. 5). These results strongly suggested that promoter DNA hypermethylation in these genes was responsible for the low expression as observed in HT-29 cells (Fig. 2).

Discussion

Recent studies have indicated that functionally active Stat6 may play an important role in carcinogenesis and prognosis of cancer (Wurster et al. 2000). Direct evidence has come from basic research that activated Stat6 induces resistance to apoptosis (Galka et al. 2004; Zhang et al. 2006, 2008; Li et al. 2008), clinical observation that Stat6 is constitutively activated in a number of human cancers (Bruns and Kaplan 2006; Ni et al. 2002), and Stat6 knockout mouse models that exhibit resistance to metastatic disease and spontaneously reject implanted tumors (Kacha et al. 2000; Ostrand-Rosenberg et al. 2000). As mentioned above, there are three naturally occurring IL-4-induced Stat6 activation phenotypes, termed as Stat6^{high}, Stat6^{low}, and Stat6^{null} (Zhang et al. 2003). Similar to Stat6$^{-/-}$ animals, the naturally occurring human Stat6^{null} phenotype shows several functional differences important to cancer cell's survival and progression including the increased expression of Th1 cytokines IL-12, TNF-α, and IFN-γ (Li et al. 2008; Zhang et al. 2004), susceptibility to apoptosis (Zhang et al. 2008; Li et al. 2008) and decreased metastatic ability (Li et al. 2008).

Widely studied human colon cancer lines, HT-29 and Caco-2, are interesting because they show very different Stat6 activities or phenotypes (Fig. 1). HT-29 cells present active Stat6^{high} phenotype which exhibits resistance to apoptosis and increased metastatic ability (Li et al. 2008). Relevant to these phenotypic features is that HT-29 cells, in comparison with caco-2 cells, express higher levels of anti-apoptotic and/or pro-metastatic genes *Survivin*, *MDM2*, and *TMPRSS4* (transmembrane protease serine 4), but lower levels of pro-apoptotic and/or anti-metastatic genes *P53*, *BAX*, and *CAV1* (Caveolin-1) (Li et al. 2008).

In effort to reveal the possible underlying mechanism(s) that may have generated IL-4/Stat6 phenotypes, we have previously investigated several possibilities, including constitutive Stat6 expression, a bi-allelic SNP polymorphism within the 3′UTR of the *STAT6* gene and *IL4RA* gene polymorphism, all of which are failed to correlate with Stat6 phenotypes (Zhang et al. 2003). We have also been unable to find differences in constitutive mRNA expression of Jak1 and Jak3 at least between Stat6^{high} HT-29 and Stat6^{null} Caco-2 cells (unpublished observations). Recently, we have focused on investigating negative regulator genes and found decreased expression of *SOCS-1* and *SHP-1*, in active Stat6^{high} HT-29 cells (Yuan et al. 2008). These findings are interesting and have led us to hypothesize that, as alterations in DNA methylation are frequent events during cancer cell's transformation (Ushijima 2005), there may be differences in DNA methylation resulting in differential expression of *SOCS-1* and *SHP-1* between HT-29 and Caco-2 cells.

Stat6^{high} HT-29 cells reproducibly express less mRNA of *SOCS-1* and *SHP-1* when compared with Stat6^{null} Caco-2 cells (Fig. 2). Using MSP assay, we have detected higher DNA methylation in the promoter regions of at least two genes *SOCS-1* and *SHP-1*, respectively, in HT-29 cells in comparison with Caco-2 cells (Figs. 3, 4). DNA methylation, unlike other epigenetic changes, does not alter the

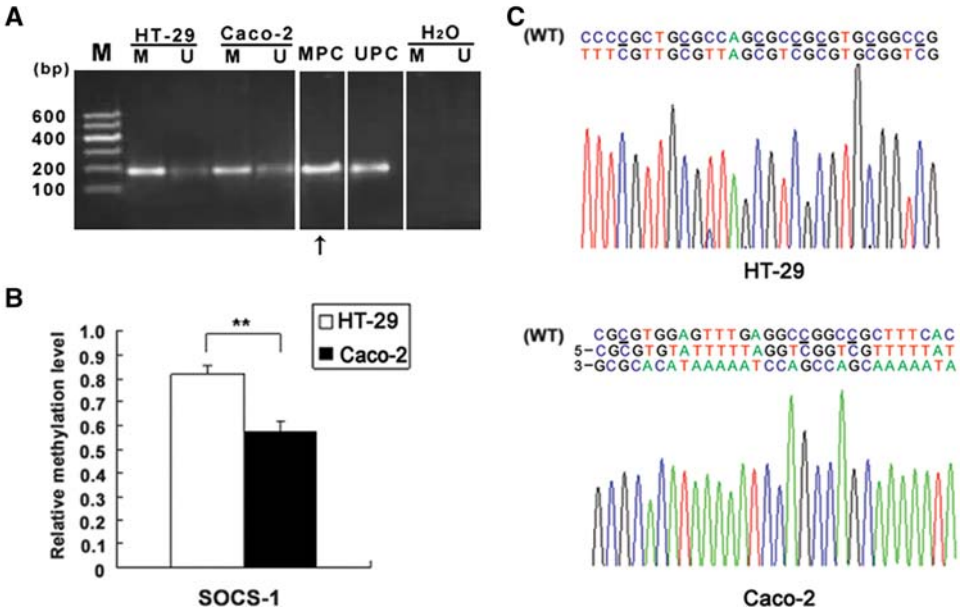

Fig. 3 Active Stat6^{high} HT-29 cells exhibit higher DNA methylation in the promoter of *SOCS-1* than that in inactive Stat6^{null} Caco-2 cells. **a** MSP detection of *SOCS-1* methylation in HT-29 and Caco-2 cells. Relative methylation levels were measured for *SOCS-1* whose OD readings over methylated product (M) were compared with that of a methylation-positive control (MPC, indicated by a *vertical arrow*), and the results were shown as a ratio of M/MPC. U and UPC indicate unmethylated product and unmethylation-positive control, respectively. As can be seen under U, both HT-29 and Caco-2 carry unmethylated products suggesting incomplete methylation of *SOCS-1*. **b** Diagrammatic comparison of methylation levels (OD ratios) obtained from three independent tests for HT-29 and Caco-2. **c** Confirmation of methylation by sequencing. Wild-type sequence of *SOCS-1* shown at *top* and the *bottom* sequence is methylation-positive sequence. Caco-2 was sequenced reversely and therefore, the middle sequence represents the complementary sequence of the sequenced DNA. After bisulfite treatment, unmethylated C is converted to "T" whereas methylated C remains as C (*underlined*). $^{**}P < 0.01$ by statistical analysis

nucleotide sequence and, therefore, is reversible (Esteller et al. 2001; Baylin 2001; Grønbaek et al. 2007). To confirm that DNA hypermethylation indeed suppresses the gene expression in HT-29 cells, we have further performed demethylation assay using 5-Aza-CdR that inhibits the activity of methyltransferase (Momparler 2005). As expected, 5-Aza-CdR treatment in HT-29 cells is able to upregulate the expression of all two hypermethylated genes *SOCS-1* and *SHP-1* (Fig. 5a, b), supporting such a hypothesis that DNA methylation is responsible for decreased expression of these genes. Furthermore, the upregulation of gene expression after demethylation is also confirmable at protein level at least for SHP-1 whose antibody was available (Fig. 5c, d). It is conceivable that, because of insufficient levels of SOCS-1 and SHP-1, Stat6 may remain highly active in cells which would show overperformed Stat6-related functions such as exaggerated Th2 response (Nelms et al. 1999), resistance to apoptosis (Nelms et al. 1999; Zhang et al. 2008; Li et al. 2008) and higher metastatic capability (Li et al. 2008). Having demonstrated that DNA hypermethylation may be a mechanism restricting the expression of Stat6 negative regulatory genes, we have further tested whether demethylation would influence Stat6-related functions such as resistance to apoptosis. Indeed, demethylation using 5-Aza-CdR in HT-29 cells reverses the apoptosis-resistant cancer cells (Li et al. 2008) to be growth-retarded, suggesting increased susceptibility to apoptosis and cell death (data not shown). In this context, our findings provide new evidence that hypermethylation results in diminished expression of *SOCS-1* and *SHP-1* which may influence cancer cell's phenotypes via Stat6 activities (Fig. 1). It should be noted that the demethylation action of 5-Aza-CdR is nonspecific and the observations in Fig. 5 may be a collective outcome due to DNA demethylation in many genes. Nevertheless, the current findings strongly suggest the usefulness of epigenetic therapy (Grønbaek et al. 2007; Issa 2007).

In conclusion, we have shown for the first time that active Stat6^{high} HT-29 colon cancer cells express insufficient levels of Stat6 negative regulators SOCS-1 and SHP-1 in comparison with inactive Stat6^{null} Caco-2 cells. The DNA hypermethylation in the promoter regions of these genes may be one, if not the sole, mechanism which is responsible for the decreased gene expression. The current findings may have important implications in the formation of varying Stat6 activities among different cancer cells (Zhang et al. 2008; Li et al. 2008). As constitutive activation of Stat6 is frequently observed in many human cancer cells (Bruns and Kaplan 2006), our findings

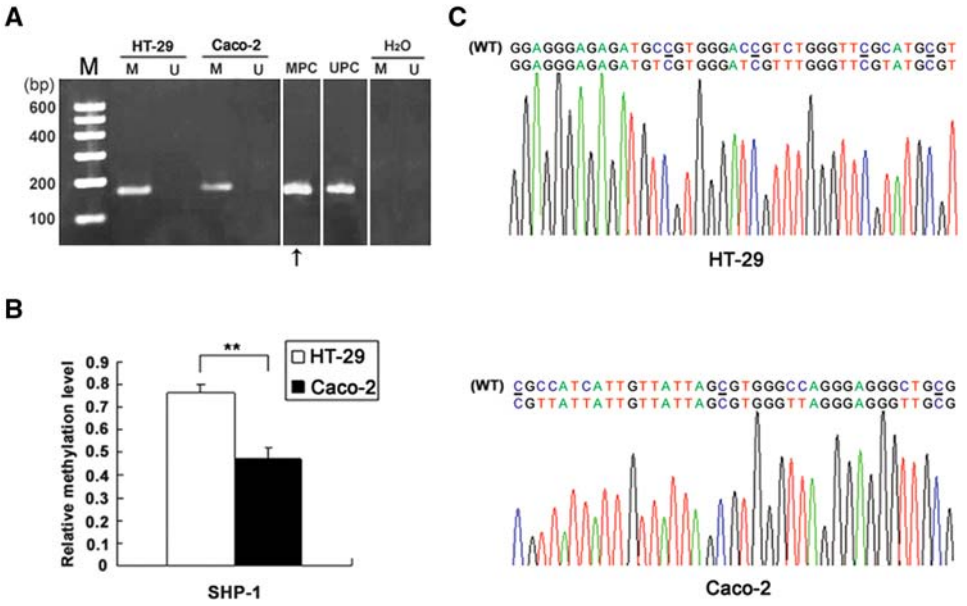

Fig. 4 Active Stat6^{high} HT-29 cells exhibit higher DNA methylation in the promoter of *SHP-1* than that in inactive Stat6^{null} Caco-2 cells. **a** MSP detection of *SHP-1* methylation in HT-29 and Caco-2 cells. Relative methylation levels were measured and presented for *SHP-1* in the same way as shown in Fig. 3. Note that *SHP-1* is completely methylated in both HT-29 and Caco-2 with varying magnitude. U and UPC indicate unmethylated product and unmethylation-positive control, respectively. **b** Diagrammatic comparison of methylation levels (OD ratios) obtained from three independent tests for HT-29 and Caco-2. **c** Confirmation of methylation by sequencing. The presentation of methylated and unmethylated sequences is in the same way as in Fig. 3 (**c**). $^{**}P<0.01$ by statistical analysis

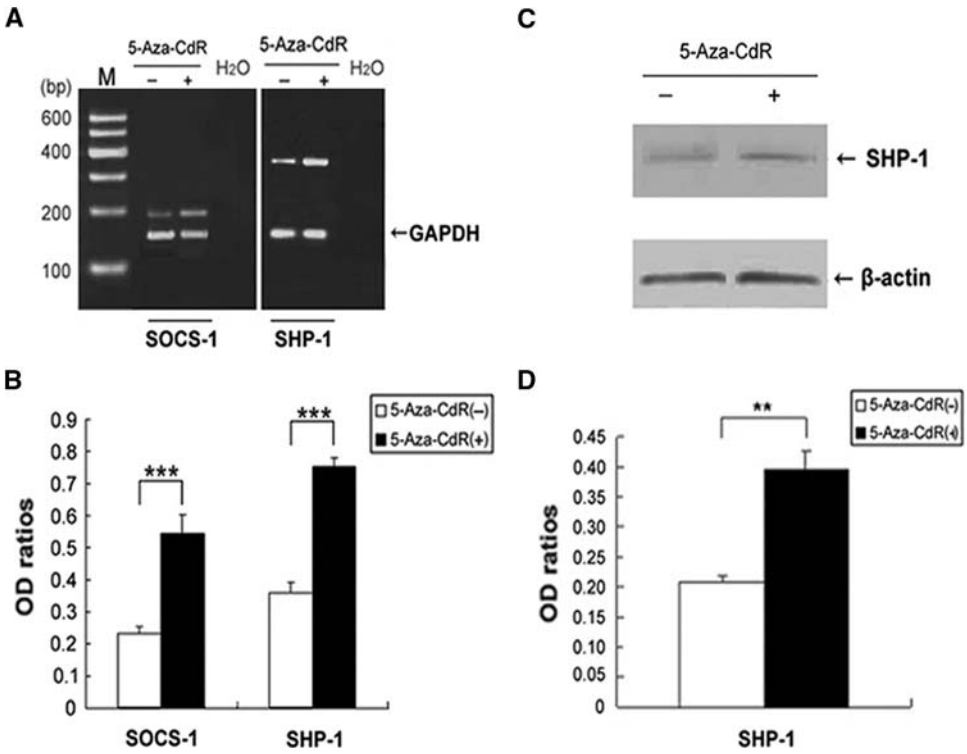

Fig. 5 Expression of mRNA and protein is recovered after demethylation in HT-29 cells. **a** Gel images of RT-PCR detection of *SOCS-1* and *SHP-1* mRNA after demethylation. The relative expression of mRNA was measured as described in Fig. 2. **b** Diagrammatic presentation of mRNA expression levels (OD ratios) from at least three independent tests. **c** Gel images of Western blots for SHP-1 and β-actin. The OD readings of SHP-1 were compared with those of β-actin and the results were expressed as a ratio of SHP-1/β-actin. **d** Summary presentation of OD ratios obtained from three independent tests. $^{***}P<0.001$ and $^{**}P<0.01$ by statistical analyses

presented here suggest that insufficient negative regulation may play a role in such uncontrolled constitutive activation of Stat6.

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